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Review Article

Antlers - Evolution, development, structure, composition, and biomechanics of an outstanding type of bone

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ABSTRACT

Antlers are bony appendages of deer that undergo periodic regeneration from the top of permanent outgrowths (the pedicles) of the frontal bones. Of the “less familiar” bone types whose study was advocated by John Currey to gain a better understanding of structure-function relationships of mineralized tissues and organs, antlers were of special interest to him. The present review summarizes our current knowledge about the evolution, development, structure, mineralization, and biomechanics of antlers and how their formation is affected by environmental factors like nutrition. Furthermore, the potential role of antlers as a model in bone biology and several fields of biomedicine as well as their use as a monitoring tool in environmental studies are discussed.

1. Introduction: why study antlers?

Bones of greatly differing types and functions were of interest to John Currey, and he strongly advocated studying the “less familiar” of these bones in order to broaden our insights into structure-function relationships of bones in vertebrates [1]. In a review about the mechanical properties of less familiar bony tissues he stated: “For many readers of this review ‘bones’ are long bones and vertebrae of mammals, often of man, the mouse and the rat. (…) It is probably good if people are reminded occasionally of the existence of other bones, and other mechanical properties” [1 p. 358]. Since his early studies [2], he understood the importance of function to explain mechanical properties and structure of the diversity of bones (and other mineralized tissues). Among the bones that attracted Currey's special attention were antlers, not the least because “functional antlers are peculiar because they are lifeless” [2 p. 414]. Antlers had the lowest mineral content of a rather large array of bones he examined, which was related to having also the greatest toughness or resistance to breaking on impact [3]. However, his studies of the less familiar bones relied on only few samples from each type, and in the case of antlers, on the erroneous assumption that functional antlers are wet structures.

As Currey repeatedly indicated, the mechanical properties of a bone are largely determined by its contents of organic matrix, mineral, and water [3–5]. Regarding antlers, he was especially intrigued by the fact that in his experiments he could not break wet specimens. That was probably one of the reasons why he was interested to perform more detailed studies on the mechanical properties of antlers, which in 2006 led to a cooperation with the research group in Albacete. This group had previously studied the mineral composition of antlers and its variation along the proximo-distal antler axis as well as the relationship of this variation with the mechanical properties of antlers. Many would probably have paid little attention to the findings of a nascent research group, but John was free from such prejudices, having the classical spirit of scientists led only by curiosity. The fruitful cooperation between York and Albacete lasted until John's death and, more than that, led to a deep personal friendship between him and the first author of this paper.

Apart from their mechanical properties, there are several other attributes that make antlers an interesting study subject for bone biologists. Thus, antlers are the fastest growing bones in the animal kingdom, making them a good model for studying bone growth and mineralization processes [6,7]. A particular advantage of antlers for such studies is their easy accessibility and periodic replacement that allows serial studies in the same individual over consecutive years. Furthermore, the

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periodic regrowth of antlers is the only example of complete regeneration of a large bony structure in a mammal [6,8–12]. Antlers are therefore of interest to regenerative medicine in its attempt to find ways of promoting the regeneration of digits or even entire limbs in humans [13–15]. The internal structure, mineral composition, mechanical properties, and histology of antlers are strongly influenced by environmental conditions. Antlers can therefore be used to compare variation in nutrient availability among different habitats [16–20], regional and temporal variation of ambient levels of contaminants with an affinity to mineralized tissues [21], or different factors leading to widespread antler breakage [20,22,23].

The aim of this paper is to review our current knowledge on the evolution, development, structure, mineralization, and biomechanics of antlers.

2. Cranial appendages in ruminants, with special emphasis on antler evolution and function

Among mammals, cranial appendages are most conspicuous and diverse in the Artiodactyla, particularly its most advanced terrestrial suborder Ruminantia [24,25]. Four of the six living ruminant families display “headgear”, known as pedicles/antlers, pronghorns, ossicones, and horns [24]. Pedicles and the antlers growing on top of them are present in the family Cervidae, pronghorns in the Antilocapridae (with the single living species *Antilocapra americana*), ossicones in the Giraffidae, and horns in the Bovidae [24–26]. Cranial appendages are missing in the extant ruminant families Tragulidae and Moschidae. The latter are unified with cervids, antilocaprids, giraffids, and bovids in the monophyletic clade Pecora, and are regarded as most closely related to the Bovidae [24–27]. In “horned” ruminants, headgear either develops only in males (Cervidae except reindeer, okapis), or, if present in both sexes, is larger in males than in females (giraffes, Bovidae, Antilocapridae) [24,25,28,29].

The cranial appendages of extant ruminants are located either entirely on the frontal bones (pedicles plus antlers, horns, pronghorns, ossicones of Okapia) or in the frontoparietal region (ossicones in adult *Giraffa*, some ossicones of older okapis) [24–26,28]. Ossicones are the simplest of these structures, consisting of a bony core covered by hairy skin. While in giraffes the integument permanently covers the entire bony core, the skin is lost from the ossicone tip in older okapis [24,28]. Pronghorns are unbranched projections of the frontal bones permanently covered by hairy skin, whose epidermis produces an annually replaced, forked keratinous sheath [24].

The horns of Bovidae consist of a permanent bony horncore that is permanently skin-covered. Also in bovids a keratinous horn sheath is produced by the epidermis (strictly speaking, the horn sheath is part of the epidermis), but, contrary to antilocaprids, the bovid horn sheath is a permanent structure. The horn sheath increases in size by the production of successive cone-shaped keratin layers. The horncore increases in diameter by periosteal apposition over its entire surface and in length by apical growth [24,26,30]. In bovid species with seasonal horn growth, the periodic production of new keratin layers is reflected by the presence of growth rings in the horn sheath that can be used for age estimation [31].

The cranial appendages of the Cervidae differ from those of other ruminants in that they are composed of a perennial proximal portion, the pedicle, and a deciduous distal one, the antler. Among extant cervids, only the water deer (*Hydropotes inermis*) lacks pedicles and antlers [32], which is regarded a case of secondary loss [24,25,33].

While pedicles are permanently skin-covered, antlers are so only during growth [6]. Antler skin is known as velvet and differs in several aspects from the skin in other head regions. Thus, velvet epidermis is thicker than that of normal scalp skin, the hair follicles in the velvet lack arrector pili muscles, and velvet is rich in large sebaceous glands [34,35]. Moreover, neogenesis of hair follicles occurs in the velvet, whose growth must keep pace with that of the underlying antler bone [36].

The bony core that is present in all types of ruminant headgear can form as a direct outgrowth (apophysis) from the cranium, as in the case of deer [6], or as an independent element (epiphysis) in the dermis of the skin that secondarily fuses to the cranium, as in the case of the giraffid ossicone [28]. Whether the bony core of pronghorns develops as an apophysis or epiphysis is presently unclear. Solounias [37] observed neither a suprafrontal ossification nor evidence for a secondary fusion of an independent bony core to the frontal bone in young *Antilocapra americana*, and therefore suggested that the bony cores develop as apophyses. Despite the great abundance and diversity of living Bovidae and the enormous economic importance of domestic bovids, there are still uncertainties regarding the development of the bovid horncore [24,25,38]. Thus, it is controversial whether during normal development a separate bony element, the so-called os cornu, is present that secondarily fuses to the frontal bone, or whether the bovid horncore develops as a frontal apophysis [38–40]. The situation is complicated by terminological problems, as some authors use the term os cornu also for unossified dermal/subcutaneous or cartilaginous structures.

There exists a longstanding controversy about whether ruminant cranial appendages have a single evolutionary origin, or whether they evolved independently in different lineages [24–26,41,42]. Cranial appendages appeared in several pecoran lineages at the end of the early Miocene and are found already in the earliest bovids, antilocaprids, cervids and giraffids known from the fossil record [25]. This fact and the differences in their morphology and development discussed above has led several authors to suggest that cranial appendages evolved independently in different pecoran lineages [25,26,43]. In contrast, a recent comparative genetic analysis showed that bovid horns and cervid antlers are very similar in their gene expression profiles and further indicated that the headgear of pecorans has a common neural-crest origin [42]. Moreover, these authors found that the gene *RXFP2*, which has a function in headgear formation, was convergently pseudogenized in two pecoran taxa, viz. the Moschidae and the water deer within the cervid lineage. Based on their results, Wang et al. [42] suggest a single evolutionary origin of cranial appendages in the pecoran lineage, followed by two independent losses in the Moschidae and Hydropotinae. According to this view, cranial appendages are an autapomorphy of the taxon Pecora, and the differences in development and morphology of pecoran headgear are the result of later evolutionary events.

Antlers show great interspecific variation in shape and size. Among extant cervid species, they range from the small spike antlers (length of 5 to 9 cm) in the genus *Pudu* to the huge (up to 140 cm long), multipronged antlers of adult wapitis (*Cervus canadensis*) [32]. The largest antlers known are those of the extinct giant deer (*Megaloceros giganteus*), with an antler span of 3.5 m in large stags and a combined antler mass of up to 40–45 kg [32,44,45].

The most apparent difference between deer antlers and bovid horns is that antlers are branched, which seems to have evolved for interlocking, enabling the typical “pushing fights” during the rut [1,2,46,47]. In contrast, horns allow for a wider array of fighting modes. Some species deliver violent bumps with the basal part of their horns [48], while the spiral horns of others could allow interlocking and pushing fights between males [47,49,50].

There exist several theories to explain why antlers are periodically cast and regenerated [6]. Some authors suggest that the (putative) permanently skin-covered antlers of ancestral deer living in temperate zones would have been vulnerable to freezing [51]. Freezing followed by necrosis, and sequestration of the necrotic parts can actually be observed in castrated deer, whose antlers remain permanently velvet-covered [6,52]. Periodic antler casting and regrowth could have evolved as a measure to ensure that complete antlers were available as display organs each year.

The main reason for their annual regeneration is, however, most likely the fact that hard (polished) antlers (i.e., antlers from which the velvet has been shed) are dead bony structures. Although some authors suggest that hard antlers are still living structures [53–55], there is clear evidence that antlers die off at or shortly after velvet shedding due to ischemia [56,57]. Being composed of dead bone, hard antlers are incapable of repairing fractures occurring during aggressive encounters, and periodic antler regeneration would be a means to ensure that a “fresh set” of weaponry is available each rutting season. Moreover, an increase in size and complexity of subsequently grown antlers is only possible if these structures are periodically cast and regenerated.

As is for instance illustrated by the formation of bony sequestra in the course of an osteomyelitis, dead bone is typically separated and detached from living bone of the body. The fact that the (dead) antlers are cast is therefore not surprising. Thus, Gruber [58] characterized antler casting as a process of “abacterial sequestration” of dead bone. It has been reported that the border between the dead antler and the living pedicle does not remain stable if antler casting is prevented by treatment of red deer stags carrying hard antlers with exogenous testosterone or oestrogen [59]. Instead, a “die-back” process down the pedicle and into the skull roof was described that could eventually be fatal. The suggested instability of the hard antler-pedicle junction could therefore be another driver for periodic antler regeneration.

It may, however, still be asked why antlers are not permanently skin-covered and composed of living bone that is continuously repaired, to avoid the physiological costs resulting from discarding and re-establishing up to one third of an individual's skeleton mass every year [60]. There are some points to consider when discussing why this was apparently not an evolutionary option. First, a full and rapid regeneration of lost parts in a complex, branched headgear might be more difficult to realize and control than periodic casting of the whole structure, followed by a re-start of the genetic program to create a completely new set of antlers. Second, a structure with only a thin layer of skin above the bone and no keratin sheath that is used for vigorous fighting would be in frequent need of repair. Third, considering the rich nerve supply and sensitivity of antlers in velvet [6], permanently viable antlers are utterly unsuited as fighting structures. Fourth, species from temperate zones carrying permanently living antlers would not only be at risk of frostbite on these structures during winter but also of exaggerated heat loss, given the high surface to volume ratio of branched antlers.

It has long been assumed that in the evolution of the Cervidae, deciduous antlers were preceded by permanently skin-covered, non-deciduous appendages or appendages in which the still living distal portions were aperiodically sequestered [25,61]. These (facultatively) perennial appendages from the early Miocene have been referred to as protoantlers [62].

The lack of a coronet or burr, a ring-like bony protuberance at the antler base, in protoantlers was regarded as evidence of their non-deciduous nature. This view was based on the assumption that in extant cervids, formation of the coronet is somehow related to the process of antler casting and regrowth. However, as was already discussed by Bubenik [63], the purported dependence of coronet formation on antler casting has never been satisfactorily explained. Moreover, antlers lacking a coronet are occasionally recorded in extant cervid species, and it has been observed that these antlers are replaced [63,64], disproving the alleged obligatory link between casting and coronet formation. A recent morphological study [61] of antlers from fossil and extant cervids, including the early Miocene genera *Procervulus*, *Ligeromeryx* and *Lagomeryx*, observed a similar structure of the basal antler surface, referred to as seal [62] or abscission area [61] in all taxa. In both living and fossil cervids, this surface was porous and rugose, denoting osteoclastic activity in the course of a sequestration process. It was concluded that antlers were cast also in the early Miocene genera and that already the earliest antlers were repeatedly lost and regenerated [61]. There is thus presently no evidence for the existence of perennial protoantlers preceding deciduous “true” antlers during cervid evolution.

