



Age estimation, growth and age-related mortality of Mediterranean monk seals *Monachus monachus*

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ABSTRACT: Mediterranean monk seals *Monachus monachus* are classified as Critically Endangered on the IUCN Red List, with <600 individuals split into 3 isolated sub-populations, the largest in the eastern Mediterranean Sea. Canine teeth collected during the last 2 decades from 45 dead monk seals inhabiting Greek waters were processed for age estimation. Ages were best estimated by counting growth layer groups (GLGs) in the cementum adjacent to the root tip using unprocessed longitudinal or transverse sections (360 µm thickness) observed under polarized light. Decalcified and stained thin sections (8 to 23 µm) of both cementum and dentine were inferior to unprocessed sections. From analysing patterns of deposition in the cementum of known age-maturity class individuals, one GLG was found to be deposited annually in *M. monachus*. Ages ranged from 0.5 to 36 yr for females, 0.5 to 21 yr for males and 0.5 to 25.5 yr for individuals of unknown sex. The majority of seals (65%) were considered adults (≥4 yr), followed by juveniles (20%, <1 yr) and sub-adults (15%, 1–3.9 yr). Thirty percent of the aged sample had died from human-related causes, such as accidental entanglement in fishing gear and direct killings. A single-Gompertz growth curve was generated for both sexes using standard length data, resulting in asymptotic values of 212.3 cm for females and 221.8 cm for males. This study represents the first quantitative glimpse of sex-specific growth in monk seals and the age structure of dead individuals in this rare species' core range.

KEY WORDS: Mediterranean monk seal · *Monachus monachus* · Teeth · Age estimation · Growth layer groups · Mortality · Endangered species · Conservation

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INTRODUCTION

Monk seals (genus *Monachus*) are phocid seals from the subfamily Monachinae, which were represented until recently by 3 species: the Mediterranean monk seal *Monachus monachus*, the Caribbean monk seal *M. tropicalis* and the Hawaiian monk seal

M. schauinslandi. Centuries of heavy human exploitation in the form of hunting, as well as habitat degradation and disturbance, have significantly impacted all 3 species. The Caribbean monk seal is now Extinct (Kovacs 2008), and the Mediterranean and Hawaiian monk seals are listed as Critically Endangered on the IUCN Red List (Aguilar & Lowry

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2008). The Mediterranean monk seal population is estimated at less than 600 individuals split into 3 isolated sub-populations inhabiting (1) the northeastern Mediterranean in the Aegean and Ionian Seas and the Cilician Basin, (2) Cap Blanc in the Western Sahara, and (3) the Madeira archipelago (Güçlüsoy et al. 2004, CMS/UNEP 2005, Johnson et al. 2006, Karamanlidis et al. 2008, Pires et al. 2008). The largest sub-population of Mediterranean monk seals is estimated at 250 to 350 individuals inhabiting the northeastern Mediterranean Sea, whereas between 100 and 150 seals currently inhabit the Cap Blanc area (Western Sahara-Mauritania) and only 30 to 35 individuals survive in the Madeira Islands (González et al. 2002, Güçlüsoy et al. 2004, Aguilar & Lowry 2008, Karamanlidis et al. 2008, Pires et al. 2008).

Mediterranean monk seals continue to face numerous threats, including human disturbance and habitat degradation (Johnson & Lavigne 1998, 1999), fisheries interactions leading to accidental mortality by entanglement in gear or deliberate killing by fishers (Panou et al. 1993, Güçlüsoy & Savas 2003, Güçlüsoy 2008, Karamanlidis et al. 2008), exposure to pollution such as organochlorine pesticides and PCBs (Borrell et al. 1997, 2007), and mortality from disease or naturally occurring biotoxins (Osterhaus et al. 1998, Harwood 1998, Hernández et al. 1998, van de Bildt et al. 1999). In 1991, MOM/Hellenic Society for the Study and Protection of the Monk Seal established the Rescue and Information Network (RINT). One of the aims of this network is to respond to stranded, injured or dead monk seals, sample carcasses and undertake necropsies. In this way, MOM has investigated the causes of seal morbidity and mortality throughout Greece for over 20 yr, and collected data and samples for population assessments (Androukaki et al. 1999, 2006, Karamanlidis et al. 2008).

Determining natural longevity, age-at-death of individuals and age structure of individuals removed by anthropogenic activities is crucial not only for understanding the dynamics of a population, but also for effectively managing and conserving that population. Counting growth layer groups (GLGs) in teeth is a reliable way to estimate age in many species of animals because they indicate chronological age, similar to calculating the age of a tree by counting the number of growth rings in its trunk (Scheffer 1950, Laws 1952, Scheffer & Myrick 1980, Myrick 1991). This approach has been successfully applied in several species of marine mammals (e.g. Bloch et al. 1993, Stewart et al. 1996, Hohn & Fernandez 1999, Lockyer et al. 2001, Mackey 2004, Murphy & Rogan 2006) because of the moderate sensitivities, long period of

registration and persistence of GLGs in dental tissue (Klevezal 1980). GLGs observed in enamel, dentine and cementum are incremental layers that start accumulating after birth. GLGs in dental hard tissues may be recognised from their cyclic repetition and must involve at least one change—between translucent and opaque, dark and light, more stained and less stained—that can be defined as a countable unit (Klevezal 1980, Perrin & Myrick 1980, Scheffer & Myrick 1980, Myrick 1991). These alterations are due to differences in the content and distribution of the mineral component in hard tissues, resulting in differences in optical density and stainability (Klevezal 1980, Luque et al. 2009).

Incremental deposition rates have been calibrated for numerous marine mammal species in captivity through the use of tetracycline, an antibiotic used as a fluorescent vital marker in teeth as it is incorporated permanently into the mineralizing tissue and can be observed under ultraviolet light (e.g. Gurevich et al. 1980, Myrick et al. 1984, Myrick & Cornell 1990). Alternatively, assessing GLGs in dental tissue of known-age individuals, or in the dental tissue of animals kept in captivity for a defined period of time, can also be used to validate incremental deposition rates. Annual deposition rates of GLGs have been identified in the majority of marine mammals, although it is still uncertain whether 1 or 2 GLGs are deposited annually in certain species, such as the beluga whale *Delphinapterus leucas* (Luque et al. 2007, Lockyer et al. 2007).

The pulp cavity is the central chamber of the tooth containing the pulp, including the root canal, and is surrounded by dentine. The pulp cavity can occlude at varying ages in different species of pinnipeds. Consequently, assessing GLGs in dentine tissue can have limited value in species where occlusion occurs early in life. Most pinniped age estimation studies analyse GLGs in the cementum, which is primarily deposited along the external root surface. As deposition of GLGs in the cementum continues throughout an individual's life, cementum layers are more reliable for assessing age (see Bowen et al. 1983, Arnborn et al. 1992, Stewart et al. 1996, Mackey 2004). Because canine teeth are the largest and relatively easiest to work with, they are the preferred tooth sample for ageing pinnipeds (e.g. Scheffer 1950, Laws 1952, Scheffer & Myrick 1980, Stewart et al. 1996).

