Width of cementum growth layer groups and its possible use in monitoring reproduction in female ringed seal

An exploratory pilot study

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Swedish University of Agricultural Sciences, SLU
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Abstract

The Baltic ringed seal (*Pusa hispida botnica*) population has faced historical declines due to both hunting and environmental pollution. It is slowly recovering but there is a need for new methods in order to monitor and assess pregnancy rates. The count of growth layer groups (GLGs) in teeth cementum have been used to age ringed seals for decades and the method is well established. This pilot study explored the possibility to measure the widths of the GLGs for subsequent use in monitoring reproduction events in female ringed seals. Canine teeth from eleven ringed seals were used (eight females, three males). The annular GLG widths were compared using a proportional width index (PWI) that corrects for body size difference between individuals and age trends. The PWI values were classified into juvenile years (<6) or adult years (>5). The variance in PWI during adult years was significantly higher in females (0.005062) than in males (0.002065, $p<0.001$). When comparing the distributions of the PWI values in females (Hartigan's dip test) the females showed a significant multimodality ($p=0.049$), forming two significantly different subgroups. This pattern may suggest the influence of pregnancy and lactation on one of the subgroups. During this pilot study, technical difficulties made comparing GLGs of known reproductive status impossible. Addressing these difficulties could be a way forward for future research.

Keywords: cementum, growth layer group (GLG), ringed seal, reproduction, recording structure, incremental line, cementum annuli

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Abbreviations

Abbreviation	Description
DCJ	Dental cementum junction
GLG	Growth layer group
KDE	Kernel density estimation
PWI	Proportional Width Index
SMNH	Swedish Museum of Natural History

1. Introduction

There are three seal species inhabiting the Baltic Sea. The grey seal (*Halichoerus grypus*), the harbour seal (*Phoca vitulina*) and, the focus of this thesis, the ringed seal (*Pusa hispida botnica)* (Almkvist *et al.* 1980). Seals are apex predators in the Baltic, and their health reflects the health of the marine ecosystem they reside in (HELCOM 2023a).

During the 20th century, the number of Baltic seals declined drastically (Harding & Härkönen 1999; HELCOM 2018). Firstly, as a result of an international campaign to decimate seals, with a bounty as incentive to hunt them (Harding & Härkönen 1999) and secondly as a result of environmental pollution. Organochlorines, such as polychlorinated biphenyls (PCB) and dichloro-diphenyl-trichloroethane (DDT), caused occlusions in the uterus leading to infertility (Helle *et al.* 1976; Helle 1980). The seals had other lesions as well, (including the occlusions) described as a “Baltic Seal Disease Complex” (Bergman 2007).

Today, the Swedish Baltic seals is monitored by population surveys to estimate the abundance (SMNH 2024a). Ringed seal reproduction is monitored by necropsies at The Swedish Museum of Natural History (SMNH), where parameters such as pregnancy, placental scars, corpus luteum, corpus albicans and pathological findings are registered (Bäcklin *et al.* 2024). The population surveys provide a general and indirect view of the result of the seals reproduction on a population level while the necropsies give a view of the present reproductive state, but no considerable information of the reproductive history of the individual. This knowledge is crucial in order to predict population changes, as mathematical modelling needs solid data on the reproductive capacity of the females at different ages.

The cementum of the teeth is extensively used for age determination in marine mammals (Stewart *et al.* 1996). The cementum is a tissue covering the root dentine surface (Berkovitz & Shellis 2018, p. 35). Its main purpose is to attach the dentine layer of the tooth to the collagen fibres in the periodontal ligament. Age determination is done by counting dark and light sections forming a pattern called growth layer groups (GLGs), incremental lines or tooth annuli (Stewart *et al.* 1996). In some bear species, the widths of these GLGs have been shown to be narrower during a breeding year than during non-breeding years (Carrell 1994; Tochigi 2018).

The aim of this study was to evaluate the possibility to use cementum GLG width to monitor reproduction events in female ringed seals from the Baltic Sea. This was done by measuring the width of the GLGs in 11 ringed seals and evaluating the patterns of the measured GLGs, comparing the widths between female and male GLGs at different stages of their lifetime.

2. Literature review

2.1 The Baltic Sea seals as an indicator of ecosystem health

The state of the Baltic Sea has been of great concern for many years due to its unique biodiversity being degraded and lost (HELCOM 2023a). This is a consequence of mostly anthropogenic pressures on the ecosystem. The key pressures are multifactorial and consists primarily of eutrophication, pollution from hazardous substances, land use and overfishing.

The Helsinki Commission (HELCOM) is an agreement entered in to by the states surrounding the Baltic Sea to protect the sea environment and its ecosystem (HELCOM 2023a). Unfortunately, there have been little to no improvement of the state of the Baltic Sea environment during 2016-2021.

The seals are the top predators in the Baltic Sea which makes them vulnerable to ecosystem changes in lower trophic levels (HELCOM 2018; HELCOM 2023b). They can also bioaccumulate harmful pollutants in their bodies. An important way to track disturbances of the ecosystem is by evaluating if the seals show signs of health and/or reproduction issues, which in turn works as a sensitive signal of diffuse or broad scale changes of the environment (HELCOM 2023a; HELCOM 2023b). If the seals were to disappear from the ecosystem, their regulation of the distribution, abundance and health of a variety of prey species would be lost (HELCOM 2023a). The seals also play a significant role for humans due to their cultural and historical importance, helping people appreciate the ecosystem and giving them a desire to protect the Baltic Sea (HELCOM 2023a).

2.2 Baltic Seal population

Since the middle of the 1970s, the Swedish Museum of Natural History (SMNH) is conducting a health monitoring program for seals in Swedish waters at the Department of Environmental Research and Monitoring (SMNH 2024b). Since 2020, the program is performed together with The Swedish National Veterinary Agency (SVA) on behalf of the Swedish Agency for Marine and Water Management (SwAM). Grey seals, harbour seals and ringed seals are necropsied for pathological findings. In addition, SMNH performs annual aerial surveys to investigate population density and distribution.

Historically, hunting has been the largest threat to the Baltic seal species (HELCOM 2018). An international extermination campaign with bounty systems were introduced in Sweden, Finland and Denmark in the late 19th century and early 20th century (Svensk fiskeritidskrift 1895 see HELCOM 2018). By the 1940s the grey seals were eradicated from Germany, Poland and the Kattegat Sea. The grey seal population in the entire Baltic Sea had gone from approximately 80,000 to 20,000 individuals (Harding & Härkönen 1999). During the same period, the ringed seal population had declined from around 200,000 individuals to 25,000. From the 1940s to the 1960s, the populations appeared to be stable. (Harding & Härkönen 1999).

During the second half of the 1960s, there was an additional drop in seal population size which caused concern (HELCOM 2018). The main reason turned out to be reproductive failure and multiple chronic organ lesions were found (Bergman 1999; Bergman 2007). In the uteruses there were stenoses, occlusions and leiomyomas observed (Helle 1976a; Bergman & Olsson 1985 see Bergman 2007). In addition, colonic ulcers (Bergman 1999), adrenal cortical hyperplasia and adenomas, renal glomerulopathy and tubular cell proliferations and arterial sclerosis were found. There were also skin, claw and skull bone lesions. These lesions are collectively referred to as the "Baltic Seal Disease Complex". During approximately the same time as the seal health was investigated, it was established that DDT and PCB pollution of the Baltic Sea were evident (Jensen *et al.* 1969 see Bergman 2007). During the seventies in the Bothnian Bay, the pregnancy rate of ringed seals was around 27-32% compared to 80-90% in areas of low levels of PCB and DDT pollution (Helle 1976a; Helle 1976b). The reduced fertility and infertility caused a new population crash of the grey seals and ringed seals, with only a few thousand of them left (Harding & Härkönen 1999). In Sweden, the use of PCB and DDT were restricted in 1972 and 1969 respectively (Olsson & Reutergårdh 1986). By the end of the 1980s, the PCB and DDT in biota had decreased and the number of seals started to increase slowly (Bergman 2007; HELCOM 2018).

The pregnancy rates have been steadily increasing but have still not reached "good status" according to HELCOM in the report from 2023 covering the years 2016-2021 (HELCOM 2023b). Pooled data from Sweden and Finland on grey seals and ringed seals show a pregnancy rate of 87% and 82%, respectively. The threshold value for "good status" according to HELCOM is 90% for grey seals and tentatively 90% for ringed seals. The same threshold was set for harbour seals, but the sample size was too low to draw any conclusions. If the abundance of seals has not reached the carrying capacity, a change in pregnancy rate will be an early indicator of changes in the ecosystem (HELCOM 2023b). This could possibly be an early warning for adverse ecosystem changes.

Historically, the ringed seal species were by far the most numerous of the seals in the Baltic Sea (Harding & Härkönen 1999). They have recovered more slowly compared to the grey seal and the number of grey seals now exceeds the number of ringed seals (Halkka *et al.* 2017). According to HELCOM, the Baltic ringed seal is now classified as vulnerable in their red list category (HELCOM 2013). The ringed seal is considered to be more vulnerable to climate change and global warming than the other Baltic seal species due to their reproduction being dependent on ice for success (SwAM 2017; Meier *et al.* 2004). The landmass north of and surrounding the Baltic Sea prevents the population to migrate north to colder climates, hence the population is "land- locked" (HELCOM 2013). It has also been proven that monitoring the ringed seals' population size is more difficult than the other seal species due to changes in moulting behaviour and ice-breakup (ICES 2023). The ringed seals are monitored by aerial surveys in April, when they are hauling out on ice to moult. Since 2013, the results of these surveys have been exceptionally high during years with early ice breakup. The ice breakup timing could be an explanation for the change in abundance of animals on the ice, but this needs further investigation. Due to these challenges, the population estimates during years with early ice breakup cannot be compared to historical population estimates, which impairs the possibility to calculate population trends. The combination of the ringed seals' red list status and the difficulties in monitoring led to ringed seal being the species of focus in this thesis.