3. Developmental biology of antlers

Antlers develop as extensions from the pedicles, which are paired permanent outgrowths of the frontal bones. The pedicles develop adjacent to the frontoparietal suture in the posterior region of a low bony ridge that extends from the supraorbital margin in posteromedial direction [65]. Their growth is triggered in the first year of life by a rise in circulating testosterone levels and can therefore be inhibited by early castration of male deer [6,66]. The formation of the first set of antlers (typically unbranched spikes) occurs as a continuation of pedicle growth and is not a regenerative event. It is with the casting of its first antlers that a deer enters into the annual cycle of antler casting and regrowth [9]. Externally, the transition from pedicle to antler growth during the formation of the primary cranial appendages is visible by a change in the type of integument from pedicle skin to antler velvet [35,65,67]. This change is a specific reaction of the skin to an inductive signal, most likely in the form of diffusible molecules, from a subdermal (periosteal/perichondral) tissue source, and constitutes an example of instructive tissue interaction [65,67,68].

As has been demonstrated by deletion and transplantation experiments, pedicle (and first antler) development is dependent on the frontal periosteum overlying the presumptive pedicle site [69–72]. Removal of this periosteum, which was termed “antlerogenic periosteum” by Goss [6], precludes pedicle and antler growth, while autologous transplantation of antlerogenic periosteum (AP) to other sites of the body causes ectopic pedicle and antler growth (Fig. 1). These ectopic antlers are covered by typical velvet and undergo casting and regeneration, denoting that the AP not just induces “unspecific” bone growth at the transplantation site, but that a specific type of appendage is formed. The results of AP transplantation also demonstrate that the competence for velvet transformation is widespread, although not

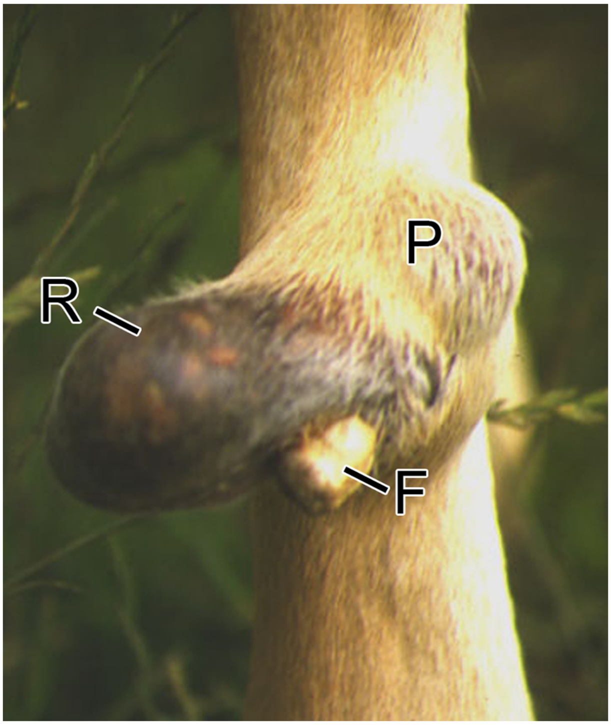

Fig. 1. Ectopic pedicle and antler growth on the left foreleg (metacarpal region) of a fallow buck (*Dama dama*) induced by subcutaneous transplantation of antlerogenic periosteum. The ectopic pedicle (P) is covered by normal leg skin. The first antler (F) grown from the pedicle has lost its velvet cover, but failed to be cast. This notwithstanding, a larger antler regenerated (R) that was covered by typical velvet skin. For details of the experiment see Kierdorf and Kierdorf [71].

ubiquitous, in the integument of deer [73].

The results of the transplantation experiments show that the AP cells are determined for pedicle and antler formation, i.e., committed to a specific differentiation pathway [65]. It is presently unknown when the AP cells become committed and whether this is a single- or multi-step process. Given the derivation of the frontal bones of mammals from cells of the cranial neural crest [74], this could either already occur in pre-migratory or migrating neural crest cells or later during skull growth. The AP is considerably thicker than the periosteum in other regions of the skull roof [75,76], and the cells of the AP's cambial layer are rich in glycogen, which is regarded to be an energy source for rapid proliferation [77]. There is experimental evidence that the AP also carries morphogenetic information for the axial orientation of antlers. Thus, when the AP was rotated 180° in situ, the subsequently formed antlers showed a reversal of their anterior-posterior axis [78]. Based on AP deletion and transplantation experiments, other authors later suggested that the morphogenetic information is primarily held in the anterior-medial portion of the AP [72].

Antlers grow at an unprecedented speed for bones. Thus, in wapiti (*Cervus canadensis*) a peak growth rate of 2.75 cm per day has been observed [79]. Maximum growth rates for the enormous antlers of the extinct giant deer (*Megaloceros giganteus*) must have been even higher. The periodic regrowth of large antlers within a period of few months constitutes a huge physiological effort and requires a rich supply of mineral elements, especially calcium and phosphorus. Experimental studies in red deer (*Cervus elaphus*) demonstrated that the mineral requirements of antlers cannot be fully met by dietary uptake during their growth period [80]. It has further been shown that during antler growth bone resorption occurs in different locations of the postcranial skeleton (especially the ribs) to mobilize mineral elements needed for antler mineralization [80–84], resembling the situation occurring in lactating females. The resorption spaces caused by this mineral mobilization process are refilled with new bone following cessation of antler growth [82] or partly already before completion of this process [84]. The seasonal shift between bone resorption and repletion in the postcranial skeleton that is associated with the antler cycle has been referred to as "cyclic bone remodeling" [83], "cyclic physiological osteoporosis" [82], or "reversible osteoporosis" [84]. Ceacero [85] reviewed the relationship between body and skeletal size and antler mass of 43 deer species. He concluded that there are physiological constraints that limit the antler mass than can be produced by a species and that these constraints are more severe in larger species

Pedicle growth initially occurs by intramembranous ossification, and in the European roe deer (*Capreolus capreolus*), a small species with a body mass of 15 to 25 kg, the complete pedicle is formed in this way [86]. However, it was shown that already during early pedicle growth a change from intramembranous to chondral (endochondral and perichondral) ossification occurs in the larger fallow deer *(Dama dama)* and red deer (*Cervus elaphus*) [75,76].

The histological organization of growing primary and regenerating antlers and the nature of the ossification process in developing antlers have been studied in different deer species [8,9,76,86–95]. These studies demonstrated that antler growth is appositional and that growth plates and secondary ossification centers, which are characteristic features of mammalian long bone growth, are not present in growing antlers. The type of bone formation in growing antlers can be characterized as a form of chondral ossification, involving both endochondral and perichondral (intramembranous) processes.

The growing tip of the antler shows a characteristic disto-proximal zonation, with a gradual transition and indistinct borders between the different zones (Fig. 2). The distalmost zone, located beneath the dermal component of the velvet, is the proliferative zone, which has also been referred as hyperplastic perichondrium [87,88]. These authors subdivided the proliferative zone in a distal to proximal sequence into three subzones, viz., the reserve mesenchyme, a prechondroblastic, and a chondroblastic layer. The latter two subzones are also referred to as precartilage zone [92]. The reserve mesenchyme is characterized by high mitotic activity, a high level of apoptosis, and the presence of small blood vessels that are continuous with larger vessels located in the inner portion of the dermis [87–89,92,93]. In the precartilage zone, the cells undergo first steps of chondrogenic differentiation.

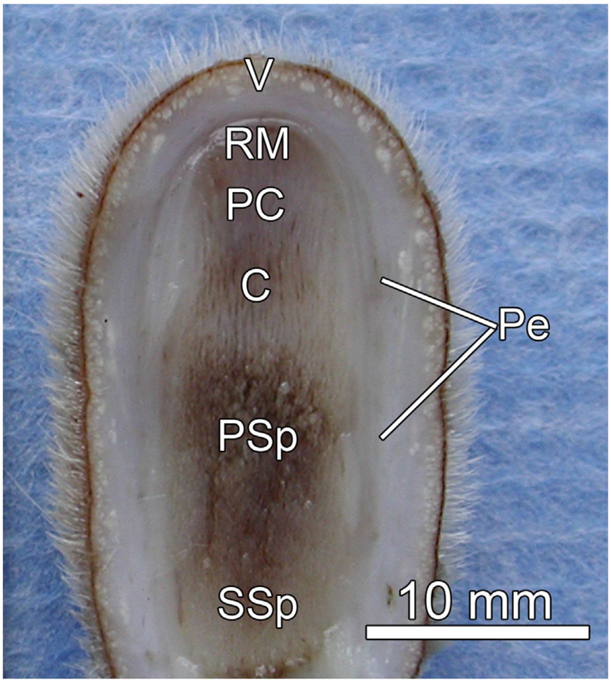

Fig. 2. Longitudinal section of the tip region of a growing primary antler of a fallow buck (*Dama dama*) to show the macroscopic appearance and approximate location of the different tissue zones. V: velvet, RM: reserve mesenchyme, PC: precartilage, C: chondrocytic zone (mature cartilage and mineralized cartilage), PSp: pimary spongiosa, SSp: secondary spongiosa, Pe. perichondrium/periosteum.

The proliferative zone is proximally followed by the chondrocytic or cartilage zone composed of hyaline cartilage, in which the chondrocytes sequentially undergo maturation and hypertrophy, and the cartilage matrix becomes mineralized (Fig. 3a and b) [87–91,96]. Like the precartilage zone, also the cartilage zone contains numerous blood vessels. The chondrocytes in this zone are arranged in interconnected vertical trabeculae (columns) that form a network around pore spaces containing a richly vascularized intertrabecular tissue (Fig. 3a and b). Within a cartilaginous column, a differentiation gradient is observed from its center to the periphery. More distally within the cartilage zone, mature chondrocytes occupy midtrabecular positions, while young chondrocytes and chondroblasts are positioned peripherally. More proximally, hypertrophic chondrocytes are found in the center of the cartilage columns, while mature chondrocytes, young chondrocytes and chondroblasts occupy gradually more peripheral positions [87–89]. Mineralization of the cartilage matrix starts in the center of the trabeculae and spreads peripherally [88,89].

Proximally, the cartilage zone is followed by an extensive zone characterized by the co-occurrence of chondroclasia, osteoclasia, and osteoblastic activity [87–89,97,98]. This zone is characterized as primary spongiosa by Banks [87] and Banks and Newbrey [88] (Fig. 3c). Within this zone, the cartilage is eroded by numerous chondroclasts, and bone is laid down at the surface of the attenuated cartilaginous trabeculae. The combined chondroclastic and osteoblastic activity leads to gradual replacement of the mineralized cartilage network by a system of bony trabeculae. In places, also the newly formed bone is eroded by osteoclasts. Especially in more distal positions within the antler, the mineralized cartilage is sometimes not completely resorbed in the replacement process, and remnants of this tissue can be found in

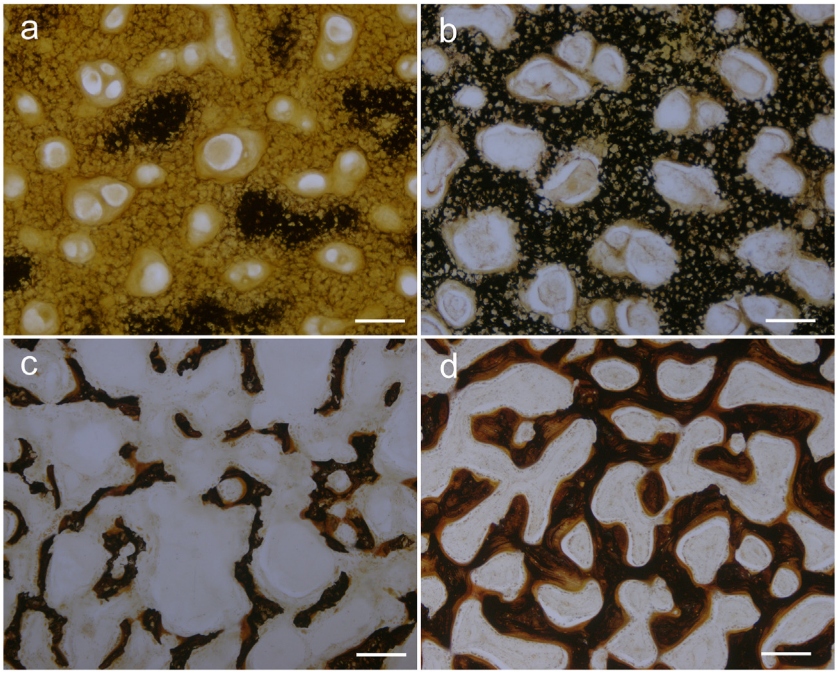

Fig. 3. Micrographs of transverse sections of developing red deer (*Cervus elaphus*) antlers showing different types of mineralized tissues. (a,b) spreading of mineralization within the cartilage; (c) primary spongiosa; (d) secondary spongiosa. Von Kossa stained sections viewed in transmitted light. Scale bars = 200 μm.