Because tooth preparation techniques can introduce biases in age estimations (Hohn & Fernandez 1999), it is vital to develop a reliable species-specific age estimation preparation method. Over the years,

approaches to age estimation in marine mammals have depended on the equipment available and the various techniques and/or procedures already utilized by each specific laboratory, including types of histological stains and storage methodologies. Previous age estimation studies on marine mammals have shown that the size and shape of a tooth will influence whether a longitudinal and transverse unprocessed (undecalcified) section, a longitudinal and transverse processed (decalcified and stained) section, or bisecting a tooth longitudinally through the pulp cavity and polishing or etching is best to read GLGs (Hohn & Lockyer 1995, Mackey 2004). For cetacean teeth, Perrin & Myrick (1980) found that GLGs were best read from decalcified and stained sections of cementum 12–14 μm in thickness or sections of dentine less than 30 μm thick. For pinniped teeth, optimum section thicknesses identified in other studies ranged from 100 to 300 μm for unprocessed cementum sections, 10 to 14 μm for processed cementum sections, and ca. 25 μm for processed dentine sections (Bernt et al. 1996, Stewart et al. 1996, Mackey 2004, Blundell & Pendleton 2008). Optimum longitudinal dentine and cementum sections were obtained from the centre of the tooth, which includes the crown and the maximum area of the pulp cavity in the former and bisecting the root canal in the latter, whereas optimum transverse sections were obtained below the gingival line.

Few studies have been conducted to date on methods of ageing monk seals (Kenyon & Fiscus 1963, Marchessaux 1989). As previous attempts at ageing Mediterranean monk seal teeth have proved difficult (A. Hohn pers. comm.), no calibration study has been undertaken to assess the annual nature of deposition of GLGs. Efforts to age Hawaiian monk seals have been conducted by bisecting a tooth longitudinally and reading the GLGs in the cementum after polishing (Kenyon & Fiscus 1963) or after softening and etching the tissue (J. Henderson pers. comm.), whereas for Mediterranean monk seals, Marchessaux (1989) obtained longitudinal sections measuring 70 μm thick from the center of the tooth and observed these sections under a dissecting microscope. GLGs were read in the dentine and cementum, though further details on the ageing methodology were not provided.

In the present study, we investigated for the first time the age structure of the Mediterranean monk seal sub-population in Greek waters using tooth samples and data collected by MOM. We explored the best method for preparing and reading teeth for age estimation and, using teeth from known age-

maturity class individuals, we identified the annual, GLG deposition rate. We estimated the age and age ranges for 45 individuals, and using these data we produced sub-population level information such as maximum age and growth patterns. We also assessed whether certain age/sex classes are associated with particular mortality events, e.g. incidental capture in fishing gear.

MATERIALS AND METHODS

Canine teeth from 45 necropsied Mediterranean monk seals (26 males, 16 females and 3 individuals of unknown sex) inhabiting Greek waters (Fig. 1) were collected by RINT. Seals died of both natural and anthropogenic causes between 1991 and 2008, though the majority of individuals were sampled after 2000 (74%). Where possible, morphometric data were obtained, including standard body length (SBL) taken using a tape measure held parallel to the animal and measured from the tip of the snout to the tip of the tail. Individuals sampled represented 4 age-maturity classes defined by MOM (adapted from Samaranch & González 2000), based on pelage colour, SBL (SBL data in the sample analysed in the present study) and reproductive status data, i.e. sexually immature and mature: (1) pups/weaners (length range = 106–130 cm, estimated age range = 0–0.5 yr, $n = 5$), (2) weaners/sub-adults (141–152 cm, estimated >0.5–1.49 yr, $n = 5$), (3) sub-adults (149–195 cm, estimated 1.5–3.9 yr, $n = 9$), and (4) adults (196–250 cm, estimated ≥ 4 yr, $n = 26$).

Tooth ageing preparation techniques

Two different tooth preparation techniques were assessed for age estimation of Mediterranean monk seal canine teeth: (1) procurement of unprocessed thick sections (360 μm) for polarized light microscopy, and (2) decalcification and histological processing of thin (5–23 μm) sections for light microscopy. We also investigated whether GLGs should be read in the dentine or cementum by obtaining thick unprocessed (undecalcified) longitudinal and transverse sections, and thin processed (decalcified and stained) longitudinal sections for comparisons.

One canine tooth was extracted from the jaw, cleaned of any soft tissue, and stored dry at MOM's research facility. Teeth samples were sent to the University of St. Andrews in Scotland for processing and age estimation, where they were initially cata-

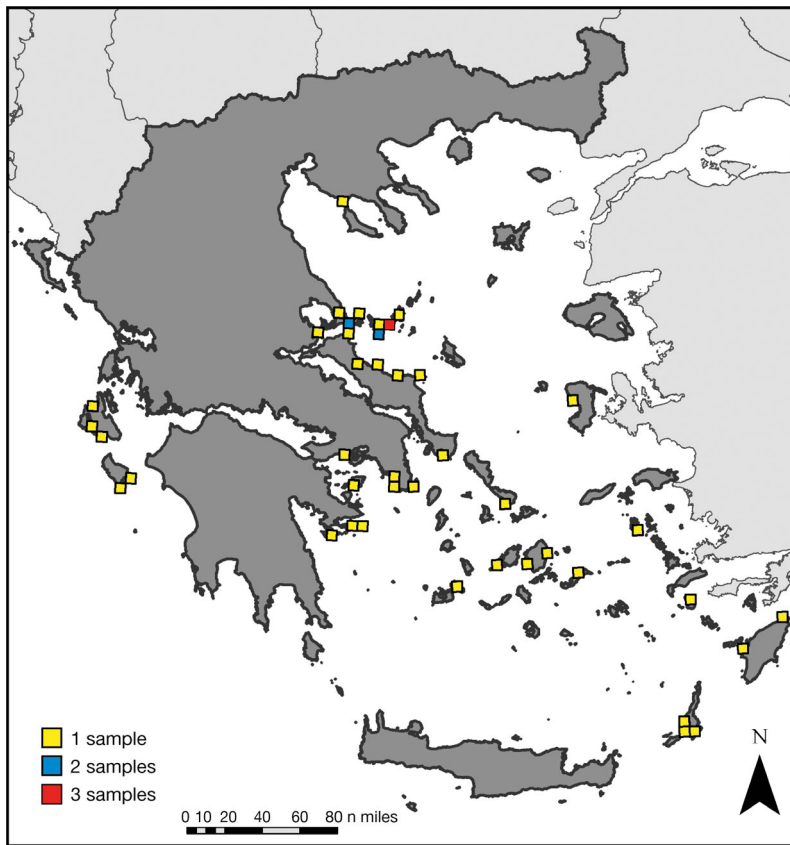


Fig. 1. Map of Greece, indicating the sampling locations of the stranded Mediterranean monk seals whose teeth were assessed in the present study

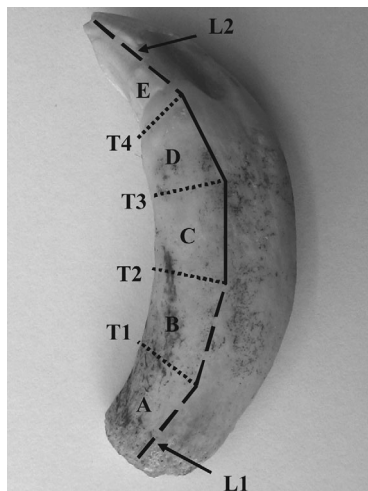


Fig. 2. *Monachus monachus*. Canine tooth from MOm ID no. 98, highlighting samples cut using an isomet saw for reading growth layer groups in undecalcified (L1–L2 and T1–T4) and decalcified and stained sections (using Segments A and E). This animal measured 220 cm in standard body length and the maximum length and width of the tooth were 55.5 and 18 mm, respectively

logued and photographed with identification labels for archival reference using a Canon digital camera. A tooth from one individual (MOm ID no. 68) had already been processed on an earlier occasion, and a thin decalcified haematoxylin stained longitudinal section was provided for ageing.

