The Application of Bone Histology for Species Identification in Archaeology; with a Photo Catalogue

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1 Species identification in archaeology

1.1 Introduction

When reconstructing the human past, one important element is the relationship between humans and animals. Animals were used for many purposes. Foremost, animals were used as a food source, whether domesticated or hunted, showing a range of different animals dependent on cultural or religious preferences and availability. Secondary animal products like hides, wool, horns and bones were used for clothing and as artefacts. In addition, animals like cattle and horses were used for traction and transport. Thus, identification of the animal species is a basic routine in archaeozoology, obtaining information about the purposes certain animals were used for.
Animals could also be a part of ritual practice, like sacrifice or burial. Cremation graves for instance can contain human and/or animal bones. In reconstructing the burial ritual in physical anthropology, it is therefore important to establish whether a bone is human or animal. This information is also relevant for the decision making process in archaeological heritage management: whether one is dealing with a deposit of some burnt animal bones or a human cremation can be essential in deciding whether an archaeological site should be protected or not.
In archaeozoology fish and bird bones can mostly be set apart from mammal bones. When dealing with fairly complete bones, the mammal species can also in most cases be identified by morphological or metrical means, with the help of reference series, atlases, and comparing measurements and indices (Schmid 1972, Brothwell 1981, Bass 1987, Cohen & Serjeantson 1996). However, when dealing with bone fragments burnt bones or worked bones, metrical means of identification can not be applied and identification has to be achieved through morphological comparison with a reference series. In such a reference series it is essential that all the skeletal parts of different species, relevant to the archaeological period, are available for comparison. Also, a reference series should display the variability found in a species due to age, sex, sizes and even pathology. Regretfully however, bone fragments can not always be assigned to a specific mammal species through morphological inspection and comparison. In those cases identification may only be possible to the general level of the category large-sized or medium-sized mammal, which can also imply a human provenance. Nevertheless, we would like to know whether we are dealing with human and/or animal bone in for example a cremation grave. In the case of animal bones, we would also like to know what animals were given as grave gifts and what kind of species were used for the making of certain artefacts (Iregren 1997, Deschler-Erb 1998, Johansen *et al*. 2000, Deeben *et al*. 2006).

1.2 Biomolecular identification methods and their application on archaeological bone

There are other methods available to distinguish between human and animal bone or to identify the species. Biomolecular methods use DNA and proteins to identify species. DNA is the genetic material found in every cell. It can be found in the nucleus (nDNA) or outside of the nucleus in the mitochondria (mtDNA). Specific combinations of the basepairs, which constitute DNA, code for certain characteristics. These parts of the DNA are called genes. All genes combined constitute the genome. Most genes carry the coded information for the making of proteins. These are organic compounds made of subunits, amino acids, arranged in a chain and joined together by peptide bonds.
The sequence of amino acids in a protein is defined by the sequence of a gene, which is encoded in the genetic code. Proteins can serve as hormones, can bind and carry substances and serve as enzymes.

A lot of work has been done on DNA extraction and analyses have shown great potential in disciplines like forensic science and archaeology. Although only minute amounts of DNA are present in ancient material (if any) the development of the polymerase chain reaction method (PCR) has ensured that these fragments can be amplified and their sequence analysed. Crouse and Schumm (1995) investigated the possibility of using nine PCR-based human STR (short tandem repeats) systems for species identification in primates. Initial results show a possibility of using STR systems in DNA as a means to distinguish orang-utan, chimpanzee and gorilla. Repetitive markers were also investigated by Guglich *et al.* (1994). Their goal was to determine the species origin of animal tissues in poaching cases with a technique that would be faster and less expensive than other available techniques. Although the developed technique of visual assessment of repetitive DNA bands, based on nuclear DNA (nDNA) does not work for a low copy number of DNA, such as found in archaeological material, the samples from wildlife forensic cases were always sufficient for analysis. Forensic wildlife identification was also the goal of the study by Murray *et al*. (1995). In a preliminary survey on the mitochondrial DNA of 15 ungulate species, sufficient species specific variation was observed to establish species origins. Deer, however, could only be identified to the genus level. Prerequisites of applying this technique, like the effect of DNA quality and the average size of the DNA fragments on the likelihood of successful amplification and identification, still have to be tested on archaeological material. Melton and Holland (2007) developed a technique based on mitochondrial DNA to identify the species origin of nonhuman casework samples in forensic science. The method has so far only been applied to hair and tissue, but was felt to be applicable to bone as well. Another assay was developed for forensic science based on DNA sequencing of two short mitochondrial DNA amplicons using pyrosequencing technique (Karlsson & Holmlund 2007). The two sequences show a high divergence factor, discriminating almost all mammals in the 28 species of European fauna investigated. The assay also was reported to work well on artificially degraded DNA and samples with low DNA concentration. Closely related pig and wild boar, different seals and deer species could, however not be discriminated. Although this data was not included in the study, the assay is said to have possibilities of differentiating between human and animal, especially primates, as well.