2.3 The Baltic ringed seal (*Pusa hispida botnica*)

The ringed seals are now confined to two main management units evaluated by HELCOM, the northern and the southern unit (HELCOM 2023c). The northern unit inhabits the Bothnian Bay and the southern inhabits the Archipelago Sea, the Gulf of Finland and western Estonia (Gulf of Riga).

The ringed seal is the smallest of the Baltic seals with a body length of 100–175 cm and a weight of 32–140 kg (SLU Artdatabanken 2024). They exhibit sexual dimorphism where the females are smaller than the males. The coat is dark grey to black-brown with light areas in the form of that gives the species its English name. They can live up to 50 years, but less than 1% reaches that age. The ones that reach adulthood usually die around the age of 25 to 30 (SLU Artdatabanken 2024). There are other sub-species of ringed seal in the Arctic with a circumpolar distribution (Reeves 1998). Most of the research have been done on the Canadian Arctic subspecies (*Pusa hispida hispida*). The ringed seal is originating from the arctic and they colonised the Baltic Sea when the ice from the late glacial period melted (Halkka *et al.* 2017). It was a favourable habitat because of the cyclic ice for breeding and even better when the polar bears disappeared. As the climate became warmer the population moved north and became isolated from the arctic population which led to the forming of the subspecies *botnica*.

The Baltic ringed seals feed on many different species of fish but mainly three spined stickleback (*Gasterosteus aculeatus aculeatus*) (74%), herring (*Clupea harengus)* (14%) and vendace (*Coregonus albula*) (3%) (Scharff-Olsen *et al.* 2019). In the stomach of ringed seals from the Bothnian Bay over 18 different species of fish were identified.

The ringed seal has strict seasonal cycles (Härkönen & Lunneryd 1992). The timing of these cycles varies with latitude and cannot be expected to be exactly similar in the different populations globally. For Baltic ringed seals there is a period from November to April when the seals are found in the ice-covered areas in the north of the Baltic. The females build lairs on the ice (Härkönen *et al.* 2008) where the parturition, lactation, weaning and mating takes place (Härkönen & Lunneryd 1992). In the end of April/beginning of May, the seals have a basking period and are moulting (shedding of fur and skin) while being hauled out on the ice. In late May, they begin to leave the areas with shallow water to start their foraging period. During June and July they are located in deeper waters (>20m) (Härkönen *et al.* 2008), foraging to replenish fat and energy for the winter (Mclaren 1958; Young & Ferguson 2013). In September and October, the seals will once again move toward the coastline (Härkönen *et al.* 2008).

2.4 Ringed seal reproduction

The ringed seal reproduction is dependent on ice to be successful (Mclaren 1958). They have adapted to relatively stable ice conditions and have historically survived on permanent ice or land-fast winter ice but are also using pack ice (ice not attached to the shoreline) (Kelly *et al.* 2010). With their claws, they dig breathing holes and the females dig subnivean lairs from underneath the ice where the pup is born and nursed for five to seven weeks in the early spring (Mclaren 1958; Smith 1991; Halkka *et al.* 2017). According to studies, conducted in the Canadian and Norwegian arctic, the female often has multiple lairs and breathing holes in proximity as a complex to protect the pup (Smith 1991; Lydersen 1995). The pups in the Baltic are born from the end of February to the beginning of March (Härkönen & Lunneryd 1992). The mortality of the pups increases with the absence of sheltering snow and stable ice conditions (Halkka *et al.* 2017). The lactation period in the arctic is long (44 days) compared to other seal species that give birth on the pack ice as well as compared to grey seals (17 days) (Smith *et al.* 1991). Preferably, the lactation should be carried out in the subnivean lair (Halkka *et al.* 2017) because it supports the pup by regulating temperature and protect it from predators (Smith *et al.* 1991). To be able to breed and moult, the ringed seals need stable ice for two to three months (Halkka *et al.* 2017). By the end of February, when the parturition period starts, the lairs need to be ready and stable ice is crucial for their construction. The ice also needs to be stable throughout the lactation period, since the break-up of ice can affect growth and condition of the seal pups. There are cases of emergency parturition on land in 2008 in the Gulf of Riga and in the Archipelago Sea, but it was unclear if the pups survived (Halkka *et al.* 2017). Successful experiments with artificial lairs and man-made snowdrift have been carried out in Lake Saimaa, in Finland, where there is a small population of ringed seals (Autilla *et al.* 2017). More studies need to be made to evaluate if that is an option to help the southern populations in the Baltic Sea, where the lack of ice is more extensive. (Halkka *et al.* 2017)

Studies of Canadian populations suggest that the female ringed seal feed beneath the ice to actively supplement her energy reserves during lactation (Hamill *et al.* 1987 see Smith *et al.* 1991). With the lairs as protection, it enables her to leave the pup for feeding (Smith *et al.* 1991). The pups are small at birth (4,5-5kg) compared to most seal species. During the lactation period the pup grows to approximately 20kg. The ringed seal pups in Svalbard are more active than those of other seal species during the lactation period and spend 50% of their time in the water (Lydersen 1995). This is probably an adaption from being heavily hunted by predators and helps the female to be able to move the pup to protect it. After the lactation period the females have lost 27% of their body weight, which is significantly less than that of the grey seal (38%) that does not hunt as often during lactation (Smith 1991; Kauhala *et al.* 2019). Ringed seal pregnancy rate declines earlier in life than for grey seal (Kauhala *et al.* 2019). For ringed seals pregnancy rates decline after 12 years of age and for grey seal it is not until the age of 25 that reproductive rates decline.

The sexual maturation of ringed seal females is defined as the time of the first ovulation by the presence of a corpus luteum and the maturation of males when sperm production has started (Mclaren 1958; Smith 1970). The male ringed seals were sexually mature at the age of six to seven in Canadian populations during the middle of the 20th century (Mclaren 1958; Smith 1970). The females matured earlier at the age of 4-5 during the same time period (Smith 1970). However, this varied between the studied areas and Smith (1970) suggested that the sexual maturation could depend on the geographic properties of the area and the hunting pressure. A light hunting pressure and an environment that enables stable ice that is ideal for breeding lairs, give an earlier onset of female sexual maturation. According to later research in Canada done by Quakenbush and coworkers (2011), sexual maturity of female ringed seals was lowered between the years 1999 and 2010 to 3.2 years. The definition used by them is when half of the females captured have ovulated. The Baltic population has, in a study performed on samples from 1981 to 2017, shown relatively early sexual maturation, with females showing signs of ovulation as early as 3 years old (Kauhala *et al.* 2018) (Half of the 10 studied 3-year-old female seals). The male sexual maturity for ringed seals in the Baltic Sea is yet to be investigated.

According to Mclaren (1958), the female ovulates before the end of the lactation of the previous pup. This was shown by females having a new corpus lutea (as well as a corpus albicans in the opposite ovary) when examined during lactation.

Determining the mating period is difficult when studying wild animals where the mating act is not easily observed. Mclaren (1958) estimated the mating period by determining the time of ovulation and the timing of presence of sperm in the testis and epididymis during necropsy. This way, the mating was estimated to occur in mid-April in the Canadian populations (Mclaren 1958). There are no studies compiling these parameters for Baltic populations and it may not be possible to extra-polate the data to Baltic populations. However, if the timing were to be compared it would mean that, due to the earlier whelping season in the Baltic, the males there should show signs of mating activity in February to April. This would also mean that the mating act would occur in the middle to end of March. Kelly and coworkers (2010) write about the mating in May and June in the Canadian population 1990 to 2006. This would extend the interval by one month.

The implantation is delayed by 91 to 107 days (3-3.5 months) and the gestation length is therefore difficult to monitor but lasts for 213 to 243 days (7-8 months) according to Reeves (1998), 240 days according to McLaren (1958) and 270 days according to Smith (1970). Smith (1970) also reported a delayed implantation of 81 days. In a review by Lydersen, C. (1995), female ringed seals in Svalbard, Norway, had a delayed implantation of 89 days, a 241-day period of gestation and on average 39 days of lactation. His review also used data from the Canadian population and extrapolated them to the Svalbard population.

To ensure favourable conditions for pregnancy, delayed implantations could act as a gate, with unsuccessful implantation if the female is not able to gain enough weight during the foraging period (Boyd *et al.* 1999). Studies on harp seals (*Pagophilus groenlandicus*) have shown that years without a pup are evident when the female's fat stores are too low, meaning that the seals did not show signs of pregnancy that year (Kjellqvist *et al.* 1995). Prey quality affects the body condition of the ringed seals in the Baltic (Kauhala *et al.* 2019) and could therefore potentially affect the reproduction indirectly.

The ringed seal females are still suffering from uterine occlusions but the rate of these is declining (Kauhala *et al.* 2019). In samples of adult females, signs of uterine occlusions were around 48% before 1997, around 23% in 1997-2006 and around 6% in 2007-2017.

2.5 Seal teeth composition and tooth structure

The ringed seal teeth and jaws are adapted for gripping fish and swallow it hole to the most extent. There are only small differences between premolar and molar teeth, collectively called post-canines (Stewart *et al.* 1998). The dental formula for the ringed seal is I (incisors) 3/2, C (canine) 1/1, PC (post canines) 5/5 (Figure 1).

Figure 1. The lower jaw of a ringed seal with arrows pointing out incisors, canines and post canines. Photo by SMNH.

Ringed seals are born with permanent teeth erupting at the time of birth or shortly after (Stewart *et al.* 1998). The permanent teeth are 54% erupted as newborns (early April, Canada) and fully erupted at approximately two months of age (Late May, Canada).