Canine teeth were quite large, ranging up to 59.5 mm in length and 22.5 mm in width. The method used to section undecalcified canine teeth was based on the protocol for grey seals *Halichoerus grypus*, harbour seals *Phoca vitulina* and ringed seals *Phoca hispida* outlined in Mackey (2004). Teeth were sectioned using a Buehler Isomet low speed saw, which was fitted with 2 Buehler diamond wafering blades (102 cm × 0.3 mm; Series 15 HC Diamond, no. 11-4244). Thick sections of 360 µm were obtained for assessment using polarized light microscopy by cutting both longitudinally through the centre of the tooth (L1 and L2) and transversally (T1, T2, T3 and T4) to the midline axis of the tooth (Fig. 2). The chosen thickness of sections was a compromise between maximising the readability of

the GLGs and maintaining the integrity of the fragile samples (preventing breakage and loss of sample material). Larger samples of the cementum and dentine, labelled A and E respectively (Fig. 2), were also taken from canine teeth for decalcification and histological processing.

Where possible, unprocessed L1 sections (bisecting the root canal/base of tooth) of the cementum were obtained from all individuals (n = 34). However, 10 teeth had partially missing roots because of complications during the tooth extraction procedure, so unprocessed T4 sections and/or decalcified stained sections of the dentine (Sample E) were used to provide an estimate of age. For 1 individual only the thin decalcified haematoxylin stained section from an earlier assessment (mentioned above) was available for ageing.

In addition to L1 sections, all unprocessed transverse sections (T1–T4) were obtained from 2 adults for comparisons, and T1 sections were obtained from a further 8 individuals (one weaner/sub-adult, 2 sub-adults and 5 adults). Samples A and E were also

taken from the canine teeth of 4 adult seals for decalcification and histological processing, along with L1 sections. For 24 seals, only L1 sections were obtained. Care was taken to preserve as much specimen material as possible for future research and reference purposes; therefore, only one half of each canine tooth was processed.

Unprocessed section preparation and reading

Sections L1–L2 and T1–T4 were examined under a Leica-Leitz DMRB light microscope (5×/0.12 or 2.5×/0.07) equipped with a polarized light filter. Sections were initially stored dry and placed on a glass slide with water for age estimation readings. Sections were subsequently permanently mounted on labelled glass slides using DPX resin, and covered with a coverslip, because of the enhanced contrast/clarity of layers within the GLGs achieved by using this procedure and the additional benefit of preserving the specimen material. GLGs were read in both the cementum (L1, T1–T4) and the dentine (L2).

Processed section preparation and reading

Samples A (root) and E (tip), obtained from 4 and 13 seals, respectively, were initially fixed in 10% neutral buffered formalin for 24 h (tissue to formalin ratio 1:10) and then rinsed in running water for at least 20 min. Samples were placed in separate labelled containers with RDO® (Apex Engineering Products), a commercial rapid decalcifying agent, at a tissue to solution ratio of 1:20. A chemical end-point test for the RDO solution prescribed by Apex Engineering was conducted periodically to determine whether decalcification was complete. However, the test was not consistent, as many samples were still rigid even though the test indicated that all the calcium had been removed. In light of this, it was deemed that manually testing the tissue was a better method of assessing whether the dental samples were decalcified. Samples were considered adequately decalcified when they became pliable with a consistency similar to that of rubber. Decalcification times varied depending on the relative size and density of the tooth, ranging from 20 min for small thin samples from young individuals to more than 24 h for samples from adults, i.e. larger, thicker, more dense samples. To ensure that excess RDO solution was removed from the tissue, samples were placed in running water overnight (a minimum of 12 h) and

then transferred to 70% ethanol for short-term storage (Perrin & Myrick 1980, Stewart et al. 1996, Mackey 2004).

Decalcified Samples A and E were halved longitudinally and the inner segment was processed using standard histology techniques, including dehydrating through increasing concentrations of ethanol, embedding tissue in paraffin wax, and sectioning at 8–23 µm using a Leitz microtome equipped with a steel histology knife. The resulting sections were affixed onto glass slides coated in 5% gelatin solution and dried overnight before staining. Initially they were stained with 0.5% toluidine blue, a solution used in earlier studies on harbour (Mackey 2004, Lockyer et al. 2010), grey and ringed seal teeth (Mackey 2004). Sections were then dehydrated, cleared in xylene and permanently mounted with DPX and a coverslip. Sections were observed under a Zeiss AxioStar Plus light microscope (×25, ×100 magnification).

Sections did not stain well using 0.5% toluidine blue and the clarity of the GLGs was poor. Thomas (1977) reported that histological stains can perform differently on mammalian dental tissue depending on the species. In light of this, a series of staining trials were conducted on thin sections from 2 individuals (MOM ID nos. 98 and 156) using the following histological staining solutions: (1) 0.05% toluidine blue, (2) Harris haematoxylin (Sigma cat. no. HHS32), (3) Harris haematoxylin with a pre-stain lithium carbonate modification, (4) 0.1% aqueous cresyl fast violet acetate and (5) 2% aqueous cresyl fast violet acetate. The optimal stain proved to be 2% aqueous cresyl fast violet acetate (duration 30 min).

Trials were also undertaken to cut thin sections of 15–20 µm using a Bright cryostat at –20°C. However, there was a limitation as to the size of specimen which would allow vertical clearance of the microtome knife. As a result, the decalcified segments of the monk seal canine teeth had to be further divided into smaller sub-samples, which were deemed inadequate for accurately ageing individuals because sections from these sub-samples did not include a sufficient portion of the cementum or dentine tissue.

Age estimation and calibration study

All undecalcified and decalcified stained sections were cross-read by 3 individuals with varying degrees of experience, including 2 experts (Readers 1 and 2) and one novice (Reader 3). Readers evaluated the tooth sections 3 times independently and then

compared their assessments to assign a best age estimate or an age range for each animal. As this was a learning exercise for the novice reader, only data obtained by the experts were used for age estimation in this paper. Pearson correlation analysis was used to compare canine-derived age estimates between Readers 1 and 2. Inter-observer agreement was further assessed by determining the Kappa coefficient with quadratic weighting (Cohen 1968). A fourth individual with expertise in processing and ageing cetacean and pinniped teeth reviewed photographic images of sections to estimate age and conduct a quality review. Ages were calculated assuming 15 October as the mean date of birth based on the prevalence of autumn (September to November) births for monk seals in Greek waters (Dendrinos et al. 1999).