Not only DNA contains information about the animal species or can help to distinguish between animal and human samples. Immunological methods based on protein sequences of e.g., collagen and albumin can also be used. Proteins, especially collagen, even have a better preservation potential than the relatively fragile DNA molecule and the analyses are less sensitive to contamination. Ubelaker *et al.* (2004) tested the possibilities of albumin for species identification when dealing with small skeletal fragments. They conducted a blind test on six known bone samples (3 human and 3 non-human) with a radioimmunoassay including antisera raised in rabbits. All the human samples were correctly distinguished from the non-human ones. One of the nonhuman samples was correctly identified as deer, setting it apart form cow, dog, goat and pig. Buckley *et al*. (2008) tested collagen as a biomarker for species of farm domesticates like cattle, sheep and pig, using MALDI-MS (matrix assisted laser desorption/ionisation mass spectrometry). Because of the survival of collagen in archaeological bone it has potential for archaeological species identification cases.

There are general problems that arise when trying to apply biomolecular methods to archaeological material. Firstly, bone can decay due to chemical deterioration of the organic phase, chemical deterioration of the mineral phase, or microbial attack (Collins *et al*. 2002). Immunological methods developed on fresh bones can show e.g. false positive results because of decayed biomolecules and micro-organisms (Brandt *et al*. 2002). Secondly, when dealing with burned bones, the burning at high temperatures severely restricts their application. PCR/DNA technology as a means to identify species in archaeology was investigated by Newman *et al*. (2002). Depending upon the age, degree of bone preservation and the quality of the DNA, some bone fragments could be identified as bison, sheep and goat. Differentiating between domestic and wild animals of the same species was less successful. Hodgins and Hedges (2001) investigated the immunological properties of fresh and old collagen. They found that relatively species-specific antigens can survive in collagen for extremely long periods of time, but there is loss of species specific characteristics in archaeological bone over time. In their test on skeletal fragments Ubelaker *et al.* (2004) included a human bone from a prehistoric site.

Although the bone was correctly attributed, it was close to the limits for identification due to its antiquity. It was concluded that protein preservation problems can occur when dealing with archaeological bones, due to age. In a study by Brown *et al.* (1995) hybridization probing of nuclear DNA using PCR was conducted on cremated bones from an early Bronze Age cemetery cairn. Strong positive signals were detected, but no actual DNA could be extracted. These findings have not been confirmed by other authors. Cattaneo *et al*. (1992, 1994) introduced ELISA (enzyme-linked immunosorbent assay) as a method for tracing the species specific blood protein albumin in human inhumations and cremations in order to differentiate human from animal bones. Albumin could be discerned in ancient human bone and although its occurrence was less than in inhumations, it could be traced in bones burned below 300 ºC. However, the burning temperature in cremated remains is in general higher (Wahl 1982). Such bone fragments are not expected to contain information for species identification at the biomolecular level, and a different method is required to identify species in this type of material. Ottoni *et al.* (2009) examined the preservation of ancient DNA in cattle bones from a medieval site. DNA preservation is not related to the presence of intact collagen fibrils and it is even possible that heating, at least below 140 ºC, can actually increase DNA preservation. However, bones burnt or cremated above 170 ºC are not expected to contain authentic DNA sequences.

1.3 The research potential of bone histology

Histology is the study of cells and tissues of plants and animals under the microscope (histos = tissue). It is also called microscopic anatomy, as opposed to gross anatomy which involves structures that can be observed with the naked eye. Bone histology has contributed to several sciences, e.g. biology, veterinarian science, medical science, palaeontology, paleoanthropology, physical anthropology, paleopathology, forensic science and archeozoology. Some examples are listed below to show not only the variety of uses, but also the general questions when dealing with bone structure variations.
In biology bone histology has contributed in various areas. Study of the bone structure gave insight into the growth of long bones in general (Amprino & Godina 1947, Enlow 1963). They found that the bone structures are dependent of growth rate of the animal. In their opinion the growth rate of a species is responsible among other less well-known factors, for the variations in the bone structure between species and differences within a species (the "rule of Amprino"). Enlow's work (1963) on the growth of long bones presented and explained the fundamental principles involved and the resulting histological structure differences within and between bones. Another fundamental work on bone structure was performed by Enlow and Brown (1956, 1957, 1958). They investigated the bone structure in various species, extinct and living. This gave insight into the histological relationships of species and the variations within a species. Following the rule of Amprino a histological study was conducted to obtain more information about the relationship between bone structures and function (de Margerie *et al*. 2004). As an example, because of the long antarctic winter, the king penguin has only a short period available to reach its adult size. This results in faster growing bone types due to a higher growth rate. Bone structure types are also influenced by biomechanical factors. Laminar primary bone seems to be an adaptation to stress caused by flapping flight (de Margerie 2002). In veterinarian sciences one of the applications of bone histology has been in assessing biomechanical stress changes the bones of horses (Mason *et al.* 1995, Martin *et al.* 1996, Batson 2000).
In palaeontology histological studies of dinosaurs have contributed to a better understanding of their physiology. Comparing dinosaur bone structure to living vertebrates whose physiology is known, a rapid, continuous growth can be deduced for some species. This points to a metabolic rate similar to large mammals and endothermy (de Ricqlès 1980). Also bone structure properties, like the organisation of canaliculi, have been investigated to solve evolutionary questions regarding the relationship between dinosaurs, birds and mammals (Rensberger & Watabe 2000). Ornithomimid dinosaurs are more like birds and ornithischian dinosaurs resemble mammals more in their bone microstructure.
Histology has also been used to obtain information on archaeological bones. Paleoanthropological studies of the bone structure in early hominid bones can give information about the individual's age and differences with modern humans in paleoanthropology. It showed that Neanderthals have a low postreproductive survival and that archaic hominids seem to have smaller osteons and less bone turnover than modern populations (Trinkaus & Thompson 1987, Pfeiffer & Zehr 1996).