The tooth structure of the permanent tooth comprises of a root in the tooth alveolar socket of the jawbone with a crown above the gum (McCann 1993). The most abundant material in the tooth is dentine, which is covered on the crown end with enamel and on the root end with one or more layers of cementum. There is a primary dentine, which is present at birth, and a secondary dentine, which deposits after birth. The secondary dentine is deposited into a hollow centre of the tooth called the pulp cavity. The pulp cavity fills up during the life of the animal until it first closes at the apical end and fills up internally. For ringed seal the cementum will grow around the apical end for approximately the third GLG (Stewart *et al.* 1996). The cementum layers grow outward to fill the gap between the expanding tooth socket and the tooth, which means that the most recent cementum is deposited on the outermost layer (McCann 1993). To the contrary, in the dentine, the most recent layer is the innermost layer.

2.6 The cementum and its micro composition

The dental cementum attaches the tooth to the periodontal ligament (Berkovitz & Shellis 2018). It consists of connective tissue that grows in concentric bands around the root of the tooth throughout the lifetime of an individual (Lieberman 1994). Together with the alveolar bone, the gingiva and the periodontal ligament, the cementum creates the periodontium which is the supporting tissue of the tooth (Berkovitz & Shellis, 2018) (Figure 2).

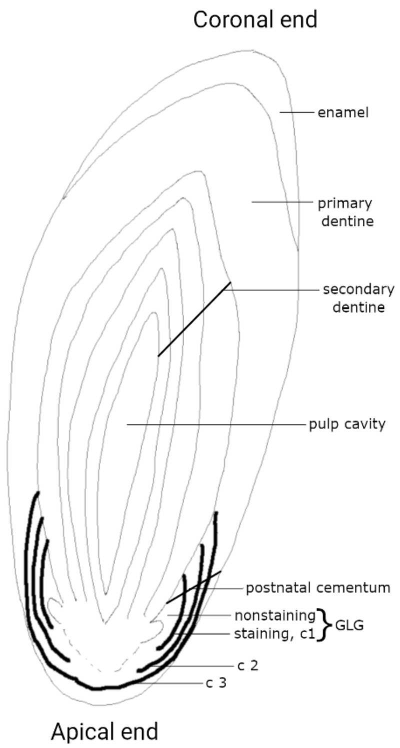


Figure 2. Schematic diagram of ringed seal canine tooth, longitudinal section (cementum annuli emphasized with thicker lines).

Drawn by Emma Grundell with inspiration from Steward et al. 1996.

Most of the studies of the microstructure of cementum has been done on human teeth but there is little evidence of major significant differences between mammalian species (Berkovitz & Shellis 2018). The centum is similar to bone structure, with a mineral component and an organic component (Klevezal 1996; Cooper & Maas 2018; Berkovitz & Shellis 2018), but unlike bone, it is an avascular tissue. The mineral component consists of crystals of mainly an impure form of hydroxyapatite. The organic component consists of 90% collagen fibres with different types of glycoproteins and mucopoly-saccharides which are called ground substance (Klevezal 1996; Berkovitz & Shellis 2018).

There is a cellular type of cementum and an acellular type. Both shows layered formation, but the cellular type also contains cementocytes located in lacunae. The cementum is formed by active cells called cementoblasts that are located on the outer surface of the cementum and as they are producing cementum, they are embedded in the tissue they create and differentiate to inactive cementocytes. The proportional distribution of acellular and cellular cementum along the root varies between species (Naji & Rendu 2022). The cementum of ringed seals is a combination of the human distribution with cellular cementum confined to the apical region of the tooth and the walrus distribution with a completely cellular cementum (Stewart *et al.* 1996).

The collagen fibres also consist of two types of fibres; intrinsic fibres and extrinsic fibres (Sharpey's fibres). The extrinsic fibres originate in the periodontal ligament and is formed by fibroblasts. The intrinsic fibres are produced by the cementoblasts on the margin of the periodontal ligament (Liebermann 1994; Cooper & Maas 2018). The extrinsic fibres are larger than the intrinsic ones (Cooper & Maas 2018). The cementum is rarely remodelled or resorbed and considered inert after synthesis (Liebermann 1994). It becomes a longitudinal record of factors affecting cementum growth because it grows from a single mineralizing front.

The cementum has a striped appearance when sectioned (Figure 3). The layers form parallel to the developing surface (Cooper & Maas 2018). These bands occur in both cellular and acellular cementum, with both intrinsic and extrinsic fibres. There is a broad translucent band and then a narrower opaque band when looking at cementum in transmitted light or stained thin slices (McCann 1993; Klevezal 1996). The two bands (one opaque and one translucent) represent one year's growth of cementum and is called a growth layer group ("GLG"). They are also referred to as incremental (the narrow dark, opaque band) lines or tooth annuli. There are two main factors resulting in seasonal bands of cementum (Liebermann 1994). The variation in relative mineralization and the variation in the orientation of the collagen fibres. The translucent bands are made under periods of more rapid cementum growth (Liebermann 1994). The opaque bands are from slower deposition of cementum during reduced tissue growth and contain more ground substance and less collagen.

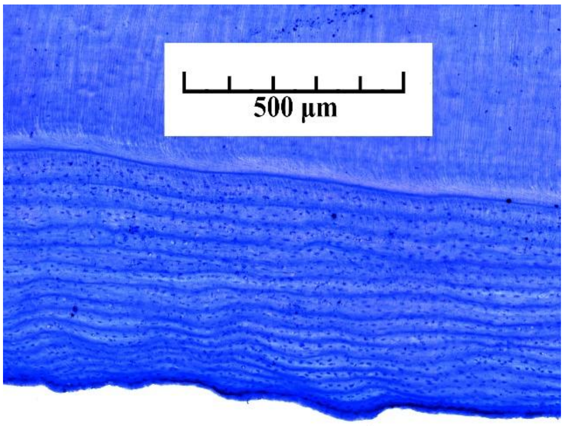


Figure 3. Striped appearance of tooth cementum.

The mineralization properties of different bands of cementum are debated between studies and through the years (Liebermann 1994; Klevezal 1996; Cool *et al.* 2002; Naji & Rendu 2022). Smith (1994) investigated cementum growth layers of American Black bears (*Ursus americanus*) and saw no difference in mineral content between the layers. They drew the conclusion that it is the collagen structure that is the difference between opaque and translucent cementum bands. However, according to Naji & Rendu (2022) the more translucent bands are periods of higher mineralization, and the darker bands correspond to less mineralized and more densely packed tissue.

The cellular, acellular, extrinsic, intrinsic and level of mineralization of cementum can combine in different ways in different species and regions of the tooth cementum. More studies need to be done on the ringed seal tooth specifically to examine the exact composition of cellularity, fibre composition and mineralization.

2.7 Age determination in marine mammals

Age determination is a highly important part of determining age specific aspects of a species life history, biology and ecology. For some species of wild animals, the age determination basis is challenging because of lack of reference animals with a known age (Stewart *et al.* 1998). In those cases, the age determination is made in so called recording structures (Klevezal 1996) which includes mineralized tissue such as bone, dentine and the cementum of teeth.

The first recording structure used in determining age in ringed seals was their claws, originally implemented by the Inuit people (Mclaren 1958). Mclaren (1958) determined that this is not a completely reliable way to age determine because claws of older ringed seals being worn down, making early bands disappear. Mclaren (1958) compared the claw method to that of using dentine. In 1996, Stewart and coworkers instead compared a dentine ageing model with one using cementum of canine teeth. They concluded that using the cementum of decalcified, stained, longitudinal sections was the best method for estimating ringed seal age compared to unstained, cross sectioned dentine calculations.

In young of the year individuals, growth layer deposits in the dentine were faster than those of cementum. In addition, cementum layers are not evident in June samples but in samples collected in the fall (Stewart *et al.* 1996). A neonatal line can be seen in the dentine in all young of the year collected in mid-April (which align with ca 2-week-old pups in Canada). This creates a lag between the cementum and dentine, which will make the most recent annulus apparent in the dentine before it is seen in cementum. This could make a difference in counting of growth layer groups, especially in spring collected seals (Stewart *et al.* 1998).

It is difficult but necessary to correctly identify GLGs to a specific year and age in order to obtain information of life-history events (Klevezal 1996; Medill *et al.* 2009). The difficulties can be due to damage during the processing of the tooth, indistinct GLGs (incremental lines), crowding of lines that decrease the age estimates and accessory lines that may cause an overestimation in age. Differences in identifying and counting GLGs and incremental lines may occur both between readers and for the same reader between reading sessions (Stewart *et al.* 1996).

2.8 Cementum width and life events

The continuous and longitudinal growth of cementum gives a promising feature for determining and timing life events and possibly reproduction events in seals (Lieberman 1994; Klevezal 1996). This is due to the cementum's persistence to change and its life-long period of registration as a recording structure (Klevezal 1996). The annual GLG pattern give a possibility of timing the changes in the tissue. The cementum structures can also be preserved in fossils.

As a first step, to investigate if cementum can be used to predict reproductive events, the sexual dimorphism could be investigated. There have been studies done on the sexual dimorphism of cementum on some species (Naji & Rendu 2022). For example, Stewart *et al.* 1996 determined a difference in the difficulty of reading and counting cementum increments/annuli of females and males in ringed seals, where males were easier to read. The writer discussed this as a possible effect of pregnancy.

There is vague indication in the literature for an impact on cementum width from female hormonal changes from possible pregnancy and lactation (Stewart *et al.* 1996; Klevezal *et al.* 1996; Medill *et al.* 2010; Tochigi *et al.* 2018). Both Medill and coworkers (2010) and Tochigi and coworkers (2018) describe a correlation between breeding and a narrowing of GLGs in cementum for bears. This is discussed mainly as an effect from increased nutritional demand but is most likely a more complex relationship.

Klevezal and coworkers (1996) describe the GLGs being less well defined, more irregular and broader before sexual maturation in some seal species. They have no evidence for an individual turning sexually mature that year but explain sexual maturation as a probable cause of the narrowing and more defined GLGs formed during later years. Söderberg (1978) also used this approach to determine sexual maturity for ringed seal in the Baltic Sea in the first half of the 20^{th} century. He based his study on a study on grey seals by Hewer (1964). The rapid decline in width and more regular cementum corresponding to older years were also shown to correlate with age at first reproduction in northern sea otters (*Enhydra lutris*) (Von Biela *et al.* 2008).