Based on knowledge of SBL, pelage colour and patterns, and date of sampling, 13 seals in the sample were assigned either as pups/weaners (estimated age range: 0–0.5 yr), weaners/sub-adults (0.5–1.5 yr), sub-adults 1 (1.5–2.5 yr) and sub-adults 2 (2.5–3.5 yr). Using these individuals of known age-maturity status, the GLG deposition rate was assessed within the cementum. Counts were conducted blind, i.e. without any reference to biological data.

Growth

The estimated ages were used in the Gompertz model to generate sex-specific growth curves and predict length and age at physical maturity. A single-Gompertz growth model was selected as it has been used in several previous investigations of marine mammal growth (Stolen et al. 2002, and references therein). In addition, it was better suited than alternatives such as the double-Gompertz model or the Richards model in which 3 and 1, respectively, additional parameters are estimated (Winship et al. 2001, Murphy et al. 2009) because of the small aged sample for both sexes and the lack of data for females between 2 and 8 yr of age. Consequently, we were unable to identify the secondary growth spurt in females, i.e. the age at intersection, necessary for the production of the double-Gompertz model. SBL data were unavailable for a number of individuals because of the state of the carcass upon examination; therefore, only a subset of aged animals could be used in the analysis. This consequently lowered the sample size for the growth model to 10 females and 18 males. Gompertz growth curves (Laird 1966, Fitzhugh 1976) were produced for both sexes to predict length and age at physical maturity:

$$S = A \exp[-b \exp(-kt)] \quad (1)$$

where S is a measure of size, A is the asymptotic value, b is the constant of integration, k is the growth rate constant and t is the tooth-based age (Fitzhugh 1976, Murphy & Rogan 2006). These 3 parameters, with standard errors, were estimated from the age and length data using nonlinear least-squares methods in SPSS v18.

RESULTS

Deposition of GLGs

In Mediterranean monk seal teeth, deposition of cementum occurs over most of the tooth surface and is thickest in the basal half of the tooth in older individuals. A series of regular GLGs were observed in the cementum of unprocessed sections, consisting of alternating light and dark layers, and when viewed under polarized transmitted light could be seen as: (1) a wide layer of varying density ranging from translucent to intermediate density, followed by (2) a narrow opaque layer. Through analysing the patterns of deposition in the cementum of the 13 individuals identified as weaners, weaners/sub-adults, sub-adults 1 and sub-adults 2, one GLG was found to be deposited annually (Fig. 3). Within this group, all individuals were assigned an age (by analysing GLG deposition) within their estimated age range (based on SBL, pelage colour and patterns, and date of sampling), except for 4 seals. MOM ID no. 138 had a SBL of 169 cm and an estimated age range of 2.5–3.5 yr, though GLGs in the cementum indicated it was only 2.33 yr old. MOM ID no. 183 was estimated to be between 0.5 and 1.5 yr of age, but an assessment of GLGs aged the individual at 1.75 yr. MOM ID nos. 146 and 154 had estimated ages of 0.5 yr, but were classified as ca. 0.75 yr based on GLG development in the cementum.

In general, GLGs in the cementum are broad near the dentine layer, though the first year is compact, and the most recent groups in older individuals are more compressed because of the translucent zone becoming more compacted and the opaque zone appearing proportionally more prominent (Fig. 4). In the oldest aged individual in the sample, a 36 yr old female (MOM ID no. 75), the tip (apical end) of the tooth was worn down and the cementum layer measured 90 mm at its widest.

In the dentine of decalcified and stained thin longitudinal sections of monk seal teeth, the prenatal dentine appears more opaque than the postnatal den-

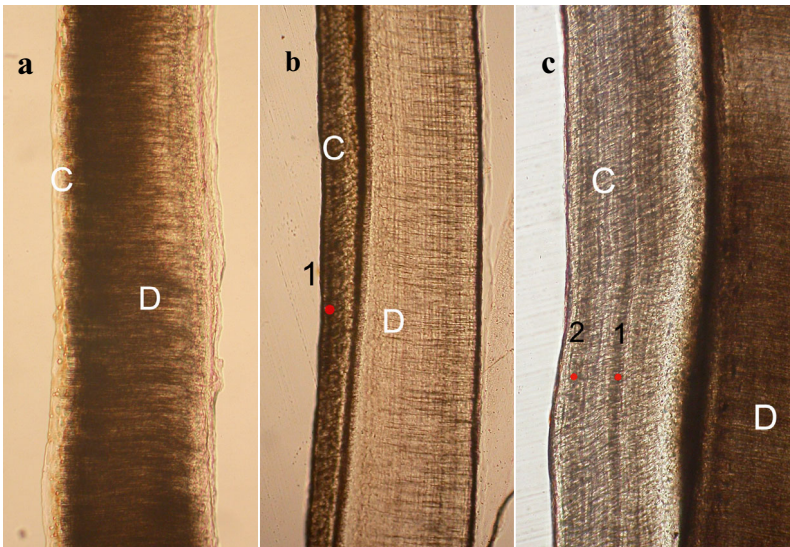


Fig. 3. *Monachus monachus*. Longitudinal L1 sections from (a) MOM ID no. 95 (sexed male, died in March), (b) MOM ID no. 124 (female, died in April) and (c) MOM ID no. 138 (male, died in January) observed under polarized light ($\times 100$ magnification). Red dots and numbers mark the presence of growth layer groups in the cementum, indicating that MOM ID no. 95 is <1 yr old, MOM ID no. 124 is 1 yr old and MOM ID no. 138 is 2 yr old. C: cementum tissue; D: dentine tissue

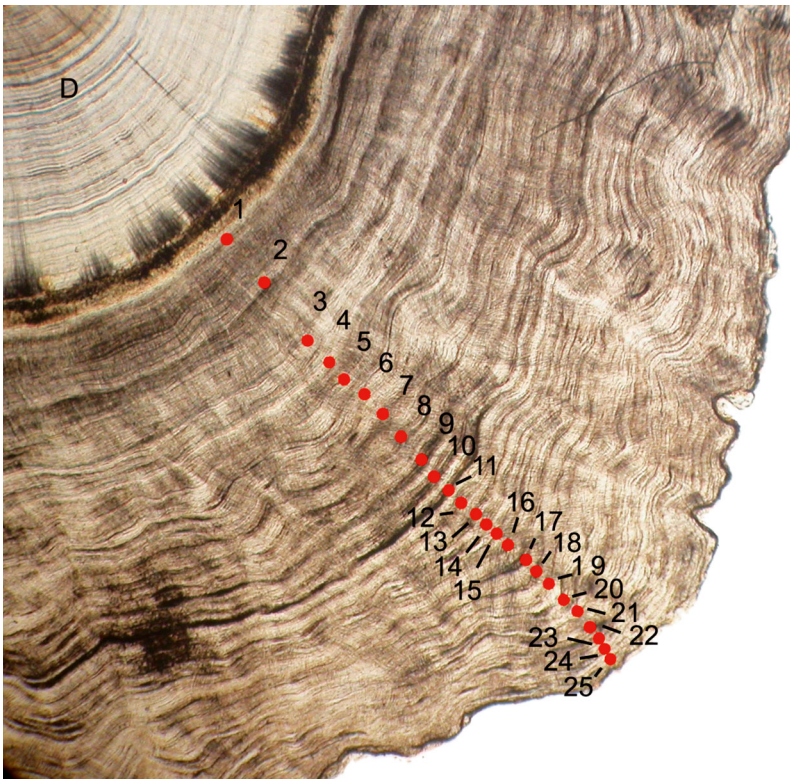


Fig. 4. *Monachus monachus*. Growth layer groups observed in the unprocessed transverse section T1 under polarized light ($\times 25$ magnification) obtained from a 25.5 yr old unsexed individual (MOM ID no. 141). D: dentine tissue

tine. The neonatal line is generally well defined, consisting of a thin translucent layer (Fig. 5). When viewed under a transmitted light, the postnatal dentine GLG consists of a thick layer that narrows with age, followed by a thin translucent layer. The thick layer has lightly layered internal structures and varies between slightly opaque and intermediate optical density.