In physical anthropology, histology can be used for various purposes: to determine the age of an individual, to establish a demographic profile of a population, to determine the minimum number of individuals, to identify specific historical individuals and to identify bone diseases. When dealing with incomplete skeletons and even more with cremated remains, age assessment can be difficult. Bone turnover gives important information about the age of an individual and helps to reconstruct past population and burial rituals (Kerley 1965, Ahlqvist & Damsten 1969, Cuijpers & Schutkowski 1993, Hummel & Schutkowski 1993, Fontijn & Cuijpers 2002). Histology can also be a valuable tool in determining the number of individuals in mixed skeletons and cremation graves (Stout & Gehlert 1979). It has even been used in identifying a specific historical individual (Stout 1986). When dealing with pathological features in archaeological bone it is not always clear whether it is indeed pathology and not changes due to diagenesis. Also the underlying cause of the pathological changes is difficult to determine. With the help of histological thin sections pathological features can be examined more in detail and as such give information about the health of an individual or even cause of death (Maat & Uytterschaut 1984, Schultz 1986). In archaeozoology histology has been used to determine the provenance of material used for making artefacts, antler versus bone and addressed the question of domestication (Lasota Moskalewska & Moskalewski 1980, Paral *et al*. 2007). Last but not least, histology has been used in forensic science to determine the human or animal origin of bone fragments (Owsley 1985).

1.4 The development of a histological identification method for archaeological bone fragments

The applications listed above show the possibilities of histology as a tool in many sciences. Specifically it demonstrates that when dealing with archaeological bones, unburnt as well as burnt, histology can be used to obtain information about humans and animals in the past. Far from being made redundant by biomolecular methods, bone histology remains a valuable alternative in species identification when dealing with archaeological bone (Cattaneo *et al*. 1999, 2001). Admittedly, the histological structure can also be heavily influenced by degradation. Soil infiltration, tunnelling by bacteria and chemical processes can be a limiting factor in microstructure recognition (Jans *et al.* 2002). Although burning can make the histological structure less visible, it has been shown that in general even burning at high temperatures of up to 800 °C still leaves the microstructure intact (Herrmann *et al*. 1990). The subsequent changes in the composition of the bone actually protect burnt bone against microbial attack because of the loss of collagen (Kars & Kars 2002: 217). Also, histology has already been used in identification cases in forensic science (Owsley 1985).

The aim of this PhD research is to investigate the possibilities of histology as an identification method in archaeology. Keeping in mind Amprino's rule and the findings on biomechanical factors, bone structure can be an indication of species. Problems with species identification in physical anthropology and archaeozoology arise when dealing with relatively small unburnt and burnt bone fragments. Therefore, the method to be developed has to be applicable within the constraints of fragmentation, degradation and burning. Firstly, the investigations concentrated on possible differences between human and animal bone. Secondly, differences between a number of animal species were looked for.
Histological analysis of bone can be conducted in two ways, by means of comparison and description (qualitatively) or by means of counting and measurement (quantitatively). Quantitative histological aging methods are, however, difficult to apply to burnt bone fragments, because the exact shrinkage is not known (Hummel & Schutkowski 1993). Because species identification problems often occur in cremated remains, it was decided to develop a qualitative method in which shrinkage percentage does not play a role. The idea of using histology for species identification is not a new one. However, earlier qualitative histological studies do not provide answers to the identification problems in archaeology (Demeter & Mathias 1928, Enlow & Brown 1956, 1957, 1958). In these studies bones from different skeletal categories in various species were investigated and compared. However, bone structure is very diverse. There are differences between species, between different bones of the same species and even within a single bone (Enlow 1966).

Therefore, a novel approach was chosen for this PhD research. It was decided to conduct an in-depth study concentrating on one bone category from a carefully selected number of species relevant to archaeology. This would give insight into the ontogenetic variability and, if possible, to infer common characteristics, which would allow a differentiation between human and animal bone, and perhaps even make it possible to tell animal species apart. Because of the range of individuals within a species and long bones within one individual needed, it was not feasible to investigate in this thesis the bone structure in all relevant mammal species. If histology proves to be a useful means of identification, other species can be additionally researched, depending on specific archaeological questions and periods under consideration.
The bone category chosen for this identification study is the diaphysis of long bones. These can cause identification problems in archaeology when fragmented, because less distinguishing features are present than in the epiphyseal parts. They are also often present because they constitute a large part of the skeleton in mammals and are sturdy. After burning, for example 58.7% of the bone fragments left is from long bones (McKinley 1989). They often constitute most of a cremation find and sometimes even are the only bone fragments left (Cuijpers 1994). Also general works on the development of and the variability within diaphyseal bone structure were available that would facilitate the development of a histological method for archaeology (Demeter & Mathias 1928, Enlow & Brown 1956, 1957, 1958, Enlow 1966). It was decided to compare human bone structure to five animal species: horses, cattle, pigs, sheep and goat. These animal species in particular were chosen because they are relevant when dealing with identification questions: reconstructing animal use and burial ritual in archaeology. These species were also chosen because of their availability in archaeological samples. As stated above, the bone structure can differ even within a bone. In order to develop an identification method for diaphyseal bones in general, different long bones within one skeleton, from different individuals of different ages, sexes and heights have to be studied. Modern animals, especially cattle and pigs are much bigger than their archaeological counterparts. Long bones from archaeological samples had to be available to exclude possible differences due to size.