The width of GLGs in females compared to males has been studied in polar bears (*Ursus maritimus*) by Medill and coworkers (2010). This research team also created a mathematical model for handling differences in tooth size and growth pattern – Proportional Width Index (PWI) and evaluated the possible sources of error in a four-way ANOVA. Medill and coworkers (2010) created a predictive model for pregnancy and lactation in female polar bears, successfully predicting known pregnancy with a 72% accuracy. A significantly reduced width of the GLG was the largest indication of pregnancy. They used females with known life history and investigated premolar teeth from 220 adult female polar bears. Although they also showed similar cementum reduction in males (40.6%) making the model not yet strong enough to determine sex in a population. No such study has been done on ringed seals and conducting one is difficult because of the lack of known pregnancy state or lactation to evaluate a predictive model (observed pups).

Tochigi and coworkers (2018) performed a study of Asian black bears (*Ursus thibetanus*) where they used the same mathematical model from Medill and co-workers (2009) to perform a LMM (linear mixed model) with PWI as a response variable and presence and absence of cubs as explanatory variables. Their analysis showed a significant negative relationship between PWI and cub presence, meaning that the GLGs were narrower when the female had cubs.

Additional incremental lines have in some studies shown a correlation with reproductive events in American black bears (Coy & Garshelis 1992 see Klevezal 1996). They showed that double incremental lines in the cementum correlated with the records of cub presence in radio-collared female bears. However, this is contradicted by other studies (Klevezal 1996).

3. Materials and methods

The seals selected for this study originated from hunting in the Bothnian bay region of the Baltic Sea (Bäcklin *et al.* 2024) (Figure 4). The hunters collect samples, including the lower jaw, during the fall (Sep-Jan) and during the spring (May-July) and send them to the SMNH for bounty. The teeth are then collected and stored at the SMNH. The SMNH hold a permit issued by the Swedish Environmental Protection Agency to perform research on collected material according to the Hunting regulation (SFS 1987:905).

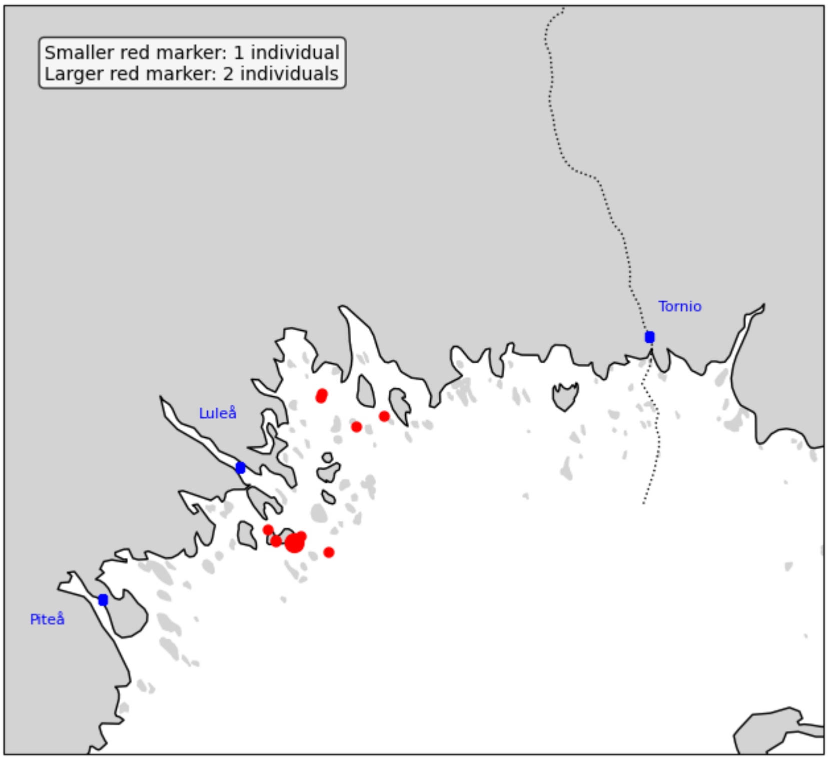


Figure 4. Northern part of Bothnian bay, Baltic Sea. Hunting locations of selected individuals (red dots). Map data © OpenStreetMap contributors – License: www.openstreetmap.org/copyright

3.1 Selection of individuals

Twelve sexually mature seals (over seven years of age) that were shot between 2019 and 2021 were selected. Females with and without signs of pregnancy during the current or previous reproductive cycle were selected. The aim was to find differences in GLG width due to reproductive status, in the most recently formed cementum (most recent year). Signs of previous pregnancy were evaluated by the presence of a placenta scar and a corpus albicans and current pregnancy status was determined by the presence of a corpus luteum or the presence of a foetus (See Table 2). Because of the long preimplantation period, current pregnancy status was stated as unknown for several individuals, but the females were presumably pregnant if they had a corpus luteum. This is because the embryo is too small to be macroscopically located at this point of the reproductive cycle. In the selection, all females died either in 2019, 2020 or 2021. There were three males selected. One died in 2019, another died in 2020 and the third in 2021.

One of the females was excluded because the stained teeth sections did not include the area preferred for measuring the GLG width. Due to time constraints, it was not possible to replace this individual.

3.2 Preparation of tooth samples and age determination

Prior to this study, the teeth were separated from the jaw by heating the jaw to 70°C in a 900L container of water for five-hour cycles, one period at a time until the teeth were separatable from the jaw and cleaned. Approximately two cycles were needed for each jaw.

The canines were sectioned in half on the distal to mesial mid-longitudinal plane when possible, using a Beuhler IsoMet Low Speed Precision Cutter with a diamond wafering blade. Due to different shapes of the teeth, the exact plane of the cut was not specifically noted. One half was decalcified in 25-50 ml RDO (rapid decalcifier) overnight for approximately 12-16 h (Apex Engineering Products Corporation, Illinois, USA) and wafered into 15-20 µm thick sections in a SLEE MTC benchtop cryostat. The slices were stained in Toluidine blue with a concentration of 0.3% (mixed in distilled water) and were put on microscope slides with the help of a brush. After mounting on the microscope slides, the sections were dried, covered in Pertex and secured with a cover glass. The slides were then left to cure for a minimum of 2-3 weeks.

The age determination was performed by two experienced readers, by counting GLGs, before the start of this study. In this study, the identification and outlining of GLGs was done with assistance of one experienced reader.

3.3 Photographing cementum

Samples were photographed at 10x magnification using a INFINITY3-6UR 6.0 Megapixel USB 3 Microscopy camera (Telefyne lumenera, USA) mounted to a transmitted light microscope (Ybotech RX50LED, China) and using the software Surveyor version 9.0.4.5 (Objective Imaging United, United Kingdom). Images were obtained from where the cementum GLGs were most clearly visible at the distal or mesial aspect of the root. The software GNU Image Manipulation Program (GIMP) version 2.10.22 was used for cropping and annotating images.

3.4 Measuring width of GLGs

Examination and measuring of GLGs in the images were done in ImageJ (National Institutes of Health, Bethesda, MD, USA). Measuring the GLGs on the apical end was avoided because there are less cementum annuli present (the root closes at three years old). The measurements should neither be done too close to the coronal end, nor the apical end (Stewart *et al.* 1996). Too close to either end will give an underestimate of the number of GLGs. See Figure 2 and 5.

Eight measurements of each GLG were done, in three photographs of different cross-sections of the same tooth at the distal or mesial aspect. The measurements and incremental lines were orthogonal. This resulted in 24 measurements of each GLG per individual.

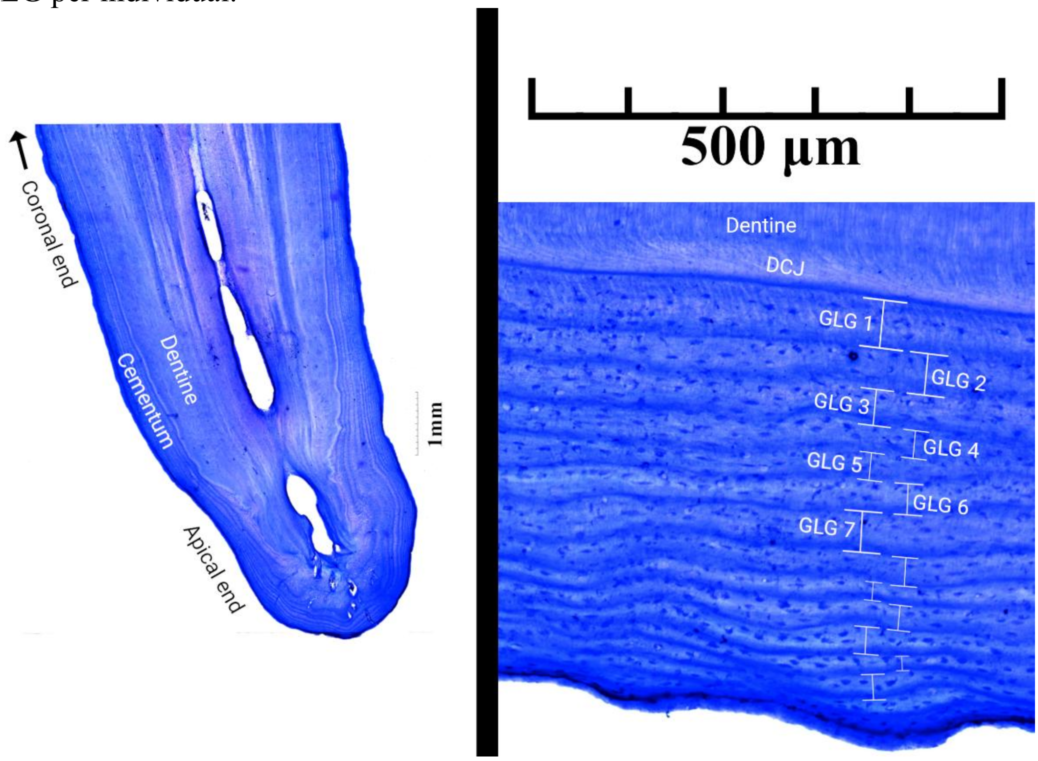


Figure 5. To the left: Longitudinal stained section of a ringed seal canine tooth root. To the right: Measurement locations of GLGs in cementum (dentine-cementum junction – DCJ).