The canine apical foramen was not fused in individuals ≤ 3 yr of age, and was fully fused in individuals ≥ 5.5 yr of age. Because of the size of the Mediterranean monk seal canine tooth, the pulp cavity was not fully occluded until at least Age 13.

Comparison of age estimations between readers

The 2 experts agreed on age in 43% of readings, and estimated aged differed by only 1 yr in a further 29% of readings and their overall age estimates were highly correlated (Pearson correlation, $r = 0.966$, $p = 0.000$). For monk seals less than 2 years of age, both readers were highly consistent (100%) in their age estimations. Where large inconsistencies existed (differing by >1 yr, $n = 6$ cases), Reader 2 estimated a higher age for individuals in all but one case. The quadratic weighted Kappa value for agreement between these 2 observers was 0.963, indicating almost perfect agreement.

Ageing methodology

For comparisons, unprocessed thick longitudinal sections L1 and L2 and processed (decalcified and stained with 2% cresyl violet) thin longitudinal sections from Samples A and E were obtained from the canine teeth of 4 adults. The decalcification and histological processes were lengthy, with 1 batch of samples taking >30 h to decalcify and several days to conduct the thin sectioning, staining and moun-

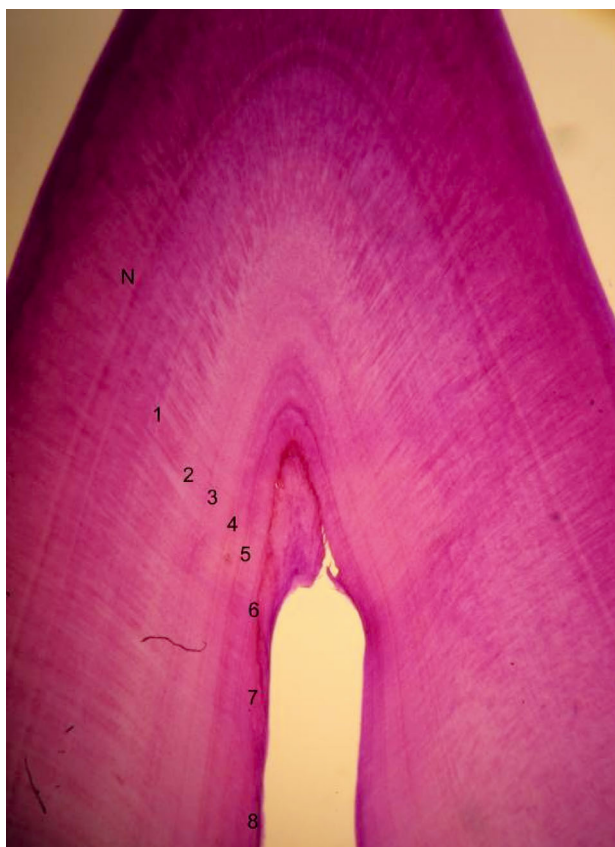


Fig. 5. *Monachus monachus*. Stained decalcified thin section from Sample E ($\times 25$ magnification), highlighting prenatal, neonatal line (N) and postnatal growth in the dentine tissue. This individual (MOm ID no. 153) was aged at >8 yr, as the sample was not fully decalcified and the root was missing due to poor tooth extraction, meaning that the age could only be approximated

ting. For a few samples that were immersed in the rapid decalcifier for an extended period, the tissue was still not fully decalcified because of the nature and density of the tooth; therefore, sections were too poor to estimate GLGs in the dentine and cementum (Table 1). The unprocessed L1 (cementum) thick section was superior to the thin decalcified and stained sections obtained from Samples A (cementum) and E (dentine) for reading GLGs. This was primarily because there was a lack of contrast within the light and dark bands of the GLGs in the stained sections of the cementum, and also the dentine. Unprocessed sections were not appropriate for reading GLGs in the dentine, and therefore age estimates from the L2 section are not presented in Table 1. Consequently, for the tooth samples with partially missing roots, decalcifying and staining Sample E produced an approximate age for individuals from dentine tissue, as long as the pulp cavity was not occluded.

In order to identify the optimum cut or region of the tooth for reading GLGs in the cementum, sections L1 and T1 were obtained from 10 individuals. In all cases, L1 sections provided the best sections for ageing because of the clarity and/or distinctiveness of GLGs. In addition, L1 sections also produced the maximum age estimate, though estimates from T1 were similar in most cases (70 %, Table 2). Longitudinal section L1 and all transverse sections (T1–T4) were obtained from 2 adults (MOm ID nos. 156 and 159) for comparisons. As seen in Table 2, cross counts of GLGs obtained from T2, T3 and T4 were less precise than those obtained from L1 and T1, and on the whole were inferior sections for reading GLGs.

Table 1. *Monachus monachus*. Age estimates from unprocessed L1 sections (cementum) and decalcified and stained sections from Samples A (cementum) and E (dentine) obtained from 4 Mediterranean monk seals. NFD: not fully decalcified

Section	Age (yr)			
	MOm ID no. 98	MOm ID no. 156	MOm ID no. 159	MOm ID no. 185
L1 (unprocessed cementum)	9	8	9	5.5
A (stained cementum)	NFD	7–9, poor section, staining not clear	>6 , poor section, staining not clear	NFD, staining not clear
E (stained dentine)	ca. 9	8, contrast not clear	NFD	>3 , NFD
Final agreed age (yr)	9	8	9	5.5

Age and growth

Forty-five Mediterranean monk seal canine samples were processed and analyzed, resulting in precise age estimates for 35 seals, and more general age ranges for a further 8 individuals. We were unable to determine an age or age range for 2 individuals because of poor tooth extraction (partially missing root of tooth) and preservation. Monk seals from our sample varied in age from 0.5 to 36 yr ($n = 12$), 0.5 to 21 yr ($n = 21$) and 0.5 to 25.5 yr ($n = 2$) for females, males and unsexed individuals, respectively. SBL ranged from 141 to 240 cm for females ($n = 13$) and

Table 2. *Monachus monachus*. Age estimates (yr) from unprocessed cementum sections (L1, T1–T4) read under polarized light. Accessory lines are a mineralization anomaly and are additional distinct layers within a GLG. Pulp stones are discrete nodules, often containing concentric rings or secondary dentine. ND: age estimate not determined