The second Chapter deals with the classification system used in this thesis. In order to describe and compare the bone structure of the species studied a classification system had to be applied. In past studies several classification systems were used, depending on the research questions. In this thesis, it was decided to apply the system of de Ricqlès (Francillon-Vieillot *et al*. 1990). This broad, open system categorises the periosteal diaphyseal bone structure into different levels. A number of bone structure types and characteristics were added to this system to make it more applicable in finding species differences when dealing with archaeological bone fragments. To facilitate the use of the developed histological species identification method, the terminology used in the present thesis is also compared with those on other studies on species classification.
Chapters 3, 4, 5 and 6 present the results of the study on diaphyseal bone structure in humans, horses, cattle, pigs, sheep and goats. In Chapter 3, the results of study on the diaphyseal bone structure in late juvenile-adult humans, horses and cattle are shown. Unidentified these bone fragments would be grouped together as “large-sized” mammals. Differences between human and animal bone structure were found. Also the applicability of these findings was tested on archaeological bone fragments in a blind test. An identification method was presented that was able to distinguish between human and animal bone, taking into account the effects of burning and degradation.
Chapter 4 describes the possibility of differentiating between horses and cattle. This can answer archaeological questions with regards to food economy, use of bones for artefacts and grave gifts. Two blind tests were conducted to formulate a distinguishing feature between these large-sized mammals allowing for identification of archaeological bone fragments, even when burned.
Another use of the large-sized mammals is traction. In Chapter 5 the bone structure in oxen (castrated cattle) is compared with other cattle and horses. An observed difference in metapodial bone structure could be very helpful in establishing the presence of oxen in cattle samples. More research on a larger sample of oxen, including archaeological bones, is needed to allow for conclusions about the different economic use of cattle and the subsequent effects on food production yields.

Chapter 6 presents the study on medium-sized mammal bone structure. The diaphyseal bone structure of children is compared with those in pigs, sheep and goats. A difference between human and animal bone structure is present and allows for identification of human vs. animal bones. Differentiating between the animal bones was however not possible, since no distinguishing differences could be ascertained. The validity and applicability of the distinguishing characteristics between human and animal bone structure within the medium-sized mammals was tested on a blind sample of archaeological bone fragments. Burning was also taken into account.
Additionally, photomicrographs of all the observed bone structure types are provided in Chapter 7. This photo catalogue provides a visual means of applying the developed species identification method by showing the different bone structure types, bone characteristics and special features in diaphyseal bone structure. In the literature several histological classification systems, each with their own terminology are found. This may cause confusion when trying to compare results or applying a method. The photo catalogue will enable visual comparison and identification of structures and therefore enhance the applicability of the devised species identification method even when using a different terminology.
Chapter 8 summarizes the most important findings of this study and its implications and opportunities for future archaeological research.

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2 Terminologies and classification systems for discerning species differences in diaphyseal bone structure

2.1 Classifying bone structure

In order to describe and compare bone structure for species identification, a bone classification system is required. Because the long bone structure has been studied in many sciences, different systems with their own terminologies have been developed for different goals. At the start of this thesis, several classification systems were compared, keeping in mind the aim of the study: to develop a histological species identification method to answer archaeological questions.
Bones function to move and support the body. They also have a protective role in the skeleton and produce red and white blood cells. Bone consists of cells, fibers and a groundmass. Macroscopically two types, spongious and compact bone, can be distinguished. In long bones, the cylindrical shaft (diaphysis) is made up of compact bone around a medullar cavity, containing the bone marrow. Spongious bone is found in the epiphyses of the long bones. Compact bone is a dynamic tissue, because long bones grow in diameter and length. During growth, however, their overall shape has to be preserved. Also compact bone is a hard tissue and, unlike e.g., muscle tissue, not capable of growing interstitially. Therefore, growth is achieved through structural remodelling (Enlow 1963). The diameter of the bone is increased by an appositional process, in which primary bone is deposited on an existing surface, e.g., by the periosteum (Table 1), accompanied by resorption of the contralateral surface. As the bone grows in length relocation of various bone regions occurs; a successive repositioning of regions into adjacent areas. Apart from this structural remodelling, also secondary remodelling of the primary bone structure takes place. This is a common process during life, due to increasing age or mechanical factors. Primary bone is resorbed by specialised bone cells, the osteoclasts that dissolve the bone mineral. The resulting resorption space is subsequently filled in by osteoblasts, bone-forming cells, resulting in a secondary osteon (Table 1). As age increases, totally remodelling of the primary osteons and remodelling of the secondary osteons into subsequent generations can occur. These remodelling processes in long bones result in various bone types, which are classified according to research aims.