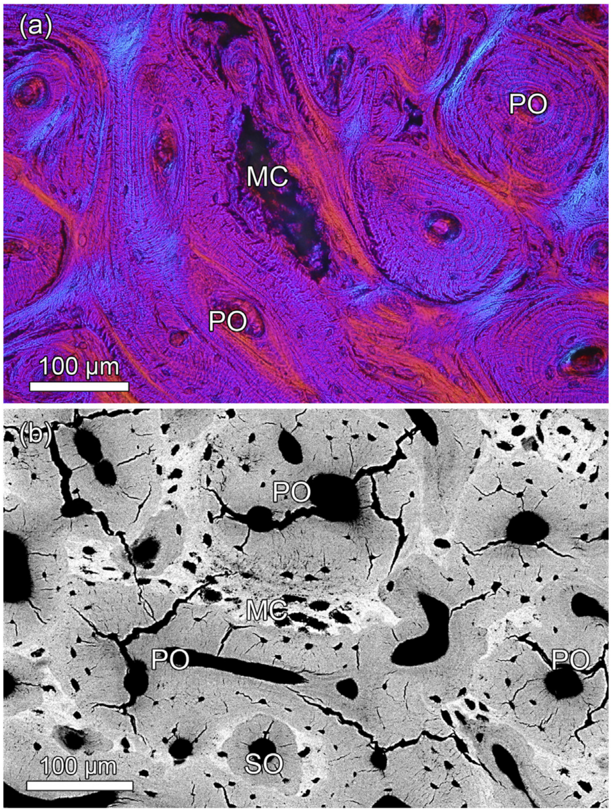

Fig. 4. Remnants of mineralized cartilage (with scalloped margins as signs of resorption) in hard antlers. (a) Light micrograph of thin ground section of a PMMA-embedded portion of a red deer (*Cervus elaphus*) antler showing mineralized cartilage (MC) within the trabecular scaffold of the main cortex; transverse section viewed in linearly polarized light with a 1λ compensator. PO: primary osteon; (b) BSE-SEM image of the polished block surface of a PMMA-embedded portion of a roe deer (*Capreolus capreolus*) antler showing mineralized cartilage (MC) as well as primary (PO) and secondary (SO) osteons in the main cortex.

the center of the bony trabeculae in hard antlers (Fig. 4a and b). These mineralized cartilage remnants typically show a scalloped surface indicative of resorption and are more highly mineralized than the surrounding bone tissue (Fig. 4b) [99].

Proximal to the primary spongiosa, the spaces between the scaffold of bony trabeculae are filled in by the apposition of lamellar bone onto the trabecular scaffolds (Fig. 3d). This zone has been referred to as secondary spongiosa [87,88]. In the periphery of the antler, the deposition of primary osteonal bone leads to a gradual compaction and the formation of a densely structured antler cortex around the interior cancellous portion [87,89,100].

The ossification process described above has been characterized as a special type of endochondral ossification, with intense vascularization of the cartilage as well as a less well defined separation of the different tissue zones and a much closer proximity of the various cell types compared to mammalian growth plates [8,9,87–90]. The antler ossification process also includes a perichondral (intramembranous) component. Thus, along the antler periphery, a bone sleeve is laid down by direct ossification from the perichondrium or periosteum [9,87–89]. It has been concluded that this process is also responsible for the formation of the protuberances that characterize the antler surface in some deer species [99].

Antlers are male secondary sexual characteristics (except in reindeer) and are often cited as an example for sexual selection in mammals [32,101]. Many different functions of antlers have been proposed; however, it is generally agreed that they serve mainly as organs of display and for intraspecific fighting during the rut [32,101]. The antler cycle of male deer is tightly linked to their reproductive cycle, and the timing of the different events within this cycle is regulated by fluctuations in the blood levels of sex hormones, testosterone in particular [6,59,102–107]. In cervid species inhabiting higher latitudes, the annual reproductive cycle itself is strictly controlled by the photoperiod [6,59,108]. Antler growth occurs during a period of low circulating testosterone levels. An increase in testosterone concentrations prior to the rut causes the intense ossification of the antlers and subsequent velvet shedding. The latter process leads to an interruption of the antlers' blood supply that principally occurs via arteries located in the vascular layer of the velvet and, in consequence, to the death of the

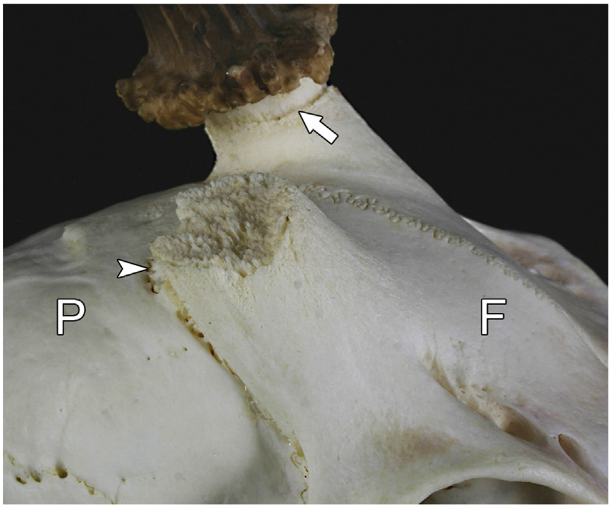

Fig. 5. Skull cap of a roe buck (*Capreolus capreolus*) that has already cast its right antler while the left antler is still attached to the pedicle. Note rough sequestration surface at the top of the right pedicle and ring-like resorption furrow (arrow) in the distal portion of the left pedicle. F – frontal bone, P – parietal bone, arrowhead – frontoparietal suture.

denuded bony antlers [109]. Subsequently, the bare bony antlers are used for fighting between males during the rut.

A drop in circulating testosterone after the rutting season triggers osteoclastic activity in the distal pedicle [86,99,110,111]. In his classic monograph on bone resorption, Kölliker [110] referred to the resorption spaces in the pedicle formed by this activity as resorption sinuses that with ongoing osteoclastic activity tend to become confluent. Osteoclastic resorption also occurs at the periosteal surface of the pedicle, causing the formation of a ring-like furrow (Fig. 5). The two processes lead to a progressive weakening of the connection between pedicle and antler, which finally causes the breaking off of the antler (plus a small amount of pedicle bone) from the pedicle (Fig. 5) [86,99,110,111].

Antler casting is followed by a short period of intramembranous bone formation at the top of the pedicle stump that partly compensates for the previous loss of pedicle bone [112]. The wound surface is rapidly covered with skin, beneath which a mass of regenerative tissue, the antler bud, is established (Fig. 6a and b). The formation of a regeneration blastema in the form of the antler bud on top of the pedicle stump resembles the processes occurring during epimorphic appendage regeneration in other vertebrates (for a detailed discussion see Kierdorf et al. [9,10,12]). In deer species, where the lowermost tine (the so-called brow tine) starts to develop very early during antler regeneration, separate proliferative zones (growth centers) for the main beam and the brow tine are recognizable already in the antler bud (Fig. 6b) [9,12,113]. During the regeneration of multi-tined antlers, the growth center at the tip of the main beam repeatedly undergoes asymmetric splitting into a larger one for the main beam and a smaller one for the respective tine (Fig. 6c).

The morphogenetic and differentiation events during antler growth are a recapitulation of those that occur during mammalian development. Thus, it has been shown that both the canonical WNT signaling pathway and the PTHrP-IHH pathway are involved in the regulation of antler regeneration [95,114,115]. Growing antlers are therefore a useful model for studying these pathways in a postnatal mammal and an easily accessible developmental system.

There is morphological, histological and experimental evidence that the regenerative tissue forming the antler bud is derived from the pedicle periosteum (PP), which itself is a derivative of the AP responsible for the initiation of pedicle and first antler growth [71,112,113,116,117]. Studies from different laboratories indicated that cells from the AP and PP possess stem cell attributes, and antler regeneration has therefore been characterized as a stem-cell-based epimorphic process [9–12,33,117–119].

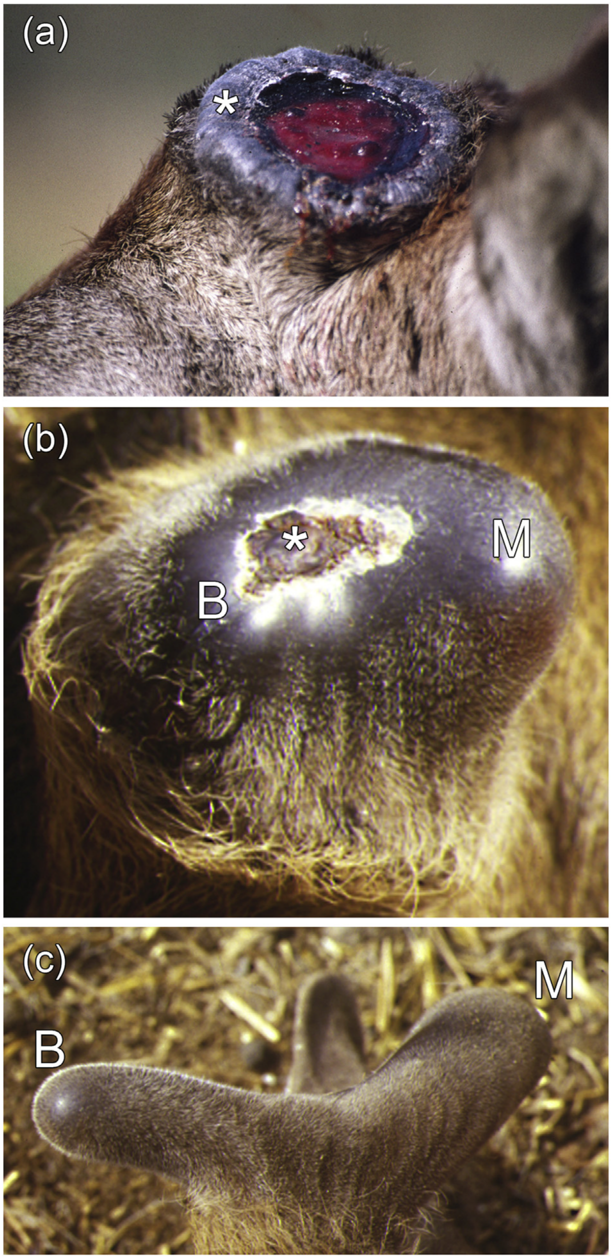

Fig. 6. Macroscopic aspects of different stages in the process of early antler regeneration. (a) Casting wound on the top of the pedicle in a red deer stag (*Cervus elaphus*). Note ring of regenerating skin (asterisk) that will cover the wound surface in centripetal direction. (b) Early stage of antler regeneration in a fallow buck (*Dama dama*). Wound healing is almost completed, but a scab (asterisk) is still present in central area of the antler bud. Separate growth centers for the main beam (M) and the brow tine (B) are discernible. Note neogenesis of hairs in the velvet. (c) More advanced stage of antler regeneration in a fallow buck, B: forming brow tine, M: forming main beam.

Cells from the AP, PP, and the reserve mesenchyme, which are collectively referred to as antler stem cells (ASCs), could in vitro be induced to differentiate into chondrocytes, osteocytes, and adipocytes [11,33,118,120], demonstrating their multipotency within the mesenchymal lineage. Moreover, it was shown that cells from the three tissue sources expressed typical markers of mesenchymal stem cells like CD73, CD90, CD105 and STRO-1 [33,118,120].

Some studies reported that antler stem cells also express the embryonic stem-cell markers Oct4, Nanog and Sox2 [11,120,121]. However, it was later shown that the *Nanog* gene expressed by these cells was actually a pseudogene [122], and a recent study failed to detect expression of any of the three marker genes in antlerogenic stem cells [42]. Therefore, ASCs are currently probably best characterized as a special type of (lineage-restricted) mesenchymal stem cells endowed with a huge proliferative potential and the capability of regenerating a complete bony appendage in a postnatal mammal.