Sample no.	MOM ID no.	Age estimate (yr) from section					Final agreed age
		L1	T1	T2	T3	T4	
1	90	9	8	ND	ND	ND	9
2	98	9	9	ND	ND	ND	9
3	115	6	5, poor section	ND	ND	ND	6, with accessory lines
4	141	25.5	25.5	ND	ND	ND	25.5
5	145	3	3	ND	ND	ND	3
6	156	8	7–9, poor section, pulp stones present	7/8, poor section, pulp stones present	6/7, poor section	>5, poor section, cementum missing in part	8
7	159	9	9	7/8, slight damage to section	ca. 7	ca. 7, poor section, cementum missing in part	9
8	177	21	21	ND	ND	ND	21
9	179	0.75	0.75	ND	ND	ND	0.75
10	185	5.5	>4, poor section	ND	ND	ND	5.5

106 to 250 cm for males ($n = 22$). The majority of seals were classified as adults (65 %, ≥ 4 yr; all aged adults were ≥ 5 yr), followed by juveniles (20 %, < 1 yr of age) and sub-adults (15 %, 1–3.9 yr). There was no significant difference between sexes in mean adult SBL (male = 218.3 cm, $n = 10$; females = 215.9 cm, $n = 7$; $t = -0.30$, $p = 0.771$).

A Gompertz growth curve was generated for both sexes using the available SBL data (Table 3). Asymptotic values were 212.3 cm (SE = 7.7) for females ($b = 0.5$, $k = 0.4$, $n = 10$) and 221.8 cm (SE = 8.6) for males ($b = 0.7$, $k = 0.3$, $n = 18$). Although Mediterranean monk seals reach sexual maturity at approximately 5 yr of age, they continued to grow for a number of years (Fig. 6). Females in our sample reached asymptotic length at ca. 10 yr of age, but males may attain it at a later age (ca. 15 yr). Growth occurred rapidly during the first 6 yr of life for both sexes. Males continued to grow for a longer period of time and attained a larger asymptotic size. Based on the estimated asymptotic values for SBL, a sexual size dimorphism (SSD) ratio (male SBL/female SBL) of 1.04 was determined. However, the small sample sizes, large standard errors and lack of data for females between 2 and 8 yr of age require these results and asymptotic estimates to be interpreted with caution.

Age-related mortality

From the limited sample of 35 animals for which age could be estimated, it can be seen that the major-

Table 3. *Monachus monachus*. Age class and standard body length range (SBL) for 28 Mediterranean monk seals (10 females, 18 males) collected between 1991 and 2008

Age class (yr)	Female		Male	
	SBL (cm)	n	SBL (cm)	n
0–0.9	141–152	2	106–130	5
1–1.9	142–164	3	150.5–159	2
2–2.9			169	1
3–3.9			181.5	1
6–6.9			167	1
8–8.9			198–205	2
9–9.9	240	1	216–250	3
13–13.9			223	1
17–17.9			230	1
21–21.9			206	1
26–26.9	206	1		
29–29.9	203	1		
32–32.9	212	1		
36–36.9	204	1		

ity of females were 0–2 yr of age, with separate clusters between 9–13 yr and 26–36 yr (Fig. 7a). Most males were ≤ 13 yr of age (90%). Using the new age estimates, MOM's defined maturity categories were updated, and individuals which had been assigned an age or age range ($n = 43$) were classified either as pups/weaners ($n = 5$, 0–0.5 yr, SBL = 106–141 cm), weaners/sub-adults ($n = 5$, >0.5–1.49 yr, SBL = 124–164 cm), sub-adults ($n = 5$, 1.5–3.9 yr, SBL = 149–181.5 cm) or adults ($n = 25$, ≥ 4 yr, SBL = 167–250 cm). Three individuals could not be assigned to an age-maturity category due to ambiguities (only large age ranges obtained) in the number of GLGs. The mean

age of adult Mediterranean monk seals declined from 18.9 (n = 9) to 13.4 yr (n = 10) between the 1990s and the 2000s, though this was not significantly different ($t = 1.30$, $p = 0.211$).

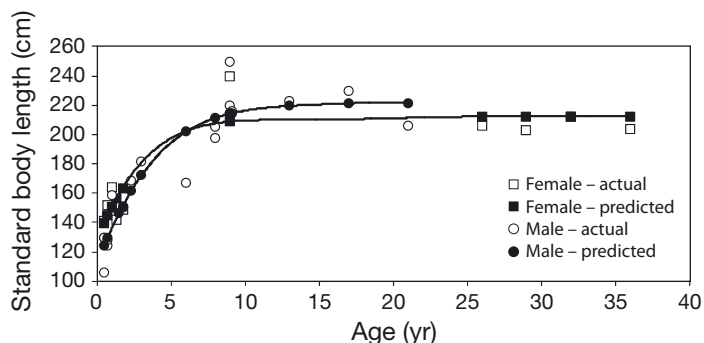


Fig. 6. *Monachus monachus*. Single-Gompertz growth curves superimposed on length-at-age data for female (n = 10) and male (n = 18) Mediterranean monk seals. Asymptotic values were 212.3 cm (SE = 7.7) for females ($b = 0.5$, $k = 0.4$) and 221.8 cm (SE = 8.6) for males ($b = 0.7$, $k = 0.3$)

Age-related mortality in the 43 necropsied seals (with age data) was investigated using 4 'cause-of-death' categories defined by MOm (Karamanlidis et al. 2008; Fig. 7b). Of the 43 individuals, 4 (9%) were reported as 'accidental deaths', i.e. incidentally caught in fishing gear, 9 (21%) as 'deliberately killed', 7 (16%) as 'non-human-induced deaths', and for 23 (54%) seals the cause of death was 'unknown'. The accidental death category contained 3 males classified as weaners and sub-adults, and 1 female of unknown age-maturity status, though based on morphological data this individual was classified as an adult. In contrast, 77% of the 'deliberately killed' sample was comprised of adults (1 unsexed, 2 females and 4 males), as well as 2 sub-adult males. All individuals classified as 'non-human-induced death', i.e. natural causes, were adults (4 females and 2 males), apart from 1 male that could not be assigned to an age-maturity category. The cause of death for the remaining individuals was 'unknown'; of these 43% were ≤ 2 yr old.