One of the earliest classifications systems was published by Foote (1916). He compared the femoral bone structure of 46 animals, including humans, mammals, birds, reptiles and amphibians. The purpose of his study was to list the variations found and, if possible, to determine their significance. In his classification system, three distinct types of bone structure, lamellae, laminae and Haversian systems, were set apart. These types were seen as “consecutive stages of differentiation of one and the same fundamental variety which underlies bone structure in all the terrestrial vertebrates” (Foote 1916: 12).
Goldbach and Hinüber (1955) developed a system to classify the bone structure in long bones for forensic use. Three categories were made. The first category, the general structure (“Grobstruktur”), is divided into bone consisting of no layers, several layers or bone with many layers. The second category is the blood vein net or pattern of vascularisation (“Blutadernetz”); for example whether the veins are longitudinal, circular or radial. The third category is the Haversian systems (“Haverssche Systeme”) or osteontypes; i.e. round, square or oval.
Enlow and Brown (1956, 1957, 1958) studied the bone tissues of the major vertebrate groups, including fossil bones, to present a histological survey. They also proposed a classification system to describe the variety of major structural types of bone tissue found in their study. Three major categories with subcategories were set apart: primary vascular (i.e. longitudinal, radial, reticular, laminar and plexiform), non-vascular and Haversian (i.e. irregular, endosteal and dense).

Table 1 Explanation of the histological terminology used in this thesis.

Periosteum	Specialized connective tissue coat that envelopes long bones, except for their articular surfaces. It contains cells that form primary periosteal bone. In this thesis the term "periost" indicates not the actual periosteum layer, because that is not present anymore, but refers to the periosteal (external) surface.
Endosteum	The bone forming layer on the inside of the bone. Depending on its location in the shaft, bone can be a result of subperiosteal or endosteal deposition. Compact cancellous endosteal bone is characterized by a convoluted pattern.
Primary bone	Initial laid down bone structure in compact bone. In the classification system used, two general primary bone categories, fibrous and lamellar, are listed. The different subtypes are defined by vascularisation: avascular, with primary vascular canals, or with primary osteons.
Secondary bone	Type of bone, also called Haversian bone, which is formed through remodelling of the primary bone structure. The different subtypes used in this thesis are defined by the amount of the secondary osteons: scattered or dense.
Simple vascular canal	Canal in primary bone that has no surrounding lamellae.
Primary osteon	Canal in primary bone that is surrounded by concentric lamellae without a reversal line.
Secondary osteon	Canal, also called a Haversian system, that is surrounded by concentric lamellae and outlined by a reversal line. It is formed through remodelling of primary bone.
Haversian canal	Canal in the middle of a secondary osteon/Haversian system. It can be longitudinal or show canals branching from it: reticular aspect of Haversian canals.
Volkmann canal	Canal connecting secondary osteons and runs perpendicular to the Haversian canal.
Fibrous bone	A primary bone type that is characterised by a haphazard organisation of its collagen fibers. In the classification system of de Ricqlès (Francillon-Vieillot *et al.* 1990), three subtypes, each with a woven component, are defined according to vascularisation. In fibro-lamellar complex bone, the woven component is combined with the lamellar component of the primary osteons.
Lamellar bone	In this primary bone type the fibers are arranged in successive thin layers. It also forms the lamellae surrounding the primary and secondary osteons. In the classification system of de Ricqlès (Francillon-Vieillot *et al.* 1990), three subtypes are defined according to vascularisation.
Orientation	Canals (primary vascular canals and primary osteons) can show different orientations. Longitudinal indicates that the canal runs in the direction of the long axis of the bone, while a circular canal runs in the direction of the circumference of the bone. A radial canal runs in the direction of the middle of the shaft and a reticular canal shows an oblique/slanting orientation. Also several arrangements, combining different orientations, can be set apart. Laminar fibro-lamellar complex bone is made up of longitudinal and circular canals. If also radial connections between the longitudinal canals occur, it is called plexiform fibro-lamellar complex bone.

It was observed that bone could be made up entirely out of one of these tissue types or that a combination of types could be present.

In their overview of skeletal mineralogy and microstructure in the major invertebrate and vertebrate phyla, Francillon-Vieillot *et al*. (1990) published a comprehensive classification system which combined several criteria of looking at the bone structure instead of a single one. Bone tissues are categorised in an open system with different levels. The first level consists of the bone matrices, periosteal and osteonal, each divided into two types. These make up the bone tissues of the second level. The bone tissues of the second level are divided into compact and cancellous. These are both divided into primary and secondary tissue types. The two types of primary compact bone tissue are lamellar and fibrous. Within these two types various kinds of vascularisation can occur, forming subtypes. Secondary compact bone tissue can be Haversian or non-Haversian. Haversian bone can show scattered osteons or a dense osteon structure.

This classification system of de Ricqlès (Francillon-Vieillot *et al*. 1990) was selected for this thesis to describe and to compare the diaphyseal bone structure in the present study for several reasons:

1. The system combines three criteria for looking at bone structure. Previous studies had concentrated on the organisation of the bone matrix (e.g. fibrous, woven, lamellar), patterns of vascularisation (e.g. non-vascular, plexiform, Haversian) or ontogenetic patterns of bone tissue formation (primary, secondary bone). It was felt that a broad system would fit the aim of the thesis best, because distinguishing features between species were looked for, whether these are caused by differences in the organisation of the bone matrix, vascularisation pattern or ontogenetic pattern.
2. The system is devised as an open system and as such gives room for adding bone structure types thought to be of discerning value for identifying the species studied.
3. Clear definitions of the bone tissue types are provided in the study of Francillon-Vieillot *et al.* (1990). They also relate their terminology to the ones used in earlier studies. This will facilitate the applicability of the system for this thesis and be helpful incorporating other studies.