4. Gross structure, microarchitecture and mineralization of antlers

John Currey repeatedly addressed the unique mechanical properties of antlers in comparison with other bone types and the adaptive value of the specific properties of antler bone [2,3,5,57]. He showed that the mechanical properties of antlers varied from base to tip [3]. Further studies (reviewed below) demonstrated that this variation is related to the combined effects of bone microarchitecture and mineralization.

When functional, the antler is a dead bone. Antler osteogenesis is completed during a short growth period (of 4 to 5 months in red deer), while the forming bone is covered by velvet. At velvet shedding, the blood flow ceases, bone formation and mineralization stops, and the antler bone undergoes necrosis. The distal antler portions are younger than the more proximal ones, which is reflected by changes in the histological structure along the longitudinal axis of mature antlers [100].

For histological examination, it is common practice to sample the hard antler at various levels along its proximo-distal axis. Usually, transverse slices are obtained at different morphologically defined positions (burr, lower, middle, upper beam) or at different distances from the antler base (Figs. 7 and 8). The slices are first made superficially inorganic by treatment with a detergent containing proteolytic enzymes [123], photographed (Fig. 8a and b), and then fixed in 70% ethanol. When growing antler is being assessed, treatment with enzyme detergent is omitted and the specimens are fixed directly in ethanol (Fig. 8e). In both cases, the slices are embedded undecalcified in epoxy resin or poly-methyl methacrylate (PMMA), and thin sections (about 50–100 μm thick) are prepared from the embedded blocks. These can be examined unstained under normal transmitted light or under linearly (LPL) or circularly polarized light (CPL) (Fig. 9) [124]. They can also be viewed following surface staining [125] (Fig. 10), in which case it is advisable to use monochromatic stains to facilitate further morphometric studies.

The hard antler can be considered a thick-walled tube made of two components: the peripheral cortex and the central spongiosa (Fig. 7a and 8). Cortical and trabecular bone differ in porosity (void space) and bone mass. Cortical bone has a low porosity, $< 5\%$ in well-formed antlers, compared to over 60% for trabecular bone. Measurements at several levels of the antler show that the cortex accounts for most of the mineralized area (mean = 73%, range 54–84%, data for red deer) present in a transverse section, and that cortical thickness decreases from proximal to distal (Fig. 7b, Table 1). Antlers from conspecifics can vary markedly in antler total area, cortical area, and cortical thickness (Fig. 7c).

Mineral density of the antler cortex is another important parameter to assess. Landete-Castillejos et al. [20], using dual X-ray absorptiometry (DXA), determined areal bone mineral density (aBMD) of the cortex in transverse antler sections. The values for antler bone are low compared to non-antler bone cortices. It was also shown that aBMD in a series of intact antlers was significantly higher ($850 \pm 10\,\mathrm{mg/cm^2}$, mean ± SE) than in broken antlers from a different year ($790 \pm 20\,\mathrm{mg/cm^2}$) (Fig. 7d). Frequent antler breakage had been caused by dietary mineral deficiency caused by climatic events (for details, see chapter 5). Furthermore, aBMD varied both between different positions within a single antler as well as between different antlers. There was a decline from proximal to distal, with a difference of $0.22\,\mathrm{g/cm^2}$ between a position located 10 cm and a position located 50 cm above the antler base.

Another suitable method for the gross analysis of the antler is peripheral quantitative computed tomography (pQCT) (Fig. 8f). Using pQCT enables the simultaneous assessment of volumetric bone mineral density (vBMD) and several bone microarchitectural parameters. Preliminary tests using pQCT recorded vBMD-values between $913\,\mathrm{mg/cm^3}$ near the antler base and $795\,\mathrm{mg/cm^3}$ near the antler tip.

In light microscopic studies of antler bone, transverse and longitudinal thin ground-sections are analyzed. Imaging can be performed both in unstained sections (Fig. 9) and in surface-stained sections, either following acid etching (e.g. 1% formic acid and toluidine blue, 1% periodic acid and silver nitrate) (Fig. 10), or using direct staining with e.g. alizarin red S (Fig. 8d) or von Kossa stain (Figs. 3 and 11). Another method to characterize the structure of antler bone is the study of polished surfaces by backscattered electron (BSE) imaging in the scanning electron microscope (SEM) (Fig. 4b) [99,132,133].

Microscopic examination shows that three cortical zones can be distinguished, viz. an outer cortex, a main (central) cortex, and a transitional zone towards the spongiosa (Figs. 9 and 13). Cortical bone is composed of two constituents that differ in microstructure and mineralization. The first-formed of these constituents is a trabecular osseous framework (scaffold) that results from the replacement of the mineralized cartilage framework in the process of endochondral ossification (Figs. 4, 10 and 11). The nature of the bony trabeculae forming this tubular framework is not entirely clear [99,100,132]. Krauss et al. [132] use the term micro-lamellar bone for this tissue to indicate an orderly collagen organization over short distances, but a lack of such an organization over longer ranges of several hundred microns. Micro-lamellar bone has been characterized as a sub-type of woven bone [134]. Histological studies by our group indicate that, depending on the location, both woven and lamellar bone can be present in the trabecular scaffold [99, 100 and unpublished observations].

The cavities lined by the trabecular scaffold are secondarily filled with primary osteons, leading to a progressive compaction of the antler cortex. (Figs. 4, 10 and 11). The primary osteons, which make up most of the antler cortex, are of highly variable shape and often contain more than one vascular canal (Figs. 4, 10 and 11) [99,132,133]. These multiple-canal osteons were referred to as “osteon conglomerates” by Skedros et al. [133]. When transverse antler sections are viewed in polarized light, the bulk of the wall of the primary osteons appears dark, indicating a longitudinal orientation of the collagen fibers (Fig. 9c). However, along the periphery of the osteons, bright seams are seen, indicating that in these areas the collagen fibers are oriented more perpendicularly to the path of the light (Fig. 9c) [132,133].

As was stressed by Skedros et al. [133], in longitudinal antler sections, both the central areas of the cortical primary osteons as well as their periphery appear relatively bright under polarized light, and thus the difference in birefringence between the two areas is less pronounced than in horizontal sections (Fig. 9d). The brightness of the osteon periphery in both transverse and longitudinal sections suggests an oblique orientation of the collagen fibers in this area. Since in longitudinal sections both the bulk of the osteonal wall and the osteon periphery appear bright, polarized light images of such sections are overall much brighter than those of transverse sections of the antler cortex (Fig. 9 c and d) [133].

Krauss et al. [132] and Skedros et al. [133] describe the presence of bright lines or hyper-mineralized seams along the periphery of many of the primary osteons in the antler cortex. These bright lines resemble the reversal lines present around secondary osteons. Krauss et al. [132] interpreted the bright lines as resting lines formed prior to the start of osteon formation, and Skedros et al. [133] suggested that the presence of these hypermineralized structures may lead to a toughening of the antler bone. Kierdorf et al. [99] and Gomez et al. [100] provided evidence for resorption at the trabecular scaffold prior to the infilling of the intertrabecular spaces with primary osteons. Moreover, BSE

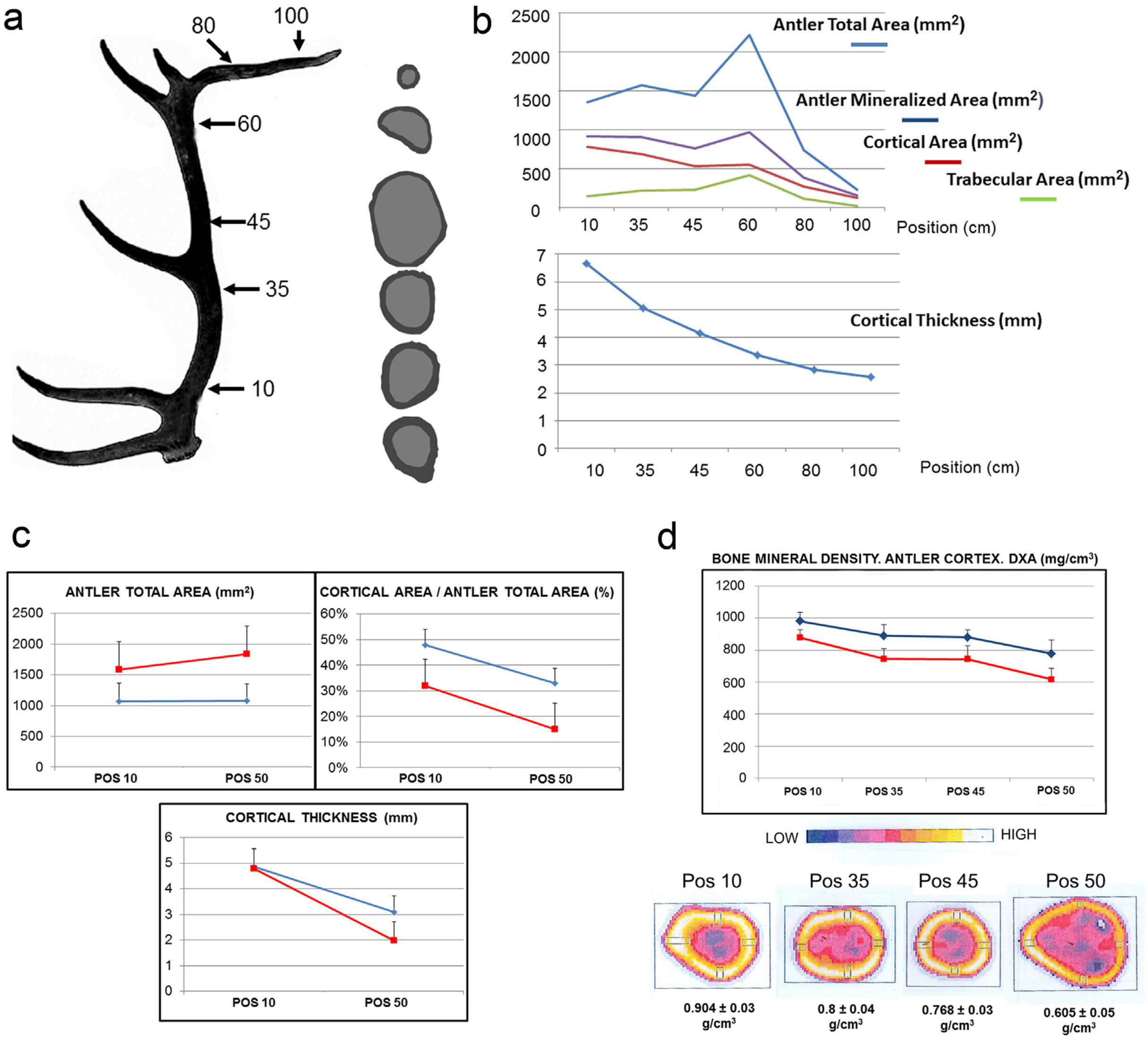

Fig. 7. Morphometry data obtained from aniline blue stained, 100 μm-thick cross-sections of hard antlers of red deer. (a) Scheme of antler and the sampled positions at different distances (cm) from the antler base. (b) Example of morphometric measurements in an antler sampled at six positions. The mineralized area (sum of mineralized cortical and trabecular areas) is a fraction of the total cross-sectional area. Thus, at positions 10 cm and 60 cm (usually sampled for histology) the percentage of mineralized area is 62.2% and 49.8%, respectively, of the total area. Note that cortical thickness decreases in distal direction along the main beam. (c) Antler total cross-sectional area (mm2), percentage cortical area of total cross-sectional area, and cortical thickness (mm) in two series of red deer antlers from Spain (*n* = 10, blue) and the Czech Republic (n = 10, red), measured at positions 10 cm and 50 cm (values indicated are means and SDs). The Czech antlers are larger in cross-section, but their cortical values are lower than those of the Spanish antlers. (d) Cortical bone mineral density (BMD), measured in cross-sectional slices using DXA at 4 positions (10, 35, 45, 50 cm from the base), of intact (*n* = 7, blue), and broken (*n* = 5, red) antlers randomly selected for histology from the study of Landete-Castillejos et al. [20]. The upper graph shows means and SDs for the four positions. The aBMD values (mg/cm^2) obtained by DXA were converted to mg/cm^3 since the slices were 1 cm thick. The lower graph illustrates the results of DXA imaging at different the positions in an intact antler. Values given are means ± SDs from four measurements in the cortical areas indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

imaging revealed the presence of scalloped bright lines along stretches of the periphery of primary osteons [99]. This suggests that at least some of the bright lines may actually represent cement lines formed at reversal sites following resorption at the trabecular scaffold, and may therefore be developmentally homologous to reversal lines in secondary osteonal bone.

Remnants of mineralized cartilage are occasionally observed within the trabecular scaffold of the main cortex of mature hard antlers [99,100] (Fig. 4a, b and 10a). These remnants, which exhibit scalloped surfaces from resorption processes, are evidence of an incomplete replacement of cartilage by bone in the process of endochondral ossification. The persistence of cores of mineralized cartilage in the trabecular scaffold must be attributed to the rapidity of the antler growth process that is abruptly stopped at velvet shedding, irrespective of the degree of antler maturation [135].