DISCUSSION

Research on tissue samples collected from stranded and bycaught marine mammals can complement field studies on live animals and provide important information to evaluate the status of populations and/or sub-populations. To date, there is a lack of information and/or data on the age structure of all 3 Mediterranean monk seal sub-populations (Aguilar & Lowry 2008). Few studies have been conducted on ageing *Monachus monachus* teeth, and previous efforts have proved challenging. In addition, no calibration study has been undertaken to assess the annual nature of the GLG deposition rate. Although the age-maturity classes of Mediterranean monk seals can be indirectly inferred from morphology and pigmentation patterns (e.g. Samaranch & González 2000), directly ageing dead and live monk seals by examining GLGs in teeth can elucidate important age-related characteristics. Apart from increasing the precision of the estimated population age structure, data can be used to identify trends in average longevity and mortality

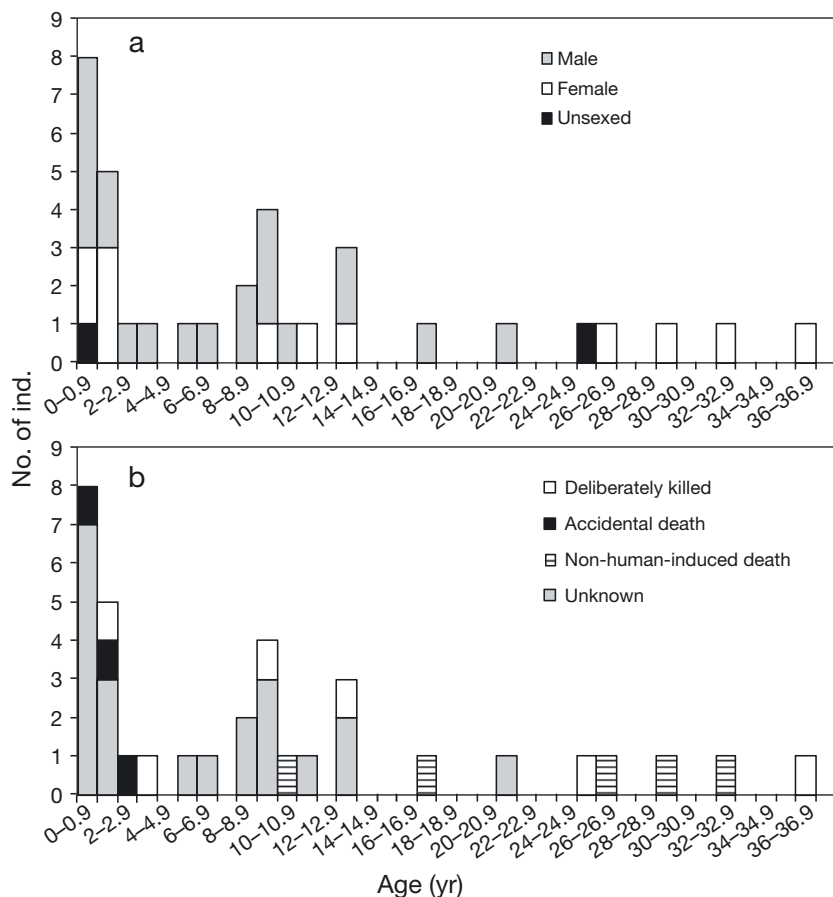


Fig. 7. *Monachus monachus*. Age-frequency distributions (n = 35, 1991–2008) of (a) the whole aged sample and (b) cause of death categories: deliberately killed, accidental (i.e. incidental capture in fishing gear), non-human-induced death (i.e. natural) and unknown. Note: this does not include 8 individuals for which only age ranges were estimated

(Klevezal 1980, Scheffer & Myrick 1980, Myrick 1991). Along with data derived from pregnancy and survivorship rates, this information can be applied to assess the current status of the Critically Endangered *M. monachus* and predict its future population growth rates (see Myrick 1991). Such investigations can provide important insights into the dynamics of the northeastern Mediterranean Sea sub-population, especially as it has been affected by deliberate killing, incidental capture in fishing gear and alterations to habitat, and can be used to develop appropriate conservation and management plans.

Age estimation

Only one GLG is laid down annually within the dentine and cementum in Mediterranean monk seal teeth, which is similar to other pinniped species such as the grey seal (Bernt et al. 1996), harbour seal (Dietz et al. 1991, Blundell & Pendleton 2008) and ringed seal (Stewart et al. 1996). In young individuals (<3 yr, see Fig. 3), GLGs in the cementum were read along the side of the root. The canine apical foramen closed between 3 and 5 yr of age, and following closure the most complete cementum deposition record was found adjacent to the root tip. This was primarily due to the greater concentration of cellular cementum around the root making the annuli broader and clearer, as noted in other pinniped species (e.g. Stewart et al. 1996). Maximum counts were obtained from the L1 section (bisecting root canal), and sections immediately adjacent (T1). The cementum was not distributed evenly along the whole root of the tooth, and sections obtained further from the root tip (e.g. T2–T4, Table 2) provided either lower or less precise age estimates—GLGs were more compact and harder to distinguish due to less cementum deposition compared with those at the root tip—thus making these sections of limited value. Although count estimates from T1 were in most cases on a par with those from L1 (Table 2), longitudinal sections bisecting the tooth were easier to obtain because of the nature and size of the tooth and the occasional difficulty in positioning it within the arm of the isomet saw, relative to the diamond blades. Thus, longitudinal sections were less likely to incur damage during the cutting process, which may affect precision and relative accuracy of age estimates. However, within the L1 section, other incremental or accessory layers were apparent because of the relative thickness of the cementum and the broader GLGs. The degree of contrast between GLGs de-

pended on the individual animal and was not consistent. To avoid any misinterpretations of annual layers, future studies should estimate age in both L1 and T1 cementum sections.

Some authors caution that it is necessary to obtain thin decalcified stained sections of the teeth in order to accurately count GLGs in both the dentine and cementum (e.g. Hohn et al. 1989, Hohn 1990, Stewart et al. 1996, Hohn & Fernandez 1999). Comparisons of unprocessed and processed (decalcified and stained with 2% cresyl violet acetate) sections were undertaken on a sub-sample of teeth only, because of the valuable and limited sample size in the present study. Advantages of the unprocessed technique over decalcification and histological processing were that it was faster, less expensive, did not require a large amount of tissue to be processed, and it has been widely used in numerous pinniped studies to date (e.g. Bowen et al. 1983, Mansfield 1991, Arnbom et al. 1992, Stewart et al. 1996, Mackey 2004). Decalcified and histologically processed sections of GLGs in the cementum (Sample A) and dentine (Sample E) were more difficult to count, i.e. less distinct, than those of unprocessed sections of cementum GLGs. This is primarily because stains did not penetrate well into the tissue, making it difficult to observe the contrasting translucent and opaque zones within the GLGs in both the cementum and the dentine. In addition, because of the nature, density and size of Samples A and E, there were uncertainties with decalcification times.

Although GLG contrast was poor, thin stained sections (from Sample E) allowed for a much better resolution of dentine growth layers compared with unprocessed dentine sections (L2). This may have been due to the thickness of the L2 section (360 µm) and the chemical composition of the GLGs in the dentine. The observation that the pulp cavity occludes with age in Mediterranean monk seals signifies that GLGs in the dentine cannot be reliably used to age older animals. Nevertheless, dentine GLGs may still be useful to age younger animals. The relatively large size of the monk seal teeth also made it challenging to embed samples in paraffin wax and, in some cases, required that partial sections be taken, i.e. full cross-sections of cementum and dentine tissue could not be obtained in older individuals.

Processing and reading the available Mediterranean monk seal teeth proved challenging and, on the whole, monk seal GLGs were not as clear and distinct in either unprocessed or processed sections as those observed in a previous study of ringed and grey seal teeth (Mackey 2004). In addition to contin-

uing investigations on the optimum decalcification and staining techniques, future research efforts should attempt to age monk seal teeth with a scanning electron microscope (SEM), which has been shown to provide the clearest GLGs in harbour seals, where GLGs are often less distinct than in other species of phocids (Mackey 2004). SEMs have also been successfully used to read GLGs in bottlenose dolphins (Hohn 1990).