4. The division of primary bone types is based on the "rule of Amprino", according to which the tissue structure of primary periosteal bone correlates with its rate of deposition (de Ricqlès 1980). Thus a correlation between adult animal size and bone structure can be inferred (Amprino 1947). This generally accepted "rule" was thought to be very useful when developing an identification method for human vs. animal bones, because humans grow relatively slow compared to e.g. horse and cattle.
5. Instead of classifying all bone structure types with osteons as Haversian bone, primary and secondary bone structure are set apart. Secondary osteons occur through remodelling of the primary bone structure and steadily replace the primary bone structure in humans (Kerley 1965). Apart from age, the amount and distribution of secondary osteons is also influenced by mechanical factors (Mason *et al*. 1995, Martin *et al*. 1996). The distinction between primary and secondary bone in the system of de Ricqlès allows for the description and comparison of bone fragments of individuals whose age is unknown and of which provenance, except for diaphyseal, is also unknown.

2.2 The development of the classification system used in this thesis

The system of de Ricqlès incorporates bone matrices and bone tissues (Francillon-Vieillot *et al.* 1990: 500). It also classifies cancelous and compact bone. To facilitate its use for this thesis and to enhance its applicability for species identification, the system of de Ricqlès was simplified, selecting only the part on bone tissues. Also, because the thesis deals with compact (Haversian) bone structure in mammals, only the part relevant to these bone tissues was used. In this adapted system, compact bone tissue is divided into primary and secondary bone types (Table 2). Primary bone structure is divided into lamellar (1a-c) and fibrous (1d-f) bone types. Within lamellar and fibrous primary bone types, diffe-rent types of vascularisation can be distinguished: non-vascular, simple vascular canals and primary osteons. Following the study of Francillon-Vieillot *et al.* (1990), these can be subdivided according to the orientation of the canals, for example reticular and radial. Secondary compact (Haversian) bone is divided into scattered and dense bone, depending whether any primary bone is still left between the secondary osteons after remodelling. During the investigations, several bone structure types were added to the open system. Also some bone characteristics were incorporated into the study (Table 3). All these additions were made to describe and compare the bone structure of the species studied, in order to find distinguishing features. The photo catalogue in Chapter 7 provides illustrations of all the observed bone structure types and characteristics.
In the study on late juvenile-adult humans, horses and cattle several additions were made to the adapted system (Chapter 3). Four types of fibrous primary bone, all subtypes of fibro-lamellar complex bone, were added: laminar/plexiform with primary osteons in a row or band (1f5), radial with primary osteons in radial rows (1f6), fibrous bone with primary osteons (1f7), and fibrous bone with primary osteons in circular rows (1f8). Another addition was a bone structure type showing a combination of the two main primary bone types, lamellar and fibrous bone. This so-called pseudo-laminar (personal communication de Ricqlès) was categorised as a subtype of fibro-lamellar complex bone. In the system of de Ricqlès, the categories of primary bone are not sharply contrasted and must be seen as a continuum (de Ricqlès 1983). Overlap can therefore occur between types. Also added to the system were two subtypes of secondary (Haversian) bone. In several thin sections, an alignment of secondary osteons was observed. A row of three or more secondary osteons, scattered or dense, was categorised as organisation of the secondary bone structure. Absence or presence of this organisation in secondary bone structure was noted. An alignment of five or more primary or secondary osteons, termed osteon banding, was mentioned as a unique characteristic of animal bones (Mulhern & Ubelaker 2001).
Two characteristics of bone structure were also included in the study. The first concerns growth layers. In the thin section of horses and cattle long bones growth layers were often observed. This refers to a difference in growth rate compared to the rest of the bone (Castanet *et al.* 1993). They are set apart by lines and can clearly be distinguished from the general bone structure. Such layers are different from lines of arrested growth, which also suggest a difference in growth rate (Herrmann & Danielmeyer 1994).

Table 2 Classification system used in this thesis, adapted from the system of de Ricqlès (Francillon-Vieillot *et al.* 1990). The figures refer to the photo catalogue in Chapter 7.