There is consensus about the scarcity of secondary osteons in antlers [99,100,132,133], which can likewise be attributed to their short lifespan that does not allow for any significant remodeling. Secondary osteons in antlers are typically small (mostly < 60 μm in diameter) and surrounded by a reversal line resulting from the patchy resorption of primary bone tissue (Fig. 4b and 10c). The secondary osteons in antler bone have been termed modeling osteons to indicate the fact that they are formed at the same time as primary osteons in other locations, i.e. while bone modeling is still ongoing [100]. Microcracks have been observed both in natural (S. Gomez, unpublished observation) and mechanically tested hard antler bone [136,137], and the role of

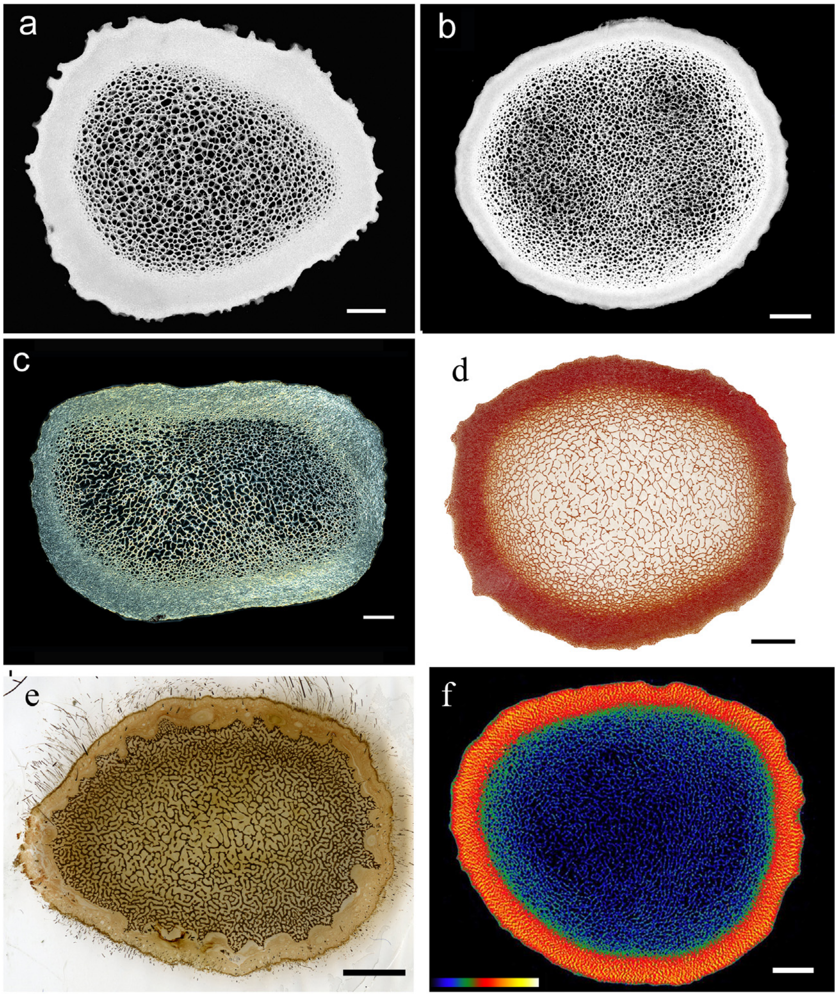

Fig. 8. Transverse sections of the main beam of red deer antlers, illustrating gross antler structure. (a,b) Two mm-thick sections of a hard antler made superficially inorganic by treatment with an protease-containing detergent at positions 10 cm (a) and 50 cm (b) above the antler base; the dense antler cortex is markedly broader in (a), and cortex and spongy core are clearly distinguishable. (c) PMMA ground-section (50 μm thick) of hard antler at position 10 cm, photographed using two circular polarizer filters, left and right handed, and an optical scanner. (d) PMMA ground-section (100 μm thick), of hard antler at position 10 cm stained with alizarin red. (e) PMMA ground section (50 μm thick) from the proximal portion of a growing first antler (day 70 of growth) stained with von Kossa. Note subperiosteal bone formation by membranous ossification along the antler periphery. (f) Peripheral quantitative computed tomography virtual section of antler at position 50 cm processed in false colour to highlight the variation in bone mineral density (blue, low; yellow, high), (original image courtesy of Dr. Willnecker, Stratec Medizintechnik). Scale bars = 5 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

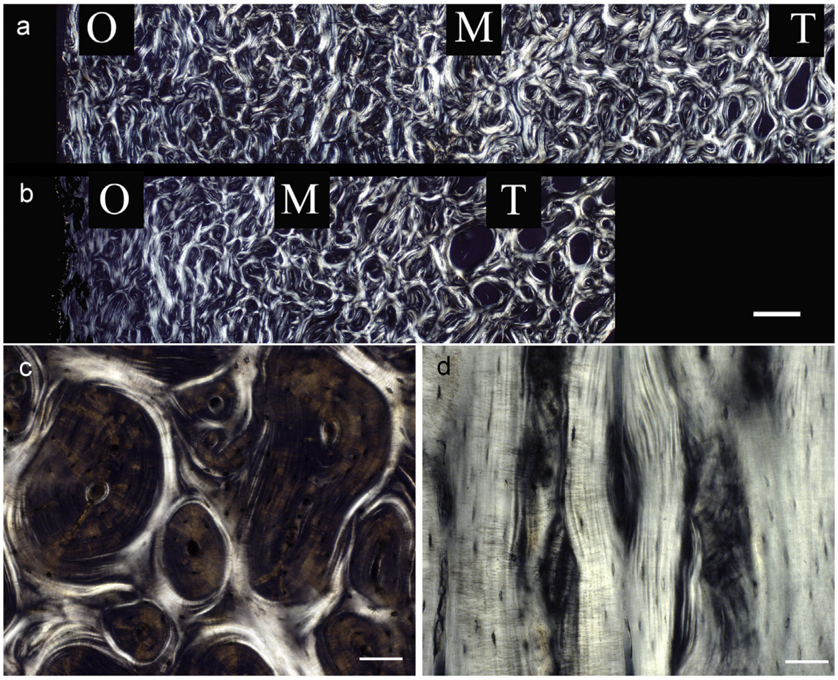

Fig. 9. Microarchitecture of the cortex of hard antlers of red deer. Circularly polarized light (CPL) microscopy of unstained ground sections. (a–b) Characteristic zonation of the cortex in an antler sampled at positions 10 cm (a) and 50 cm (b) above the base. Three zones can be distinguished: an outer cortex (O), a main (central) cortex (M), and a transitional zone (T). Scale bar = 200 μm. (c,d) The two principal components of the cortex, trabecular bone framework (scaffold) and primary osteons as seen in transverse (c) and longitudinal (d) sections. Scale bars = 50 μm.

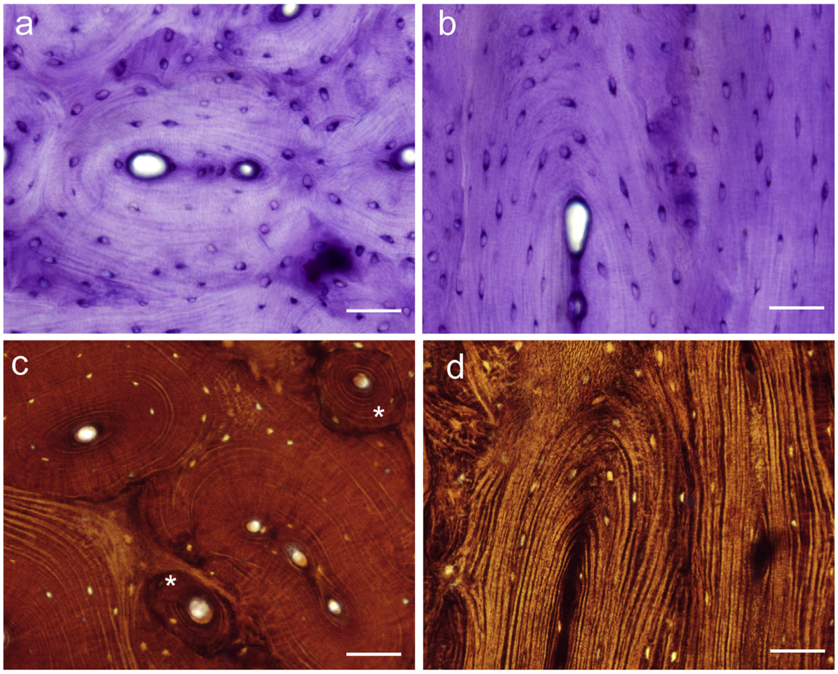

Fig. 10. Microarchitecture of the cortex of hard antlers of red deer. Bright-field microscopy of surface-stained transverse (a,c) and longitudinal (b,d) ground sections. Trabecular scaffold and primary osteons are clearly distinguishable, (a,b) toluidine blue staining following pre-treatment with 1% formic acid, (c,d) silver nitrate staining following pre-treatment with 1% periodic acid. In (a) a remnant of mineralized cartilage (deeply stained) is observed. In (c) two secondary (modeling) osteons are present (asterisks). Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microcracking as a mechanism increasing the fracture toughness of antlers has been discussed in detail [136,137].

The term cortical porosity as used here refers to non-mineralized areas within the antler cortex, including the vascular canals and the osteoid seams of the primary osteons. It does not encompass microporosity (osteocyte lacunae and canaliculi). Cortical porosity of mature antlers is low, but can vary greatly if the formation of the primary osteons is not completed. Fig. 12 shows an example, in which porosity varies widely across the cortex, being lower in the central portions compared to the outer cortex and the transition zone. In the outer and transitional zones of the cortex of hard antlers, often broad seams of unmineralized osteoid are found [99,100] (Fig. 11d), indicating

Table 1

Range of values (minimum and maximum means) published in previous studies [18, 20, 126-130] for the structural characteristics, mechanical properties and mineral profile of antlers in adult red deer. Antler basal position corresponds to the position immediately above the burr. Antler distal position corresponds to the sampling position just below the crown. Dashes indicate lack of data.

Variables	Total antler	Antler basal position	Antler distal position	Internal bone (femur)
Average cortical thickness (mm)	5.1-6.3[c]	5.7-7.4[d]	3.3-5.4[e]	4.8[6]
Average cortical area (%)	0.43-0.50[g]	0.56–0.59[d]	0.40–0.48[4]	–
Density of cortical bone (kg/dm^3)	1.55–1.70[e]	1.62–1.77[e]	1.49–1.70[e]	–
Ash content (%)	51.0–62.2[b]	61.3–63.9[e]	58.0–62.6[e]	72.5[f]
Young's modulus of elasticity (E), (GPa)	6.9–17.3[b]	14.2–18.7[d]	13.2–15.3[d]	22[f]
Bending Strength (BS), (MPa)	103.7–343.0[b]	268.5–284.7[d]	233.2–257.0[e]	271.6[f]
Work to peak force (W), (kJ m^{-2})	22.2–42.9[e]	43.1–43.9[d]	38.2–42.3[5]	9.3[f]
Impact energy absorption (U), (kJ m^{-2})	15.8–21.8[d]	15.4–19.7[d]	15.2–22.5[d]	–
Microhardness (kg mm^{-2}) (Vickers)	24.6–33.6[e]	33.1–36.7[e]	27.4–32.7[e]	59.6
Ca (wt%)	19.6–34.4[a]	19.6–21.2[e]	19.7–20.5[e]	21.1[h]–27.7[f]
Mg (wt%)	0.49–0.96[a]	0.48–0.58[d]	0.50–0.56[d]	0.38[h]–0.45[f]
Na (wt%)	0.53–1.15[a]	0.57–0.64[d]	0.50–0.62[d]	0.53[h]–0.66[f]
P (wt%)	9.5–11.7[d]	10.0–12.3[d]	9.7–11.3[4]	13.0[f]
B (ppm)	1.8–4.2[e]	1.9–4.8[e]	1.9–4.5[e]	–
Co (ppm)	0.30–0.34[b]	0.4[e]	0.3[e]	–
Cu (ppm)	0.38–0.80[e]	0.39–0.80[e]	0.39–0.90[e]	0.23[f]
Fe(ppm)	3.4–46[a]	3.4–4.5[e]	4.4–7.5[e]	1.6[f]
K (ppm)	431.9–864[g]	559.9[e]	473[e]	–
Mn (ppm)	0.30–0.68[b]	0.38[e]	0.4[e]	0.26[f]
Ni (ppm)	0.40[e]	0.44[e]	0.50[e]	–
Se (ppm)	1.7[e]	2.7[e]	2.3[e]	0.4[f]
Si (ppm)	59.6–120[a]	48.2[e]	58.6[e]	–
Sr (ppm)	344.3–754.3[e]	374.8–875.3[e]	351.2–761[e]	251.1[f]
Zn (ppm)	52.0–87.0[a]	69.3–77.3[e]	67.3–83.6[e]	60.1[f]

[a] Landete-Castillejos et al. [18]

[b] Landete-Castillejos et al. [20]

[c] Landete-Castillejos et al. [126]

[d] Gambín et al. [127]

[e] Cappelli et al. [128]

[f] Olguín et al. [129]

[g] Estévez et al. [130].