All of the teeth used to estimate age in the present study were canines. Although canine teeth have been primarily employed for ageing pinnipeds (Scheffer 1950, Laws 1952, Scheffer & Myrick 1980, Stewart et al. 1996), incisors and premolars have also been used in studies of Antarctic fur seals *Arctocephalus gazella*, southern elephant seals *Mirotunga leonina* (Arnbom et al. 1992), grey seals *Halichoerus grypus* (Bernt et al. 1996) and harbour seals *Phoca vitulina* (Blundell & Pendleton 2008). A thorough investigation into the utility of using incisors for ageing monk seals would be worthwhile as there may be some advantages, i.e. they are smaller than canines and therefore easier to decalcify and section, although it has been reported that incisor-based estimates can be less accurate than canine estimates in some seal species (Bernt et al. 1996) compared with others (Blundell & Pendleton 2008).

Drawing population-level inferences

Although the present study was limited by a small sample size, it provides preliminary information on the age structure of Mediterranean monk seals that died in Greek waters between 1991 and 2008. It should be noted though that sampling will have had inherent biases, and the present study did not account for carcasses that were lost at sea, or live animals that may have had adverse interactions with humans and survived. Previous studies have suggested that Mediterranean monk seals may live up to 20–30 yr in the wild (Sergeant et al. 1978, IUCN/UNEP 1988). Marchessaux (1989) aged 23 canine teeth collected largely from the Atlantic (83%, Cap Blanc sub-population) between 1959 and 1986 and reported a maximum age of >20 yr. The maximum age determined in the northeastern Mediterranean sub-population in the present study was 36 yr, although the oldest aged male was only 21 yr. Only 20% of the whole age sample was >14 yr of age, and the oldest individual, a female measuring 204 cm in SBL (MOM ID no. 75), was deliberately killed in August 1993. Within the aged sample, maximum

SBLs were 250 and 240 cm for males and females, respectively. Samaranch & González (2000) reported that male SBL (mean = 251.9 cm, $n = 37$) was significantly larger than that of females (mean = 242.4 cm, $n = 39$) in the Cap Blanc sub-population, unlike the present study. Mean adult SBL for both sexes in Cap Blanc and maximum body size (270 cm for males) is also much larger than that of the northeastern Mediterranean sub-population (mean male adult SBL = 218.3 cm; mean female adult SBL = 215.9 cm). Further study is required to evaluate the differences in SBLs between these 2 sub-populations, and to assess whether it is an issue of sample size.

The growth curves generated by the Gompertz growth model should be seen as providing only a preliminary perspective on growth in Mediterranean monk seals. Asymptotic length was estimated at 212.3 cm for females and 221.8 cm for males. Previous studies on the Cap Blanc sub-population reported possible moderate polygyny or promiscuity within the species and, although they are land breeders, copulations occur in the water and males defend aquatic territories (Marchessaux 1989, Reidman 1990, Samaranch & González 2000). The low SSD ratio (1.04) and lack of secondary sexual characteristics, apart from sexual dimorphism in pelage colour (Samaranch & González 2000), suggest a lack of intensive aggressive interactions between males for access to females. Nevertheless, pronounced sexual dimorphism is ineffectual for seals that copulate in water (Reidman 1990).

In general, mammals exhibit a U-shaped mortality curve, with an initial period of high juvenile mortality, followed by a phase of relatively low mortality and concluding with a rapid increase in senescent mortality (Caughley 1966, Siler 1979, Barlow & Boveng 1991). Owing to the small sample size within the present study, we were unable to fit a mortality curve to these data. However, as can be seen in Fig. 7b, the age profile of dead *Monachus monachus* in our study represents an age-frequency distribution broadly characteristic of a mammalian species, with a peak in mortality of juveniles (<1 yr of age). Within the Cap Blanc sub-population it has been reported that pup survival is low, with less than 50% surviving during their first 2 mo of life (to the onset of their moult) and most mortalities occurring within the first 2 wk (Gazo et al. 2000, Aguilar & Lowry 2008)—storm surges that enter breeding caves pose a significant risk to pup survival (Dendrinos et al. 2007, Pires et al. 2008). Mediterranean monk seal pups are generally weaned at approximately 4 to 5 mo of age (Pastor & Aguilar 2003, Aguilar et al. 2007). In

Hawaiian monk seals, low survivorship of juveniles and sub-adults because of nutritional stress has been identified as one of the main threats impeding the recovery of that species (Baker 2006, NMFS 2007). The aged-sample in the present study showed an under-representation of individuals between 2 and 8 yr of age, which included a lack of females within this age range. This reflects either a sampling bias in the present study, or a true disruption to the normal age and sex distribution in this depleted sub-population. Of the individuals sampled between 2 and 8 yr of age 66% died from unknown causes.

Fisheries-related mortalities pose a serious threat to the Mediterranean monk seal (Panou et al. 1993, Androukaki et al. 1999, 2006, Güçlüsoy & Savas 2003, Güçlüsoy 2008, Karamanlidis et al. 2008). A clearer understanding of the sex and age classes involved in fisheries interactions coupled with an analysis of stranding network efforts may help authorities develop appropriate mitigation plans to reduce future conflicts. Males composed the vast majority of aged individuals (69%) killed accidentally or deliberately by human interactions. Of all aged individuals in the accidental death category 75% were either juveniles or sub-adults and, although this suggests a propensity for young individuals to be captured in fishing gear, this is clearly a small and unrepresentative sample. In addition, this should not be interpreted as suggesting that adult seals are not at risk of adverse human interactions, as they comprised 77% of the 'deliberately killed' sample. Using a much larger sample size of 96 necropsied Mediterranean monk seals sampled by RINT between 1991 and 2007, of which a sub-sample was aged within the present study, the primary cause of mortality for adult seals was deliberate killing (50%), whereas sub-adults primarily died from accidental causes (46%) such as accidental entanglement in fishing gear, and 93% of pups died from natural causes (Karamanlidis et al. 2008). Further, information obtained from a questionnaire on accidental ensnarement of Mediterranean monk seals in this region documented that 92% of individuals observed were sub-adults (Karamanlidis et al. 2008). These results suggest that sub-adult monk seals may be particularly prone to entanglement as they may be less cautious and less experienced than adults when approaching nets, with static gear posing the highest threat (Karamanlidis et al. 2008).

In conclusion, the observed age-frequency distribution within the present study is different to that of a typical mammalian stable age distribution, apart from a peak in mortality of juveniles (<1 yr). This may

reflect sampling biases due to negative human interactions and/or the actual underlying age structure in this severely depleted sub-population. Although a larger sample size coupled with data on age-specific birth rates for females would allow for life table parameters to be calculated along with survivorship curves, the extremely low abundance estimate of the northeastern Mediterranean Sea sub-population of ca. 300 individuals impedes the possibility of undertaking these analyses. Future research incorporating the available samples and data sets from all 3 sub-populations of Mediterranean monk seals across the species' entire range will help identify larger-scale trends, and can help management authorities update and implement various conservation initiatives in place for this Critically Endangered species.

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