Primary (periosteal) bone types
1a: lamellar non-vascular (Figs. 35a and 35b)
1b: lamellar simple (primary) vascular canals
1b1 longitudinal (-)
1b2 circular (-)
1b3 reticular (Fig. 36)
1b4 radial (Fig. 37)
1c: lamellar with primary osteons
1c1 longitudinal primary osteons (Figs. 38a and 38b)
1c2 longitudinal primary osteons with radial canals (-)
1c3 longitudinal primary osteons with reticular canals (-)
1c4 longitudinal primary osteons and radial simple vascular canals (-)
1c5 longitudinal primary osteons in circular rows (Figs. 39a and 39b)
1d: fibrous non-vascular bone (Fig. 40)
1e: fibrous bone with simple (primary) vascular canals
1e1 longitudinal (-)
1e2 circular (-)
1e3 reticular (Fig. 41)
1e4 radial (Fig. 42)
1f: fibrous bone with primary osteons (fibro-lamellar complex)
1f1: laminar (Figs. 43a and 43b)
1f2: plexiform (Fig. 44)
1f3: reticular (Figs. 45a and 45b)
1f4: radial (Fig. 46)
1f5a: laminar/plexiform with primary osteons in circular rows (Fig. 48a)
1f5b: laminar/plexiform with primary osteons in a band (Fig. 48b)
1f6: radial with longitudinal primary osteons in radial rows (Fig. 47)
1f7: fibrous bone with longitudinal primary osteons (Figs. 49a-c)
1f8: fibrous bone with longitudinal osteons in circular rows (Fig. 50)
1f1/1a-c: pseudo-laminar (Fig. 51a)
1f/1a-c: pseudo-fibro-lamellar complex (Fig. 51b)
secondary (periosteal) bone types
2a1: scattered osteons
2a1a: scattered osteons with no organisation (Figs. 52a and 52b)
2a1b: circular rows of scattered osteons (Figs. 53a and 53b)
2a2: dense osteons
2a2a: dense osteons with no organisation (Figs. 54a and 54b)
2a2b: circular rows of dense osteons (Figs. 55a and 55b)

(-) these structures were not observed in the reference series.

From the classification of the bone structure types, however, it can not be discerned whether the bone type is generally occurring in the bone, or is found exclusively in a growth layer. A distinction was therefore made by indicating the bone structure type found in growth layers by an asterisk. This makes it possible to set them apart from the bone types constituting the general bone structure in a thin section. A characteristic that was also noted was the longitudinal and reticular aspect of the Haversian canals in secondary bone (HC1 and HC3).
Normally, secondary osteons with longitudinal Haversian canals, sometimes connected by Volkmann's canals, were observed. However, in secondary bone also Haversian canals displaying several branching connecting canals, giving the bone structure a reticular aspect, occur.
Comparing the bone structure of horses and cattle (see Table 10 in Chapter 4), two subtypes were added to one of the fibro-lamellar complex bone types.

Table 3 Characteristics added to the system. The figures refer to the photo catalogue in Chapter 7.

organisation of the secondary bone structure (Figs. 53a, 53b, 55a and b)
longitudinal and reticular Haversian canals (Figs. 54a, 54b, 60a and b)
growth marks: growth layers (Figs. 56a and b) and lines of arrested growth (Fig. 57)
porosity (Fig. 58)
composition of fibro-lamellar complex bone (Figs. 59a-d)

In their study on calves and foals Mori *et al.* (2003) suggested that a row of longitudinal primary osteons was a bone structure unique to foals. In order to investigate this possible discerning characteristic, laminar/plexiform fibro-lamellar complex bone with primary longitudinal osteons (1f5) was subdivided into primary osteons in a row (1f5a) and primary osteons in a band (1f5b) to fit the description; see Chapter 7 (Laminar and plexiform are lumped together in these fibro-lamellar complex subtypes). Two characteristics of the bone structure were also incorporated into the study. First, the distribution of the two components, lamellar and fibrous, within fibro-lamellar complex bone was noted. Within a lamina, the fibrous and lamellar component did show the same thickness. But also a predominance of either the fibrous or lamellar component was observed. Secondly, an enlargement of the vascular canals was tested for its validity as an identification tool. In the study by Deschler-Erb (1998) "porosity" is mentioned as a characteristic of the bone structure in horses. This "porosity" does not refer to the number of resorption canals present, which is a remodelling feature, but to the size of the simple vascular canals, for example, or the circular primary osteons in fibro-lamellar complex bone. Although in itself a primary feature that connects secondary osteons, enlargement of the Volk-mann's canal was not regarded as porosity.
In the study on the oxen no additions were deemed to be necessary (see Table 16 in Chapter 5). In the photo catalogue of Chapter 7 the metaphyseal bone structure in one of the oxen metapodia is shown as a special feature in order to provide a reference to distinguish between diaphyseal and metaphyseal bone when the provenance of the long bone fragment is unclear.
In the study on medium-sized mammals, only one change was made to the classification system. Sheep, goats and pigs showed many variations of pseudo-fibro-lamellar bone; not only a combination of lamellar with laminar fibro-lamellar complex bone, but also lamellar with reticular fibro-lamellar complex bone and laminar/plexiform with primary osteons in a row (Chapter 7). There-fore, pseudo-laminar bone, a subtype of fibro-lamellar complex bone, was replaced by a broader subtype: pseudo fibro-lamellar complex bone (Table 21 in Chapter 6). To illustrate the difference between diaphyseal bone structure and bone structure found in the metaphyseal part, the meta-physeal bone structure in a pig is shown as a special feature in the photo catalogue (Chapter 7).