3.5 Data preparation

3.5.1 Calculating proportional width (PW) and proportional width index (PWI)

By using proportional GLG width (PW) instead of actual widths, it is possible to account for possible size difference between teeth by calculating a proportion of the width (Medill *et al.* 2009). It normalises the size difference between individuals making them comparable. This is done by dividing the actual width of the GLG with the sum of all previous GLG widths according to Equation 1 and 2 where $x = width\ (\mu m)$ and $i = specific\ GLG$. The second equation accounts for the first GLG which does not have a previous one to depend on.

$$PW_i = \frac{x_i}{\sum_1^i x_i} \qquad \textit{Equation 1}$$

$$PW_1 = \frac{x_1}{x_1 + x_2} \qquad \textit{Equation 2}$$

In several species, it has been found that there is a declining trend in cementum deposition with age, leading to GLGs narrowing with age (Klevezal 1996; Stewart *et al.* 1996; Medill *et al.* 2009). In order to see other patterns in cementum, this trend needs to be normalized. After normalization, the expected narrowing trend is removed and possible fluctuations in GLG width due to other factors can be investigated. This also facilitates the use of statistical methods on the measurements. Medill and coworkers (2009) developed a normalization method using indices, called proportional width index (PWI) to account for the general difference in width at various ages and removing the trend. PWI is calculated by dividing the PW values to an age specific and sex specific mean (described by $\overline{PW}$ in equation 3). By log transforming the data, skewness is removed. This is done according to equation 3.

$$PWI_i = log_{10}(\frac{PW_i}{\overline{PW_i}} + 1) \qquad \textit{Equation 3}$$

3.5.2 Exclusion and classification

There were two technical issues which created artifacts. The first was a folding on the edge of the cementum sections on the microscope slide (Figure 5). The second was a difficulty with determining where the GLG stopped, and the periodontal ligament started. Due to these artifacts, accurately measuring the last GLG closest to the periodontal ligament was impossible. The last PWI (GLG measurement) of each individual was therefore removed from statistical analysis but kept for the raw data and PWI visualisations (Figure 6-7, 8-9 respectively). Eleven PWI values were discarded due to this. The remaining PWI that were used for statistical analysis were classified into juvenile and adult PWI by assuming sexual maturity at six years of age (juvenile <6 years old, GLG number five and below, adult >5 years old, GLG number six and above). It is possible for females to have a pup before the age of 6, but this rather high threshold was chosen to make sure that only PWI values from sexually mature years were included in the adult category. Of the 113 PWI values used for statistical analysis, 26 PWI from male teeth were classified as being formed during the seals' adult years and 15 as juvenile years. For females, the corresponding numbers were 32 PWI as adult years and 40 PWI as juvenile years.

3.6 Statistical analysis

Histograms are a helpful tool to visualise frequency distribution, spread and range of values as well as data skewness. To visualize the frequency distribution, sex and maturity specific histograms were plotted using 10 bins between 0.18 and 0.44 with seaborn and matplotlib (Waskom 2021; The Matplotlib Development Team 2024).

For further analysis of the distribution, Kernel density estimation plots were used to estimate the smooth distribution pattern comparing juvenile PWI and adult PWI in both males and females. Scott's bandwidth method and a Gaussian kernel were used as parameters with a grid size of 200. Plotted with seaborn and matplotlib (Waskom 2021; The Matplotlib Development Team 2024).

Kernel density estimation (KDE) plots visualise a smoothed distribution and range, as well as skewness (Chen 2017). KDE plots excel when showing multi-modality. Multimodality is when more than one peak occurs, suggesting distribution sub-groups within the data. They function as if they add a smooth function (a kernel) around each data point and then sum value of the functions, leading to the plot. For comparison, imagine if you laid out each data point on a line and poured an equally small amount of sand on every point. That would give you a density estimation plot using the sand as a kernel.

A Hartigan's dip test (Hartigan & Hartigan 1985) was performed to investigate if multimodality occurred in the PWI values from adult years. This was done on males and females separately (using package diptest (Maechler 2024) in program R).

To investigate sex differences in PWI, a linear mixed model was used with PWI as dependent factor and individual as a random factor to control for multiple individual samples (using package lme4 [Bates *et al.* 2015] in program R). Sex differences in PWI was tested both on the entire lifespan and separately on adult PWI values, using the same kind of linear mixed model.

Differences in variation of PWI were tested using a non-parametric Levene's test (using package car (Fox & Weisberg 2019) in program R), by comparing PWI during juvenile years and adult years for both males and females.

4. Results

4.1 Study population

Individual parameters describing the selected seals are shown in Table 1 and reproductive data based on necropsy findings can be found in Table 2.

Table 1. Selected individuals.

Individual	Year of death	Month of death	Age	Sex	Body length cm
A2019/05391	2019	6	8	Female	122
A2020/05511	2019	6	14	Female	140
B2022/00620	2019	5	9	Female	135
A2020/05393	2019	9	13	Male	130
A2021/05260	2020	5	9	Female	110
A2021/05271	2020	9	10	Female	129
A2021/05288	2020	5	10	Female	125
A2021/05270	2020	10	14	Male	145
B2022/00084	2021	5	9	Female	135
B2022/00083	2021	5	11	Female	135
A2022/00009	2021	9	14	Male	152

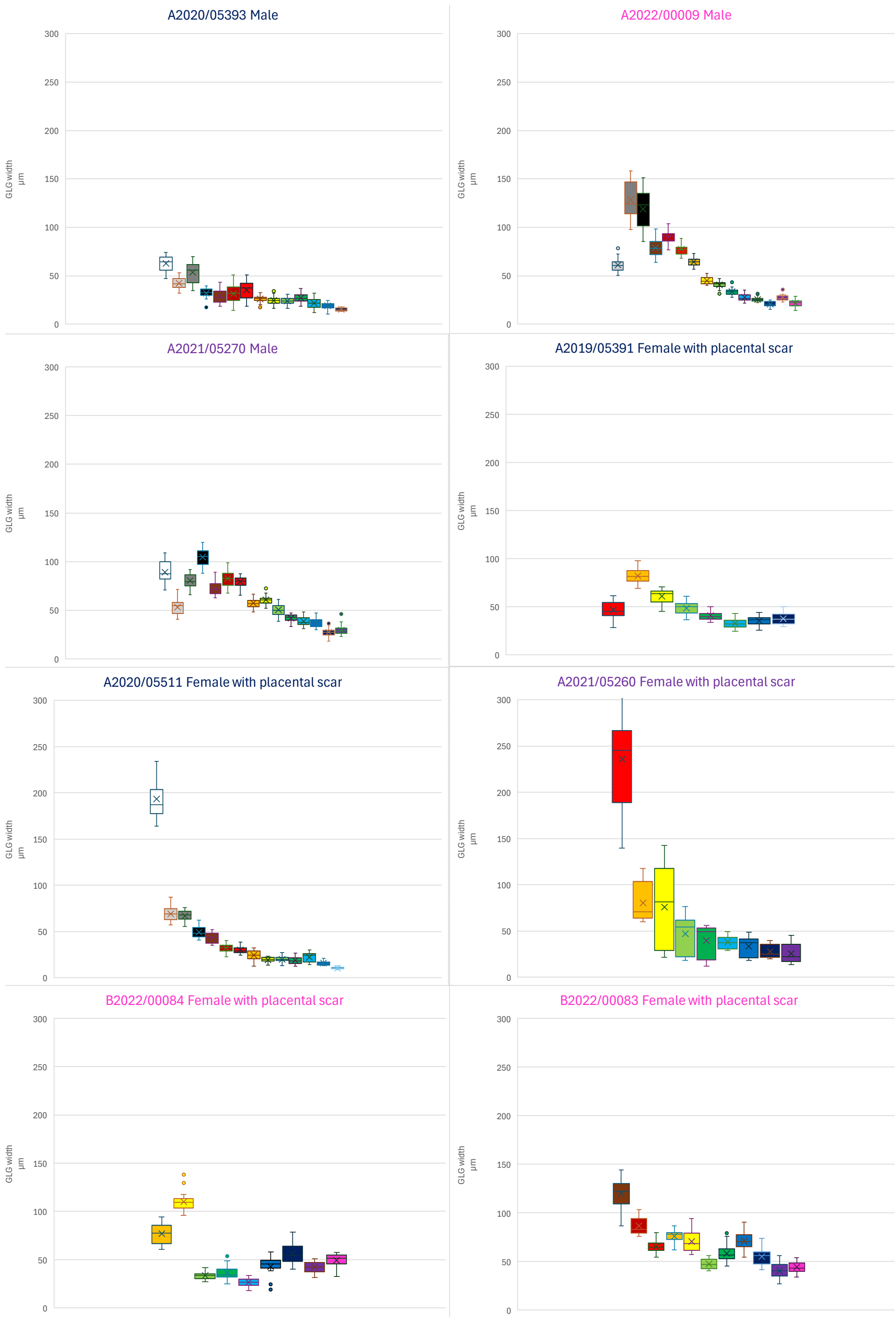
Table 2. Pregnancy and postpartum signs of selected females.