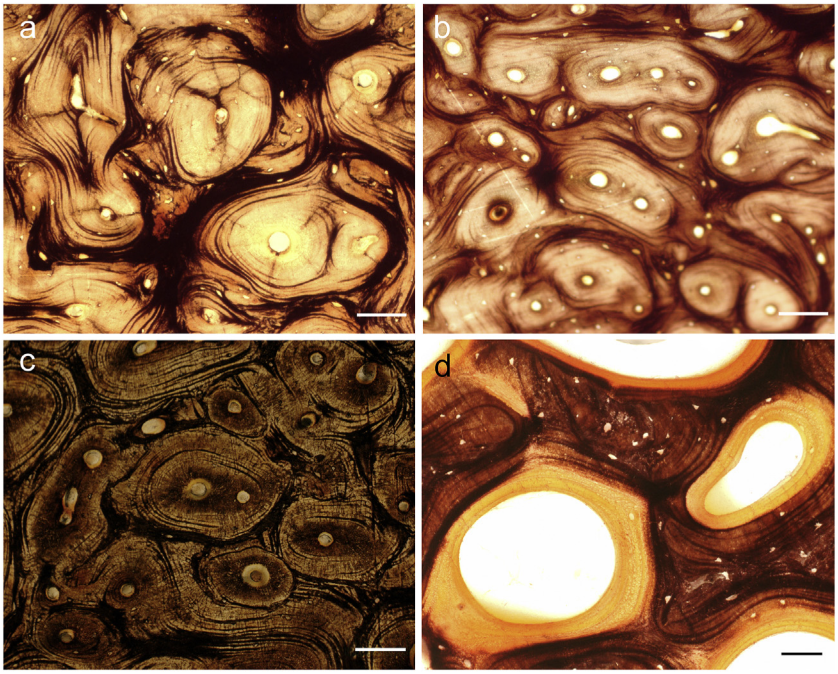

Fig. 11. Microarchitecture of the cortex of hard antlers of red deer. Bright-field microscopy of von Kossa surface-stained transverse ground sections. (a) Trabecular scaffold and primary osteons in the main cortex as seen in a section obtained from position 10 cm. (b) Corresponding region at position 80 cm. Note smaller size of primary osteons compared to (a). (c) Primary osteons in the main cortex of an antler from a deer with poor nutrition. (d) Wide osteoid seams (indicative of an osteomalacic condition) in the transitional zone of the antler cortex from a deer with poor nutrition. Scale bars = 50 μm.

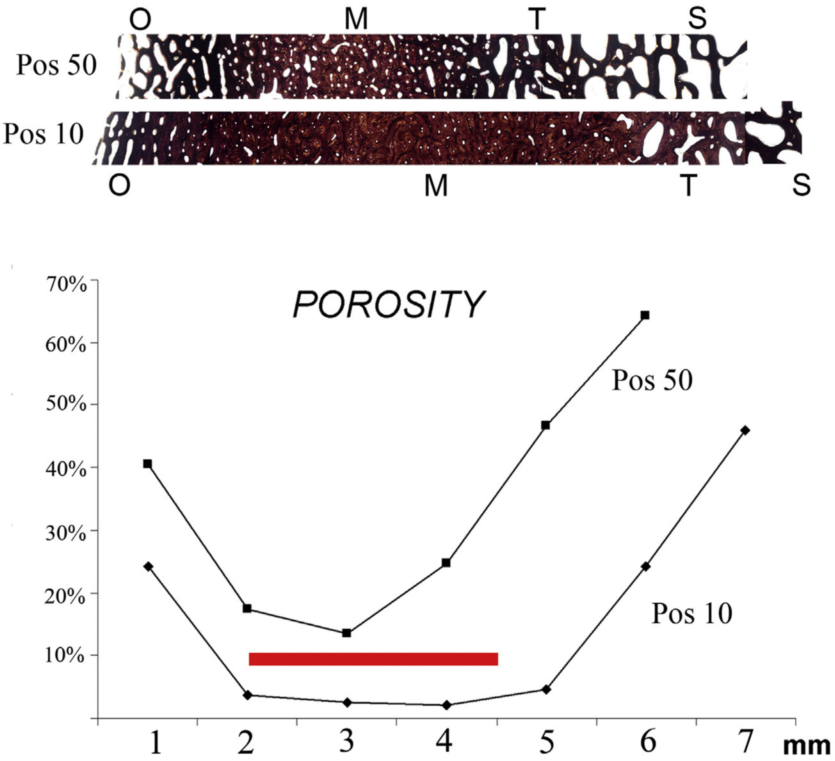

Fig. 12. Variation of cortical porosity along the main beam of a hard antler from a red deer. The upper image shows two von Kossa-stained sections of the antler cortex at, respectively, 10 and 50 cm from the antler base. Prior to converting into 1 bit images, the non-mineralized areas (= pores, comprising vascular channels and osteoid seams) on the original photographs were manually filled with white. The graph below shows the variation in cortical porosity for the two positions, measured at 1 mm intervals from the outer cortex to the spongy part. The region from which the bars of cortical bone used for mechanical testing were obtained is indicated in red. The porosity of the bars and their mechanical behavior strongly depend on the cortical region from which they were cut. O, outer cortex; M, main (central) cortex; T, transitional zone; S, spongy bone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

incomplete primary osteon formation suggestive of an osteomalacic condition.

Currey [138] compiled the principal mechanical characteristics of 32 bones types from different species. Of these bones, the antler cortex of red deer had the lowest mineral volume fraction. The mineral content of antler bone is mostly determined in homogenized tissue samples, and does not take into account the spatial distribution of the mineral in the tissue. Information on this can be obtained by using mapping methods such as microradiography or BSE imaging in the SEM. Marked heterogeneity of mineralization within the antler has been observed, the degree of mineralization being highest in the remnants of mineralized cartilage [99] (Fig. 4b).

CPL-microscopy of undecalcified sections (of mostly 100 μm thickness) is considered the method of choice to determine the orientation of collagen fibers in bone [139]. However, in such rather thick sections, individual collagen fibers are not resolved. To overcome this problem, thinner sections (< 15 μm thick) must be analyzed. The sections are decalcified for 40 min using a 2.5% phosphomolybdic acid solution (pH 1.4) and then exposed to black light until they become bluish-gray. Examination of these sections under bright field microscopy shows that the non-collagenous proteins were stained, but not the collagen fibers. Fig. 13 gives an example of the antler cortex observed both in cross

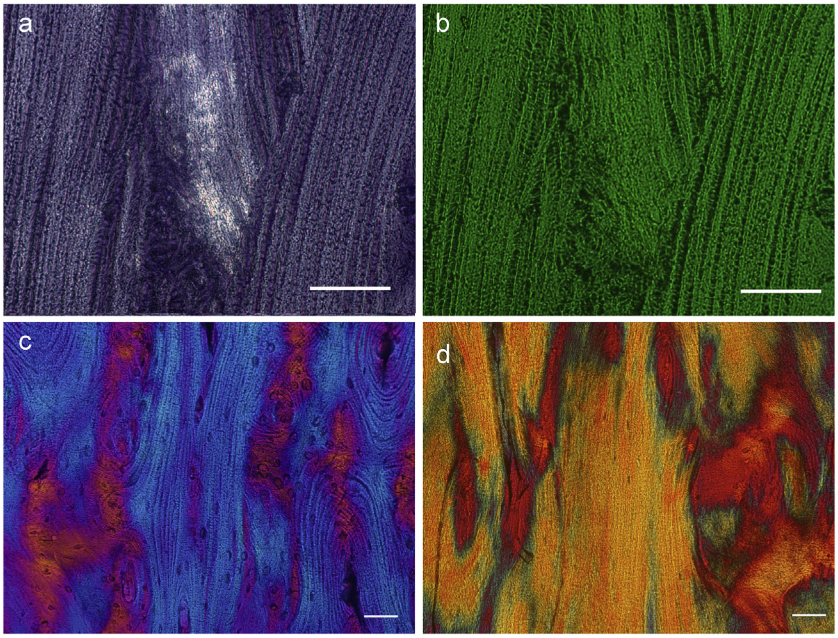

Fig. 13. Orientation of collagen fibers and mineral packages in longitudinal semithin ground sections of hard antlers of red deer. (a, b) Primary osteons and trabecular scaffold (center of images) as seen in a decalcified (phosphomolybdic acid) and stained section. (a) Cross-polarization microscopy (LPL). (b) Monochromatic polarization microscopy using a ¼ λ compensator and a 546 nm bandpass filter. (c,d) Polarization images using a 1 λ compensator whose slow optic axis is placed in N-S direction (always at 45° relative to the orientation of the polarizer and analyzer) to study the sign of birefringence in collagen and mineral. In primary osteons, opposite polarization signs are observed for collagen fibers (+), which display a blue colour by constructive interference (c; decalcified section), and in mineral packages (−) showing orange colour by destructive interference (d; section rendered inorganic by NaOCl treatment). Note that in both images, the trabecular scaffold shows different interference colors than the primary osteons, indicating variation in the preferred orientation of collagen fibers and mineral packages. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

polarization (Fig. 13a) and in monochromatic polarization (Fig. 13b) as detailed in Bromage et al. [125]. The bluish-gray colour of the stain becomes black in monochromatic polarization, contrasting with the green polarization colour of the collagen fibers. Using this method, the path of the collagen fibers can easily be observed. In longitudinal sections analyzed by LPL with the use of a 1λ compensator, the collagen fibers show a blue colour and their predominantly longitudinal orientation is clearly visible (Fig. 13c).

The bone mineral is a carbonated hydroxyapatite of microcrystalline character, whose crystal size escapes the resolution of light microscopy. However, when very thin sections that have been rendered inorganic by treatment with NaOCl are examined between crossed polarizers, it is possible to observe the predominant orientation of the mineral packages at a micron-scale. In longitudinal sections viewed using a 1λ compensator, the primary osteons show an orange colour (Fig. 13d). These images demonstrate a preferred longitudinal orientation of the hydroxyapatite mineral, indicating that it has the same orientation as the collagen fibers.

Formation of the antler cortex has been studied in red deer by fluorochrome labeling of growing antlers [100]. Fig. 14 illustrates the dynamics of osteon formation as revealed by the labels from tetracycline injections given at different times during antler growth (casting day = day 0). In the lower third of the antler beam, primary osteon formation starts around day 70 of growth and is almost completed by day 120. With increasing distance from the antler base, these events occur progressively later during the antler growth period. The temporal sequence of the different developmental steps along the proximo-distal antler axis is illustrated in Fig. 15. The labeling study also clearly showed that the mineralization process in the antler stops at velvet shedding.

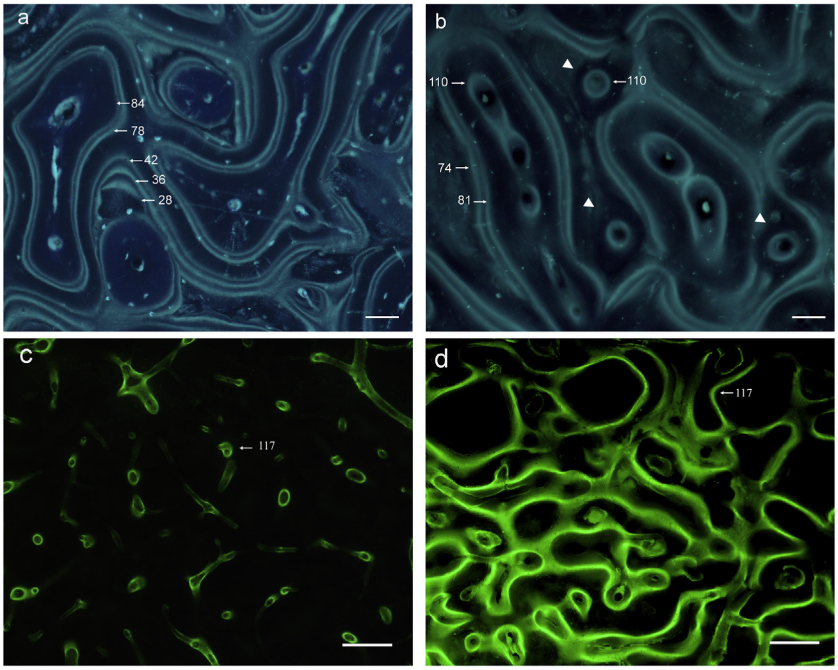

Fig. 14. Dynamics of bone tissues formation in the antler cortex as revealed by fluorochrome labeling of growing red deer antlers. Epifluorescence microscopy of transverse thin PMMA sections of hard antlers (a) Cortex of the lower beam. The deer received tetracycline injections on days 28, 35, 42, 78 and 84 (day 0 = antler casting) during antler growth. The trabecular scaffold is marked by the injections on days 28, 35 and 42, while the primary osteons are marked by the injections on days 78 and 84 (numbered arrows). (b) Cortex of the lower beam. The deer received tetracycline injections on days 74, 81, and 110 to label exclusively the forming primary osteons (numbered arrows) and secondary osteons (arrowheads). Note that the arrowheads indicate three secondary osteons which were marked on day 110. This labeling day is indicated only in one (the uppermost) of them. (c,d). The deer received a calcein injection on day 117 of antler growth. (c) Cortex of the lower beam. The primary osteons have completed their formation. (d) Cortex of the upper beam. The primary osteons are still forming. Scale bars = 50 μm.