2.3 Comparing the terminology used in this thesis with other studies on species identification

When reading studies on bone structure, different terminologies are found, depending on the classification system used. This can lead to confusion and even mistakes when trying to interpret and compare findings. In order to facilitate the applicability of the results described in this thesis, the used terminology will be related to some relevant articles on histological species identification. Furthermore, the photomicrographs in Chapter 7 provide a visual means of identifying bone structure types. This will clarify similarities and differences, enabling species identification even when using a different terminology than the one applied in this thesis.
Mori *et al.* (2003) compared the laminar bone structure in young calves and foals. They made no differentiation between laminar and plexiform tissues. Although, in the present study laminar and plexiform bone are subtypes of fibro-lamellar complex primary bone, plexiform can be interpreted as laminar bone with additional radial connections within the continuum of primary bone categories. Mori *et al.* (2003) observed rows of cylindrical osteon-like structures with Haversian canal-like canals between the concentric hypercalcified lines. Studying their figures, this feature equals a subtype of 1f5, laminar/plexiform with longitudinal primary osteons in a row. The cylindrical osteon-like structures equal primary osteons. The hypercalcified, bright lines mark a lamina (see Chapter 7).
In Mori *et al*. (2005) the long bones of young calves, pigs, and sheep were compared. Pig bones showed a wire-netting bone with laminar bone units, in contrast to sheep bone which only showed laminar bone structure. Wire-netting bone with laminar bone units is termed plexiform bone in the present study. Pseudo-osteons equal primary osteons.
In their paper on the Donner Family Campsite, an archaeological project investigating the camp of Californian emigrants who became snowbound in the winter of 1846-1847, Robbins and Hanks (in press) compared material from this site with bone fragments of large mammals, cervids, bovids and canids.

They present a system in which a division into three categories of bone is made: woven, primary and secondary bone. Within the category of primary bone several types are mentioned: lamellar, plexiform (laminar), primary osteons and lamellar woven bone. This division is different from the one used in this thesis. Primary bone is divided into fibrous and lamellar bone. In all three fibrous bone subtypes a woven component can be found. The subtypes of fibrous primary bone are defined by different types of vascularisation. Also, primary osteons are not seen as a different subtype next to lamellar and plexiform, but are incorporated into lamellar and fibrous primary bone. Subtypes of fibro-lamellar complex bone, like laminar and plexiform, are categorised on the different arrangements of the primary osteons. What exactly is meant with lamellar woven bone is not explained in their paper.

Martiniakova *et al.* (2007) published an article on species determination based on quantitative and qualitative characteristics. They used the classification system developed by Enlow and Brown (1956, 1957, 1958). Compact bone is divided into three categories: primary vascular, non-vascular and Haversian. Primary vascular bone is subdivided into 9 subtypes, among them lamellar, laminar and plexiform. In the system of de Ricqlès, the first two categories, primary vascular and non-vascular, are lumped as primary bone. This is subdivided into lamellar and fibrous, both of which can be non-vascular or vascular. The third category of compact bone described by Martiniakova *et al.* (2007), Haversian bone, is subdivided into irregular, endosteal and dense. Irregular Haversian bone tissue corresponds to scattered osteons in this thesis. Endosteal Haversian bone, in which Haversian bone is restricted to the endosteal margin, is not set apart as a separate category of secondary bone in the present study. In general, it consists of densely packed osteons and is therefore labelled as dense secondary osteon structure. Therefore, in the thesis, dense secondary osteon structure incorporates both endosteal and dense Haversian bone as defined by Enlow and Brown (1956, 1957, 1958). In their study, Martiniakova *et al.* (2007) mention a unique characteristic in cow bones. From the provided figure it can be deduced that the non-vascular bone tissue type corresponds to fibrous non-vascular bone (1d) in the classification system used in this thesis.

In their review article on histological identification methods, Hillier and Bell (2007) distinguished between two bone tissue types within compact and cancelous bone: woven and lamellar. Compact bone is further divided into primary, secondary and avascular bone. Primary bone is defined as newly formed bone, which contains primary osteons. Several types of primary bone are mentioned, among them laminar and plexiform. The first difference with the present system concerns woven bone. Although, in both systems woven bone is seen as a rapidly formed bone tissue that is produced, e.g. during initial growth in a foetus or infant and during tissue repair, its position within the classification system differs. In this thesis a distinction is made between fibrous and lamellar primary bone types. All three subtypes of fibrous bone contain a woven component and are set apart by different kinds of vascularisation. It is not clear whether woven bone is seen by Hillier and Bell (2007) as non-vascular fibrous bone, in this thesis a subtype of fibrous primary bone, or as the general primary bone type, fibrous bone. Secondly, the definition of primary bone differs. Contrary to Hillier and Bell (2007), in the present classification system primary bone does not always contain primary osteons. It can also be non-vascular in appearance or contain simple vascular canals. Following, avascular bone is not seen as a separate type next to primary bone. Thirdly, there is a difference in the definition of laminar bone. Laminar is defined by Hillier and Bell (2007) as bone tissue exhibiting seasonal banding. Individual bands are referred to as a lamina of bone, which can be composed of woven or lamellar bone tissue. In the system of de Ricqlès a lamina consists of a combination of both lamellar and fibrous bone. The fourth difference lies in the division of secondary bone structure, which Hillier and Bell (2007) divide into three groups: irregular, endosteal and dense. This is according to the classification system of Enlow and Brown (1956, 1957, 1958).

Irregular corresponds to scattered secondary osteon structure; endosteal and dense osteon structure are lumped in the present thesis as dense secondary osteon structure, as explained above. Hillier and Bell's (2007) subsequent, important overview of the literature on bone types found in several species illustrates the problems encountered when dealing with different terminologies, e.g. pseudo-osteons containing fibrous bone. The observations made in the reviewed articles can, therefore, not always be related to the ones made in this thesis.

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