Individual	Placenta scar	Corpus albicans	Corpus luteum	Current pregnancy status	Signs of previous pregnancy
A2019/05391	Yes	Yes	Yes	Unknown[1]	Yes
A2020/05511	Yes	Yes	m.d.[2]	Unknown[1]	Yes
B2022/00620	No	No	Yes	Unknown[1]	No
A2021/05260	Yes	Yes	No	Not pregnant	Yes
A2021/05271	No	No	Yes	Pregnant	No
A2021/05288	No	No	Yes	Unknown[1]	No
B2022/00084	Yes	Yes	Yes	Unknown[1]	Yes
B2022/00083	Yes	Yes	Yes	Unknown[1]	Yes

[1] Presumably early in pregnancy, in the ringed seals preimplantation period.
[2] Missing data

4.2 Variation in GLG width

The GLG widths from juvenile years have a generally larger variation as seen in the box plots in Figure 6. When further looking at Figure 6, it seems that most adult females had larger variations in the GLG width than adult males (larger boxes and whiskers in the box plots above five years). One female had more variation in the measurements of GLG width than the other seals, as indicated by the generally larger box sizes in Figure 6 (A2021/05260).



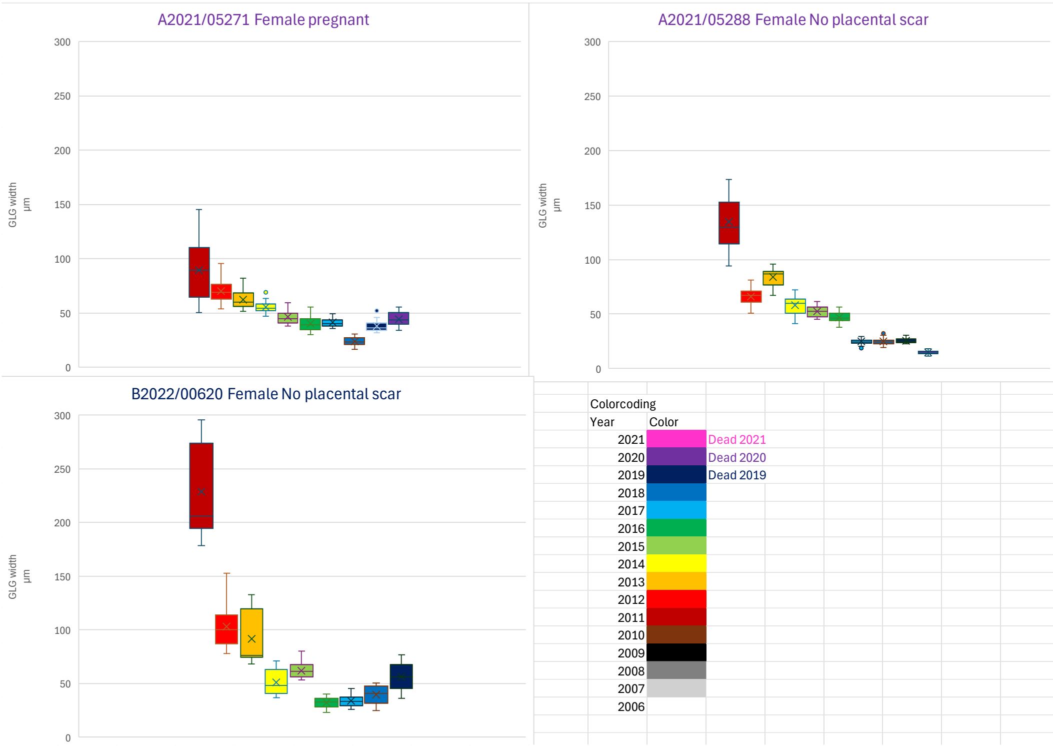


Figure 6. Box plots of GLG widths by GLG number, per individual. Box=first to third quartile, x=mean, line in box=median, whiskers=minimum value and maximum value but no values outside of 1.5x interquartile range from box. Outliers are outside of 1.5x interquartile range from box and are plotted as dots.

4.3 Relationship between GLG width and GLG number

The GLG width shows a distinct visual pattern (Figure 7) of less growth the older the seal gets. This tendency is apparent for both females and males.

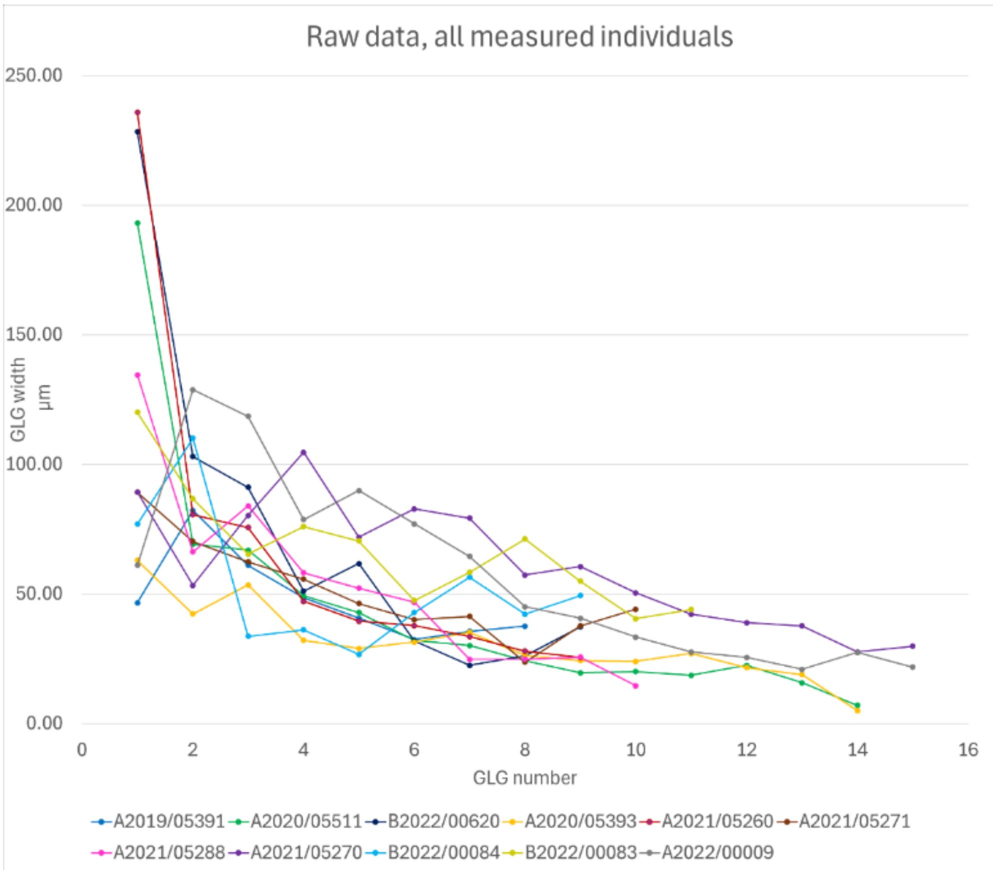


Figure 7. Mean of GLG width measurements plotted against GLG number (age) per individual

4.4 Normalizing the trend by using Proportional width index (PWI)

The GLG widths were normalised into PWI to account for the general decrease in width at higher ages and thus removing the age-trend seen in Figure 7. Indeed, when plotting the PWI values, the trend is no longer evident (Figure 8).

PWI values for GLG 2 was greater than for GLG 1 for some individuals and for others it was the other way around, creating an X-shape in the left-hand side of Figure 8.

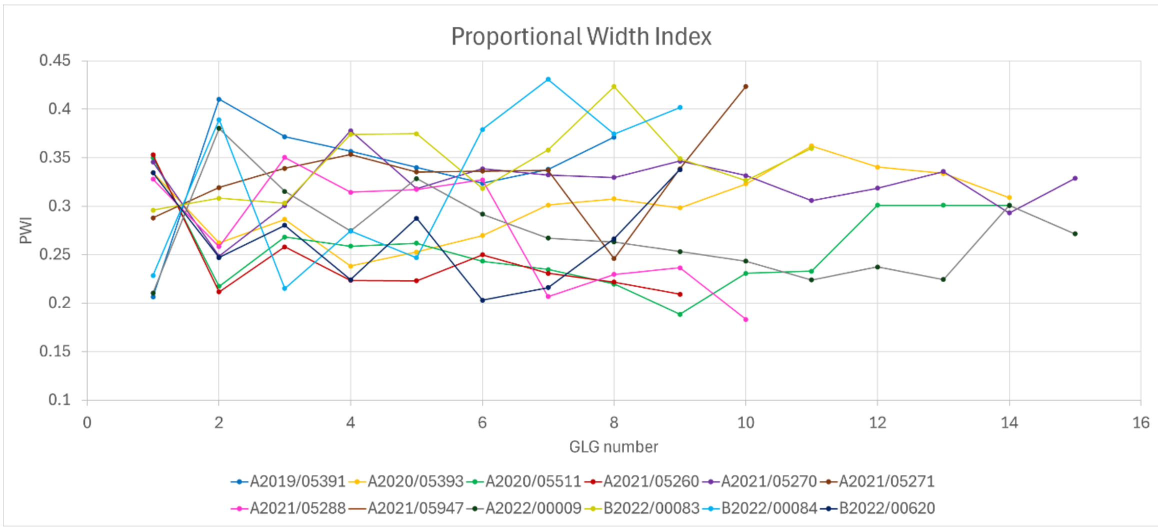


Figure 8. PWI value per GLG number (age) for all individuals

When plotting the relationship between year and PWI value there were no evident recurring trends (Figure 9). However, it appears that between 2006 and 2008, all PWI values first decreased and then increased. Note that this pattern seems to be common in seals born other years as well, and can also be seen in Figure 8, where half of the individuals showing a decreased PWI between the first and second year. Except for 2006-2008 there is no other year where all individuals show the same pattern (Figure 9).