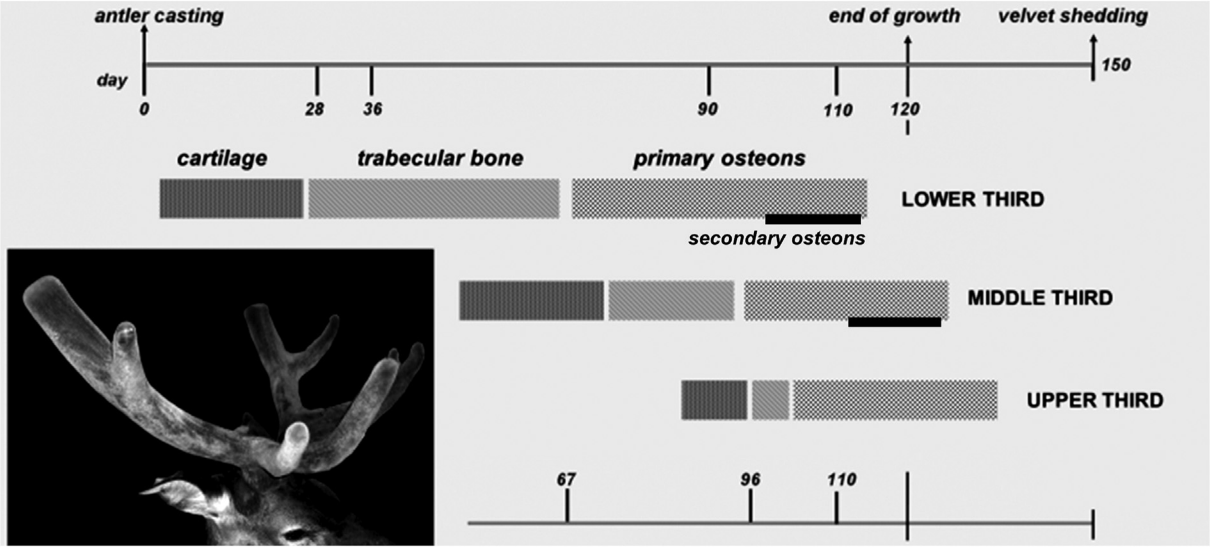

Fig. 15. Temporal sequence of formation of trabecular bone (scaffold), primary osteons and secondary (modeling) osteons in the antler cortex along the proximo-distal axis of red deer antlers. Modified from Gomez et al. [100].

Measurements of the extent of the mineralizing surfaces predict an increasing demand for calcium during formation of the antler cortex that peaks around day 100 of antler growth. Mineral apposition rate (MAR) is higher in lamellar bone of the trabecular scaffold (average of 3.2 μm/day) than in primary osteons (average of 2.15 μm/d). The values for antler primary osteons are quite similar to that measured in secondary osteons from the femur of a one-year-old red deer (2.3 μm/day, Gomez unpublished data). Lower values (average of 0.89 μm/day) were recorded for secondary osteons (modeling osteons) in antler.

Bone mineralization is classically described as a biphasic process, consisting of the initial deposition of calcium phosphate mineral, followed by the gradual maturation of the bone mineral, which includes an increase in the number and size of the crystals as well as their crystallographic perfection [140]. Secondary mineralization is a very slow process, and it takes months to years until the maximum degree of mineralization is reached. Given the short lifespan of antlers, this means that antler mineralization cannot be as complete as that of non-antler bone. Gomez et al. [131] studied the mineralization dynamics of primary osteons using micro-PIXE samples of antlers that had been labeled with calcein (Fig. 14c and d). They found differences along the beam, the recently deposited mineral areas in the upper third of the beam containing less Ca and P than were present at more basal positions. The Ca/P mass ratio in the antler was relatively high (2.37, 2.38, and 2.43 in, respectively, the lower, middle, and the upper antler position), suggesting a less "mature" mineral. Kierdorf et al. [141], using X-ray fluorescence analysis, had previously reported a significantly higher Ca/P mass ratio in first antlers (2.21 ± 0.10, mean ± SD) than pedicles (2.12 ± 0.05) of yearling red deer.

Information on the sequence of mineralization can also be obtained using Zinc histochemistry (Fig. 16). Zinc is one of the most important trace elements in bone, as it acts as a co-factor of metalloenzymes involved in mineralization. Zinc is unevenly distributed in bone, showing higher concentration at the mineralization front [142–144]. In hard antlers, zinc histochemistry reveals the zinc distribution present at velvet shedding. Zinc staining showed a variable distribution of the element in the antler cortex [126]. Completely formed primary osteons did not show zinc staining (Fig. 16a), while forming osteons showed zinc staining at the mineralization front (Fig. 16b and c). However, osteons with broad (osteomalacic) osteoid seams had no zinc staining, indicating that mineralization had already stopped. Studies of sections treated with EDTA, which causes partial Zn-extraction [142], showed that some zinc staining remained in osteons (Fig. 16d). This suggests that, in addition to zinc ions, there is a pool of zinc bound to proteins, such as alkaline phosphatase, which is always present in mineralizing tissues of the calcium-phosphate type [142,143].

5. Mineral profiles in antlers

The gross composition of antlers (hydroxyapatite, collagen, non-collagenous proteins and water) is basically similar to other bones [17,18,100,138,145]. Antlers contain more organic matrix and less mineral than other bones [138], and, in consequence, require the greatest energy to fracture of all of them [17,138]. As is typical for biological apatites [146], the bone mineral of antlers contains various macro- and trace elements in different concentrations [127,128,130,141,147]. An overview about structural characteristics, mechanical properties and mineral profiles of antlers (and femora, for comparison) from adult red deer is given in Table 1.

It has been demonstrated that antlers from three deer species reared in captivity and fed the same diet, significantly differed in copper content but not with respect to other elements (Co, Zn, Pd, Cd), indicating little interspecific variation in mineral profiles [148]. Several studies showed that antlers are well suited as indicators of environmental pollution by contaminants that are taken up by forming bone [21,149–154]. Others suggested the use of element levels in antler bone as an index of assimilation in nutrition studies [148,155,156]. In fact, the high variability of antler mineral profiles found among studies [127,128,130,147] might reflect the numerous factors that influence it. The research group from Albacete has demonstrated that the chemical composition of antlers conveys important information on nutritional and environmental conditions and may therefore be a useful tool in game management.

The chemical composition of spike antlers is related to body size and differs between high and low quality antlers [17]. Antlers undergo apical growth, and show variation of mineral composition and mechanical properties along their long axis [17–19]. A trend of decreasing bone density and mineral content along the long axis was later also observed in the horncore of a bovid species, the eland (*Taurotragus oryx*) by Cappelli et al. [50]. This was associated with regional variation in the mechanical properties of the horncore.

Landete-Castillejos et al. [17] found differences between antler base (burr) and top of spike antlers in ash percentage and concentrations of Ca, P, K, Fe, and Zn despite the fact that the animals were continuously fed ad libitum. Consistent with the hypothesis that antler chemical

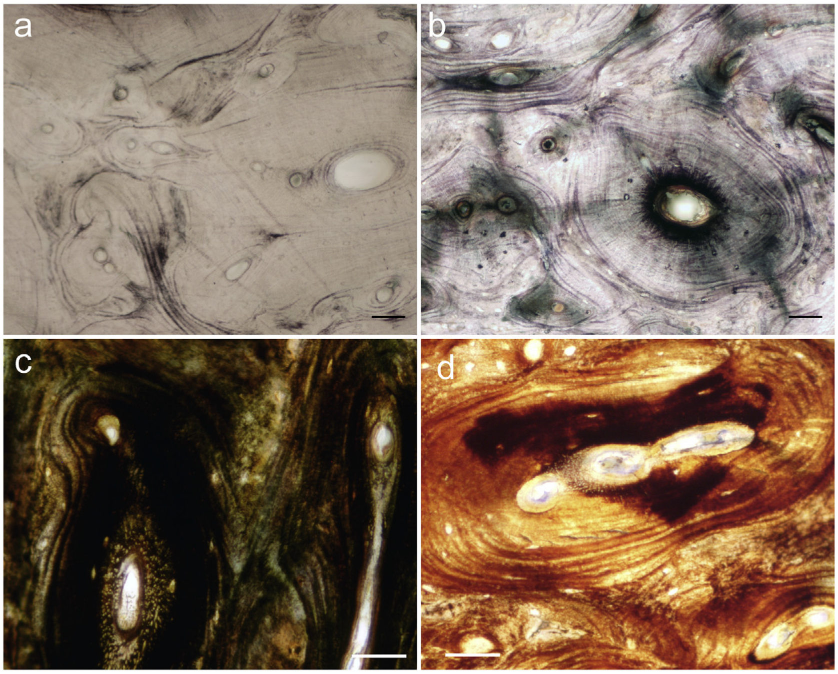

Fig. 16. Zinc histochemistry of the antler cortex in hard antlers of red deer. Zinc histochemistry reveals the Zn-distribution (dark deposits) present at velvet shedding. (a) Completely formed primary osteons show no Zn-staining. (b,c) Primary osteons that were still mineralizing at velvet shedding (from a deer with poor nutrition) show variable Zn-staining, both at the mineralization front (b,c) and in the osteoid (c). (d) Location of Zn-staining in a mineralizing primary osteon after partial Zn-extraction using EDTA. The zinc shown is considered to be bound to proteins (alkaline phosphatase). Scale bars = 50 μm.

composition is an index of male quality, also an influence of body size and growth on ash percentage and contents of Ca, P, K and Na was found.

Another study compared bone mineral composition along the antler shaft between two red deer groups, (1) a captive, well-fed and health-managed herd and (2) free-ranging deer with lower food quality and no health treatment [18]. Significant differences were observed between the two groups, indicative of greater physiological exhaustion in the free ranging deer. The results suggest that the mineral composition of antler bone at least partly reflects the physiological effort made to build it. Similar observations were later also made in a study on Corsican red deer (*C. elaphus corsicanus*) by Cappelli et al. [147]. A decline of bone density from the base to the tip of the main shaft (−17.6%) was accompanied by a reduction in mineral content (Ca, −2.4%; Mg, −3.6%; Cu, −17%; Mn, −4%; P, −2.6%).

A strong influence of diet on antler composition was also found by Estévez et al. [130] who compared red deer from the experimental farm fed ad libitum with food containing 16% crude protein and appropriate amounts of mineral elements (Ca, Mg, Na, K, Zn, and Fe), and conspecifics from a public game estate located in a suboptimal habitat. Chemical analyses showed that the diet of the farm deer had higher levels of Na, Mg and K than that of the wild deer, and that the antlers of the farm deer had significantly higher levels of these elements than those of the wild deer. Differences in dietary mineral supply are also reflected in antler structure. Thus, Landete-Castillejos et al. [126] found a five-fold increase in porosity in top parts of the antlers from wild red deer with poor diet compared to well-fed farm animals.

A study of similar design was recently performed by Cappelli et al. (under review) in roe deer. They compared animals from a Spanish game estate with year-round good food availability and continuous mineral supplementation and a group from a Czech hunting ground with food competitors (fallow deer), reduced food availability during winter and only discontinuous mineral supplementation. The Czech roe deer had smaller antlers than the Spanish ones, with lower contents of sodium and manganese and a lower Ca/P ratio. However, contrary to what had previously been observed in red deer [18,19], no clear trends in mineral composition were observed along long axes in the roe deer antlers, which is probably related to their relatively small size.