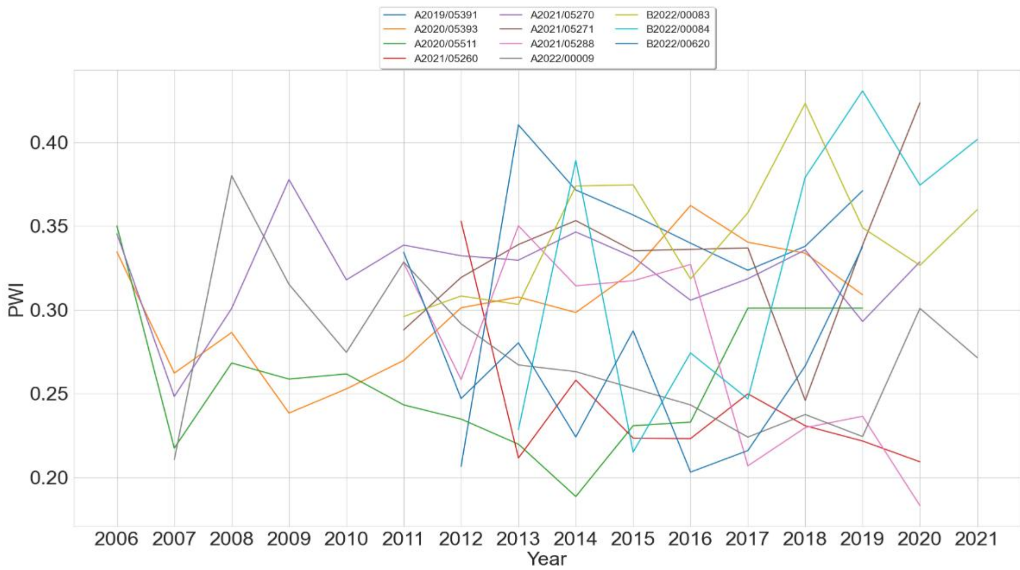


Figure 9. PWI values per year for all individuals.

4.5 Proportional width index - Distribution, variance and sex-/maturity effects

When investigating the distribution of the PWI values, a more uniform distribution can be seen for PWI values from juvenile years compared to adult years (Figure 10). The female distribution of adult PWI values (orange, histogram to the right) indicates two distinct subgroups of PWI values around 0.2-0.25 and 0.30-0.36, respectively (two peaks). This is not seen for the male PWI values or the PWI values corresponding to juvenile years. The grouping of female PWI values from adult years around 0.30 aligns with the adult PWI values of the male seals. In addition, this grouping is supported by the KDE plots in Figure 11. As confirmation of this visual finding, the Hartigan's dip test revealed a significant difference between the two subgroups of female PWI values from adult years and thus indicated the female distribution was multimodal (D=0.09, p=0.049), while the male distribution was unimodal (D=0.06, p=0.606).

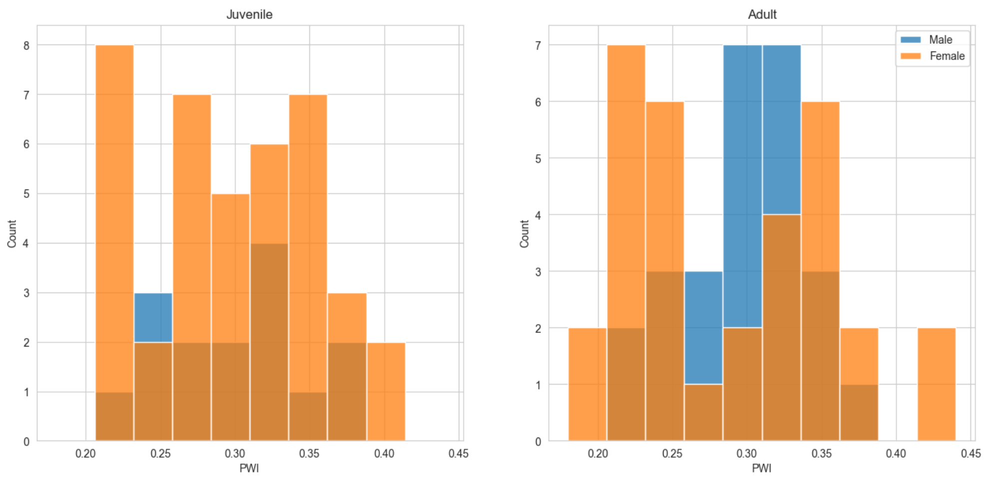


Figure 10. Histograms for juveniles and adults. Blue=male, orange=female.

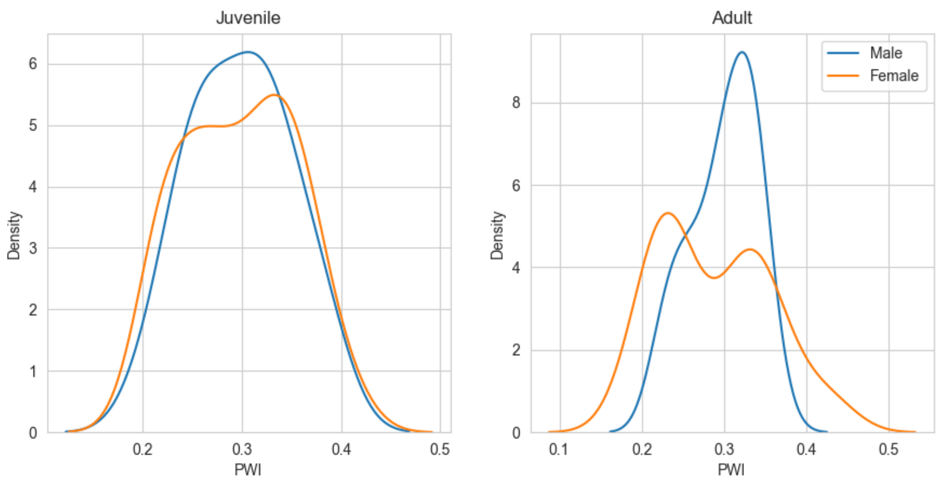


Figure 11. Kernel density estimation for juveniles and adults. Blue=male, orange=female.

The linear mixed model showed no overall statistical difference in PWI values between males and females (*p*=0.107). In addition, no statistical difference was found when comparing male and female PWI values formed during adult years (p=0.282).

By using a non-parametric Levene's test, no difference in variance of the PWI between the sexes was found for PWI values corresponding to juvenile years (*p*=0.964). However, females had a significantly larger variance than males during adult years (*p*<0.001). Mean, standard deviation and variance of PWI can be found in Table 3.

Table 3. Descriptive statistics of proportional GLG width index (PWI).

Sex	Maturity	Mean	Std. dev	Variance
Female	Juvenile	0.2828	0.05616	0.003154
Male	Juvenile	0.3337	0.06292	0.003959
Female	Adult	0.2762	0.07115	0.005062
Male	Adult	0.3237	0.04544	0.002065

5. Discussion

The main findings from this study were: (1) The female ringed seals had a bimodal distribution of their PWI values (GLG widths) formed during their adult years and (2) female PWI values from adulthood had a larger variance than male PWI values from adulthood.

5.1 Study population

Due to visibility issues with the outermost GLG (the most recently formed layer of cementum), the plan to compare GLG widths corresponding to a known pregnancy status had to be abandoned. Instead, the PWI values were analysed for differences between sexes and sexual maturity. If the original idea had been to compare males and females, it would have been of value to prioritize an equal number of individuals of both sexes in the study groups.

5.2 Variation in GLG width

During measurement execution, the GLGs of male teeth were easier to outline compared to the GLGs of female teeth. The female GLGs were less distinct, less straight and less smooth compared to the males' GLGs. This led to some difficulties in determining exactly where the female GLGs started and ended. Stewart and coworkers (1996) performed a study where they did not measure the GLG width but counted them for age determination and found that the variation in cementum counts of females were greater than for males between two readers. They also described similar difficulties outlining the female GLGs and speculated that this could be the result of pregnancy and lactation (i.e. hormonal stress).

In this study, the GLGs up to approximately 4 or 6 years of age were generally wider than GLGs formed during adult years (as seen in Figure 6). This is similar to findings in previous research on grey seal (Hewer 1962 see Klevezal 1996), northern sea otters (Von Biela *et al.* 2008) and ringed seal (Söderberg 1978).

The variation in the widths during the repeated measurements per GLG, is partly induced by measurement error and partly due to true variation. The true variation is present because of the cementum biology where the cementum is not equally formed along the length of the tooth (Steward *et al.* 1996). This should be addressed by measuring on the same aspect of the tooth and at the same distance from the apical end to attain less variable values. This source of variation was partly handled, in this thesis, by using a mean value for PWI calculations and by not measuring at the apical end and instead measured on the side of the sections.

Medill and coworkers (2009) evaluated potential error sources when measuring GLGs in polar bears. They concluded that the aspect of where the measurements were performed on the tooth (distal or mesial), contributed the most to variation compared to individual bear, the selected tooth (upper/lower jaw and left right tooth) and the depth of the longitudinal section. Therefore, this is something to prioritize in continuing studies of GLG width in ringed seals.

In this study, there were technical issues with the last measurement, consisting of difficulties in determining were the GLG stopped, and the periodontal ligament started, as well as the cementum folding near the edge of the teeth sections on the microscope slide. According to Stewart and coworkers (1996) the staining creates an "edge-effect" which makes the interface between cementum and periodontal ligament look similar to a GLG. An improvement to distinguish interface between the periodontal ligament and the cementum could be investigated further in future studies. The folding issue have been addressed by personnel at the SMNH with samples now being prepped by coating the microscope slide with 5% gelatine to stop folding of the edges from occurring.

5.3 Relationship between GLG width and GLG number

There is an obvious slope appearance of the plotted GLG widths in Figure 7. This describe a decreased cementum deposition with age and is similar to previous studies on ringed seal (Steward *et al.* 1996) polar bears (Medill *et al.* 2009), brown bears (*Ursus arctos)* (Mundy & Fuller 1976 see Klevezal 1996), grey seals (Hewer 1962 see Klevezal 1996), and walruses (*Odobenus rosmarus*) (Mansfield 1958 see Klevezal 1996; Krylov 1965 see Klevezal 1996).