Varying weather conditions and resulting variation in the composition of their plant diet can also affect antler quality in deer. Thus, a study by Landete-Castillejos et al. [20] demonstrated variation in antler bone composition and mechanical properties between consecutive years in a red deer population due to the occurrence of extraordinary late winter frosts in one year. This late-frost event probably influenced the mineral composition of the plants eaten by the deer. Similar effects of weather conditions on antler quality, exercised via availability or composition of food, have also been reported by other authors [157–161]. The most comprehensive study of how weather conditions can indirectly affect antler quality was prompted by an extraordinary frost event in February of 2005, the time when plants are sprouting in central-southern Spain. This event was associated with a high frequency (55%) of antler breakage in red deer from the studied game estate [20]. In addition, reductions in antler mass (30%), impact energy absorption (27%), and work to fracture (10%), and changes in mineral composition (e.g. increased Si content, decreased contents of Mn, Na, and Zn) of antler bone compared to antlers grown after a standard winter were observed. Interestingly, the red deer from the experimental farm of the University of Castilla-La Mancha at Albacete, which had been exposed to the same weather conditions, showed none of the above changes in the quality of their antlers. This was attributed to the fact that, contrary to the animals from the game estate, the deer at the experimental farm experienced no change in the quantity or quality of their diet. It was assumed that, as a response to the cold stress, plants had increased their Si uptake (a typical stress response) and reduced their Mn uptake, which in turn affected the chemical composition of the antlers grown by the wild deer that fed on these plants. These observations prompted an experimental study in which Mn was administered (injection) to red deer stags fed a balanced diet [128]. This treatment led to an increase in antler bone concentrations of Mn, Ca, Na, P, B, Co, Cu, K, Mn, Ni and Se and a decrease in Si. Manganese supplementation furthermore caused an increase in the impact energy and work to fracture of the antler bone. A further study found that composition and mechanical properties of antler bone differed between wild deer in natural parks under public management and deer from game estates or farms receiving food supplements [162].

There is thus ample evidence to suggest that overall mineral composition of the antler and its variation along the antler's long axis reflects availability from both diet and body stores. It has further been shown that mineral composition, structure, particularly the amount of cortical porosity, and mechanical properties of antlers are correlated, and that antler quality is strongly influenced by diet quantity and quality during antler growth and the state of body reserves. There is also evidence to suggest that, in addition to Ca and P, also other mineral elements (like Mn) affect the growth, composition and mechanical properties of antler bone [20,128]. Copper may also be among those elements, as it plays an essential role in the normal maturation of collagen [163]. Copper deficiency may delay cartilage mineralization with negative effects on skeletal growth [164], and affect Ca and vitamin D3 metabolism [165]. Insufficient Cu constitutes one of the most common trace element deficiencies in deer [166]. As Cu deficiency is associated with bone fragility [167], insufficient Cu uptake during antler growth could lead to an increased risk of antler breakage. A study by Gambín et al. [127] assessed the effects of parenteral Cu supplementation on the antlers of yearling and adult red deer fed a balanced diet. They observed an only moderate increase in cortical thickness of antler bone in the Cu supplemented animals, which probably indicates that the normal diet already contained sufficient amounts of that trace element.

6. Mechanical properties of antler bone

The most important parameters used to characterize the mechanical properties of the bone material (data for antlers of adult red deer are summarized in Table 1) are (1) Young's modulus of elasticity *E* (a measure of material stiffness), (2) bending strength (*BS*, the maximum stress held or minimum load per unit of volume required to break a specimen), (3) work of fracture *W* (the work necessary to break a specimen), and (4) impact energy absorption *U* (the energy required to break a specimen in impact) [2,136,168]. John Currey was intrigued by the fact that antlers had the greatest impact energy absorption of all bones studied, which he attributed to the low mineral content of antler bone [46]. Initially, he considered that antlers are wet bones when used in fighting. This erroneous assumption might have been caused by the appearance of antlers cut immediately after velvet shedding, when their spongy core is still wet. The view that hard antlers not only possess a wet core but are also still living structures, is indeed held by some researchers [54,55]. A more recent study, however, clearly showed that at the time when they are used for fighting the antlers are dry structures, whose residual moisture is in equilibrium with the humidity of the surrounding atmosphere [57]. Only shortly after velvet shedding, the antlers still had an increased moisture content, but within a short period they dried out [57]. These findings are inconsistent with the view that hard antlers are living structures with a sufficient blood supply to keep even the antler core wet.

The studies also demonstrated that dry antlers are actually mechanically better suited for their role in fighting than wet bones [5,57]. Compared with wet bone, dry antler had a considerably higher bending strength and a much higher work to fracture. This was accompanied by an only small reduction in stiffness (12% lower Young's modulus) of the dry antler compared with wet bone. Corroborating earlier results [46], it was further shown that wet antler can barely be broken on impact [5,57], although biologically this is irrelevant because when used for fighting the antlers are dry. The low modulus of elasticity of wet antler renders it unsuitable for this function, because in the pushing fights between males it would tend to be distorted by bending rather than to serve as a tool to “brush aside” a rival. Thus, while most internal bones, particularly long bones, have evolved to being stiff and resist loading, antlers are specifically adapted to their role in fighting, combining stiffness and resistance to impact.

As already stated above and mentioned also by others [e.g., Picavet and Balligand 145], the mechanical properties of antler bone are, among other factors, related to its histological structure. Various histological parameters, such as bone porosity, osteocyte lacuna number, primary and secondary osteon populations, and collagen fiber orientation have been assessed in cortical osteonal bone [169]. Of these, collagen fiber orientation seems to be the most important one [169]. Collagen fiber orientation can be determined quantitatively in sections under polarized light [139], thus providing a good index for correlation with mechanical properties.

The porosity of a bone is strongly related to its mechanical properties, particularly material stiffness [170]. It has been shown that a small increase in porosity leads to a disproportionately large loss in bone strength [171,172]. One of the key structural features of hard antlers that may affect their mechanical properties and the frequency of breakage is therefore their degree of cortical porosity. Several studies demonstrated a relationship between the quantity and quality of their diet and the porosity and mechanical properties of antlers in red deer [18,19,126].

Antler bone, regardless of whether present in a young or old individual is always a developmentally young bone, composed largely of primary bone tissue with only few secondary (modeling) osteons. In contrast, due to the remodeling process, most other bones of adult mammals are composed of a mixture of bone packages that differ in their developmental age. Antler bone may thus constitute a simpler model than internal bones to assess the effects of nutrition on bone structure and biomechanics. On the other hand, due to the limited lifespan of antlers, long-term (chronic) effects of dietary regimes or other external factors on bone parameters cannot be studied in antlers.

There is increasing evidence that bone mechanical properties are affected also by other factors than mineral density, Ca content and porosity [173,174]. Thus, e.g. the mechanical role of cement lines (reversal lines) around secondary osteons is discussed [175,176], or, in the case of antlers, the possible equivalent function of the hypermineralized seams around primary osteons [133]. Landete-Castillejos et al. [19] observed an effect of nutrition on the chemical composition of antlers in red deer that was associated with mechanical properties of the antlers (greater values of *E*, *BS* and *W* for the better-fed and health-managed deer). Statistical evaluation of the data suggested an association between bone chemical composition and mechanical properties of antlers independent of their ash content [19]. There is also evidence to suggest that mineral composition, cortical thickness and mechanical properties of antlers are influenced by Mn uptake [20,128]. This, and the observed effects of Cu supplementation on antler quality [127] are of potential interest to both game managers and deer farmers.

7. The potential importance of antler studies for bone biology

The above sections provide a broad overview of a peculiar type of bone. The reasons for studying antlers are manifold and go far beyond mere zoological curiosity. Thus, the study of antler development, structure, composition, and biomechanics may provide important information for many fields of bone biology. As antlers are the only example of complete regeneration of a large bony appendage in a mammal [9,10,12], a better understanding of this process may help to find ways of stimulating the regeneration of digits or entire limbs in humans.

Antlers are the fastest growing bones in the animal kingdom, and in the red deer stags in prime condition can produce antlers of up to 23 kg combined bone mass during a growth period of about four months (Landete-Castillejos, unpubl. Observ.). It may be assumed that antler growth rate is close to the maximum of bone growth that is physiologically possible in a mammal. This probably makes the bone growth process in antlers, and the resulting antler structure, composition, and biomechanics, particularly sensitive to effects of nutrition (both deficiency and supplementation) or pharmaceutical products (e.g. anti-resorptive drugs). However, when using antler bone as a model for mammalian bone, one must keep in mind the specific properties of antlers when extrapolating results to (ordinary) internal bones.

Antlers grow appositionally, and different stages of the growth process are simultaneously observable from base to top in forming antlers [100]. Several studies found gradients in chemical, structural, and mechanical parameters and variation in physiological processes along the antler's long axis [17,18,100,126]. Studies using antlers as a

Fig. 17. Abnormally shaped velvet antlers of a red deer. The main beam is bent downward, which is attributed to insufficient mineralization caused by a high parasite load.

model for mammalian bone must pay particular attention to this variation from base to top.

Antlers have been used in environmental studies to assess ambient levels of certain contaminants [21]. Their formation during a species-specific, seasonal growth period makes antlers "naturally-standardized" monitoring units [21]. The analyses of larger time series of antlers from a certain region can therefore be used to reconstruct temporal changes in environmental contamination with bone-seeking contaminants like fluoride and lead [150–152]. A promising field for future studies is the analysis of stable-isotope signatures in antler bone, such as the determination of the ratios of the stable Pb isotopes 204, 206, 207, and 208 for source apportionment of lead in the environment.

Antlers are also useful structures for studying effects of factors (in addition to nutrition) that influence bone formation. For example, antlers with downward bent beams are occasionally observed (Fig. 17), which in the German hunting literature have been given the telling name "Gummigeweihe" ("rubber antlers") [63,64]. Their formation is attributed to mineral deficiency, the action of gravity on the insufficiently mineralized antlers causing their bending. Impaired mineralization has been related to habitat conditions (areas with poor mineral supply) or heavy parasite (e.g. lungworm or liver flukes) loads.

Antler growth opens the way also to potentially interesting studies not concerned with the antler itself. The great demand for mineral elements (calcium in particular) during antler growth gives rise to a cyclic remodeling in the postcranial skeleton in association with the antler cycle [83], which has also been referred to as cyclic physiological osteoporosis [82,177,178]. Comparing the physiological process in deer with the skeletal disorder osteoporosis in humans may provide interesting insights into possibilities of attenuating or treating that disorder. Borsy et al. [177] studied gene expression patterns in deer rib bones during antler growth-related bone resorption and repletion and other periods of the year. These expression patterns were compared with those in femoral bones of humans with osteoporosis and non-osteoporotic controls. The study found that some of the genes upregulated during physiological osteoporosis in deer could be used as additional markers of osteoporosis in humans.

Several studies analyzed gene expression patterns in the antler growth region to identify the functional regulation of rapid antler growth [95,178–180]. The results from these studies can be used to stimulate bone growth and mineralization processes in humans, e.g., during fracture healing. In general, the antler is a useful model to study cell metabolism and proliferation at the upper limit of fast growth. Thus, some authors [181,182] studied the fast nerve growth that occurs during antler regrowth and the factors involved in the regulation of this exceptional case of repetitive nerve regeneration.

Only very few cases of bone tumors have been reported in antlers [183], and it has been suggested that developing antlers are particularly resistant to tumor formation [184,185]. Zoo records furthermore show that cancer incidence rates in deer are lower than in other mammalian taxa [33]. A recent study showed that gene expression profiles in growing antler bone are more similar to those of osteosarcomas than to normal bone tissue, indicating similarities in growth programs between antlers and tumors [42]. It was, however, also found that many tumor suppressor genes are strongly expressed in developing antlers, which is considered a protective mechanism against tumor formation. There is thus evidence that antler growth combines oncogenic and tumor suppression pathways in a way that enables rapid growth and prevents tumorigenesis. It has also been observed that velvet antler extracts showed anti-cancer properties both in cultures of prostate cancer cells and in xenograft models [186,187]. It remains to be shown whether these findings can open up new ways of cancer therapy in the future.

The ultimate goal of regenerative medicine is to find ways of stimulating the regrowth of amputated digits or limbs in humans, which requires both rapid cell proliferation and strictly controlled pattern formation. At the same time, tumorous transformation has to be avoided. The study of the tight developmental control of antler growth can likely provide important clues for achieving these objectives.

In summary, deer antlers are unique structures because they are periodically regenerated, they are the fastest growing bones in the animal kingdom, and their growth is coupled to an intense bone remodeling in the skeleton. These features and, of course, the special mechanical properties of antlers, triggered the interest of John Currey into these structures. As we have tried to show, antlers have a huge potential to serve as a model for the regulation of bone growth and mineralization processes as well as in other fields of biomedical research.

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