5.4 Normalizing the trend by using Proportional width index (PWI)

When looking at Figure 8, the X-shape between GLG1 and GLG2 stands out and could potentially be due to the comparatively larger variation in GLG widths seen in GLG1 and GLG2 (Figure 6 boxplots) or due to living conditions in the pups first year affecting cementum growth. Though when looking in Figure 9 it is evident that seals born the same year do not always follow the same pattern for GLG1 and GLG2. It is more likely that pups born the same year would have been exposed to similar conditions from the environment (prey quality/quantity, weather conditions etc.) and thus share the same pattern. This tells us that it is less likely that it is the living conditions giving us the X-shape in Figure 8. There can still be individual factors affecting the living conditions such as how well the mother took care of the pup. This will in turn, affect the first year´s cementum deposition by creating favourable or unfavourable conditions for the pup.

However, the GLG closest to the dentine (GLG 1) proved difficult to measure due to the dentine cementum junction (DCJ) being irregular, creating a large true variation in the measurements which can be seen in the box plots (Figure 6). This tells us that interpreting the X-shape needs to be done with caution.

A disadvantage with using PWI values will be that the PWI value for a GLG width is calculated by using the previous GLG widths. Because the variations in the GLG widths (based on 24 measurements per GLG) are larger in the first GLG numbers (Figure 6, box plots) this will affect the calculation of all the subsequent PWI values, and any errors will therefore propagate.

5.5 Proportional width index - Distribution, variance and sex-/maturity effects

The mathematical model of PWI values was created with polar bears as model animal (Medill *et al.* 2009). In polar bears, one would look for decreased GLG width, indicating the presence of offspring (Medill *et al.* 2010). This is because they have two to three years of interbirth interval, since cubs are weaned after 1.5 to 2.5 years of age. This will create a longer reproduction cycle than ringed seals. Studies on American and Asian black bears have substantiated the studies of polar bears with similar results showing a decreased GLG width of breeding years (Coy & Garshelis 1992 see Von Biela *et al.* 2008; Carrel 1994; Tochigi *et al.* 2018).

In contrast, the ringed seal has a theoretical capacity to carry one pup per year (HELCOM 2023b) and may thus be expected to be under the influence of pregnancy and lactation every year under optimal conditions. This would result in a smaller GLG as a rule after sexual maturation and a wider GLG during a year without a pup. The absence of a pup by an increased GLG width are searched for instead of decreased GLG width as for bears. This is an opposite pattern (more years with pup) but similar effect (less growth of GLG) of breeding compared to the bears.

In this study, the variance in PWI values corresponding to adult years in female was significantly higher than the variance in PWI values corresponding to adult years in male. In addition, the PWI values of female ringed seals corresponding to adult years were found to form two distinct distributions (subgroups) which was supported by the Hartigan's dip test. Based on the previously described assumption that the seals have narrower GLGs during breeding, the subgroup with lower PWI could possibly correspond to years with a pup and the subgroup, with higher PWI, years without a pup. The rightmost peak in the KDE plot (higher PWI value in adult part of Figure 11) has a lower density, which would suggest that years without a pup are less prevalent than years with a pup. This is supported by the pregnancy rate calculated by HELCOM being more than 50% (82%) between the years of 2016 and 2021 (HELCOM 2023b). The fact that these subgroups exist is a promising feature because it could mean that there is a possibility to distinguish between years (and GLGs formed) with a pup and years without a pup, making further analysis of reproductive rate from teeth cementum possible. Nevertheless, further investigations need to be done, substantiating the assumption that ringed seals have a similar effect (narrower GLGs) on the GLGs as for bears when under breeding conditions. The variation between females and males in the adult PWI group could potentially be explained by pregnancy, hormonal changes and lactation. It goes hand in hand with the idea of two populations of values as indicated by the KDE plots and statistically supported by Hartigan´s dip test.

5.6 Future research

When investigating the literature concerning cementum formation in several species, there are still a lot of questions that needs to be addressed in future research. The factors that influence the GLG formation, the collagen fibre orientation, as well as the mineralization of incremental lines need to be more thoroughly investigated. What part of cementum growth that is genetically controlled vs. environ-mentally or hormonally controlled are other questions that needs to be explored.

Pregnancy and lactation have a lot of associated physiological changes that may affect growth of the GLGs. For example, body condition, nutritional status and hormonal impact. The hormonal regulation changes considerably and may lead to changes in mineralization and growth rate. Naji & Rendu (2022) consider hormonal influences of the cementum, together with other external factors, to be present. They claim the hormones are not controlling the number of GLGs (that they predict are genetically controlled) but the quality (width, mineralization density, collagen fibre orientation). Smid and coworkers (2004) also showed that cellular cementum is highly dependent on growth hormone in mice. In ringed seals breeding and lactation affects foraging behaviour (Smith *et al.* 1991), possibly leading to nutritional status changes which in turn, could lead to changes in cementum growth rate or changes in collagen fibre orientation (Lieberman 1994). These are all interesting aspects for further research.

The exact composition of ringed seal cementum also needs to be more thoroughly studied because the relative proportions of different components vary between species (Cooper & Maas 2018). The exact composition of cellularity, fibre composition and mineralization are not yet explored for ringed seals other than the pattern of cellularity being assessed histologically by Stewart and coworkers

(1996) to be a mixture of walrus and human. The human pattern has the cellular cementum only at the apical end and the walrus has an entirely cellular cementum. Newer, modern, more technically advanced methods like different types of mass spectrometry could give more insight to both mineralization properties, trace elements, and fibre composition (Ristova *et al.* 2022). When these properties are investigated other possibilities of distinguishing between GLGs emerge.

This study uses samples from seals shot during both fall and spring. The deposition of cementum of the latest (current) year will have grown more in the seals that died in the fall, than in the seals that died in the spring. In future studies this is something that needs to be taken into consideration. If the last (most recently formed) GLGs are to be used in future studies, the selection would (1) contain only spring samples, (2) contain only fall samples or (3) be stratified accordingly, thoroughly separating spring samples from fall samples.

In this study the PWI values are compared between individuals and sexes. The PWI values are constructed for this comparison and therefore it is reasonable to use them as such (Medill *et al.* 2009). However, for future evaluation of the PWI values an individual approach could be of value. Using this approach, the individuals are their own control. To investigate possible years without a pup, years with significantly wider PWI values would be searched for. In other words, analysing wider GLGs in relation to other GLGs in the same individual, not in relation to the population. PWI values are then used for normalizing the age-related declining trend in GLG width, but this approach would not require the size normalization also obtained by the PWI calculation. In addition, when studying events during adulthood, the variable GLGs formed during the first juvenile years could be avoided to improve accuracy.

Due to lack of studies of the Baltic ringed seal population (especially regarding reproduction), the literature review is to some extent an extrapolation of Canadian and Norwegian populations. Environmental (latitude, seasonal, geological) and genetic factors may contribute to differences between various populations not yet explored. For example, the sexual maturity timing might differ between populations and through time as described by Smith (1970) and Quakenbush and coworkers (2011). Kauhala and coworkers (2019) described the age of sexual maturity of ringed seal females in the Baltic Sea, however, the same data is still missing for males. Therefore, this study is adapted to the Baltic Sea population by using the female sexual maturity timing for determining the classification boundary (when a PWI value is considered juvenile or adult). The focus was to only have sexually mature females in the adult PWI group. When conducting further studies, the maturity of male ringed seals in the Baltic Sea may also be of value.

5.7 Conclusion

The need to discard the last PWI values hindered the possibility to evaluate different reproductive statuses for females. However, this pilot study found that the PWI values in females have a larger variance and different distribution than in males for PWI values formed during adulthood. The PWI values for cementum formed during the adult years in females formed two subgroups where one may be indicative of pregnancy and lactation and the other subgroup indicative of years without a pup. These findings support the possibility of different PWI values for different reproductive states in females and warrants further investigation of the use of cementum layers in studying the life history of ringed seals. There is still a question if the sensitivity of these measurements is enough for a predictive model. More research needs to be done on preferably a larger sample size and with modification in preparation techniques and measuring of the most recently formed GLG.

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Popular science summary: Can seal teeth tell the story of reproduction?

What can teeth tell us? The cementum is a tissue at the root of the tooth that grow a new layer each year during the individual's entire life. Much like a tree with its growth rings. According to some researchers, cementum can be called a recording structure and can store information about life events. This is because the layers do not change after they are formed so they can capture and store information about that part of time. We wanted to investigate if the width of these growth layers could tell us if a female seal had a pup the year the growth layer was formed. This proved harder than we expected but we still managed to get some hints for the future.

The Baltic ringed seal is a small seal, mostly inhabiting the northern parts of the Baltic Sea. Ringed seals have, together with other Baltic seals, faced horrible consequences of human ignorance. Firstly, we shot almost all seals for bounty and then we polluted the environment and made them ill. For ringed seals the problems do not end here. They are also sensitive to the global warming because they have their pup in lairs on the ice. When the winter ice is disappearing, they might not be able to successfully breed. During winter, when they live out at sea, they are difficult to monitor and study. Therefore, researchers and decision makers need indirect tools to find information about reproductive events and to understand the status of the population.

From a sample of eleven hunted ringed seals, sent to the Swedish museum of Natural History, a canine tooth was removed, sliced and photographed under microscope magnification. The width of the growth layers in the cementum were measured on the photographs, starting from the year when the seal was born (the innermost layer) until the year it died (the outermost layer). Statistical methods were used to compare the widths of the cementum growth layers to find differences between males and females. To study the overall pattern of layers, they were plotted and thoroughly examined. The most interesting layer (the outermost) that we wanted to compare with known pregnancy data, was also the most difficult growth layer to measure and that data had to be discarded.

This study found that females had much more varied widths during their adult years than males. It seemed that they had either layers of similar widths as males or, more commonly somewhat narrower growth layers. The adult males did not have this variation, which indicates that there are one or more unknown factors that affect the width for females in a way that do not affect the widths for male ringed seals. This pattern could be explained by the females giving birth to a pup some years and not others. Therefore, we concluded that future studies are warranted to find the best methods to study how reproductive events affect cementum formation in ringed seals.

So, what can teeth tell us? The teeth have their own language, we only need to learn how to understand it.

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