Micro-anatomical record of cortical bone remodeling and high vascularity in a fossil giant rat midshaft femur.

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ABSTRACT

Rat cortical bone does not typically undergo secondary (Haversian) remodeling. Haversian organization of rat bone has been mainly observed in experimental settings following biomechanical or dietary manipulation. Here, we report an observation of cortical secondary osteons within a histological femur cross-section from an extinct (late Quaternary) form of Timorese giant rat (Murinae gen. et sp. indet). The medio-lateral midshaft diameter of its femur, used as a measure of bone size, is 6.15 mm and indicates a heavier than normal skeletal frame. We compare this sample to bone histology in a small rat's midshaft femur of 2.33 mm diameter. A complete lack of Haversian bone remodeling characteristics are noted for the smaller sample, which is dominated by radial vascular canals. The giant rat shows clear secondary osteons and diffuse vascularity mainly composed of tightly packed longitudinal canals across its cortex. It appears that rat cortical bone can undergo bone remodeling, and is organized in a highly vascularized manner, in insular giant cases. Our findings from Timor align with results reported in experimental rat model skeletal biology literature and other insular fossil rat material. Where macro-anatomical examination is limited, histological observations on fossil rat limb bones have the potential to aid reconstructions of life history and skeletal growth aspects in these rodents.

KEYWORDS: bone histology, femur, secondary osteon, bone remodeling, Murinae, Haversian canal

"*The longitudinal and transverse canals in the compacta of the long bones have stimulated the interest of anatomists since Clopton Havers (1691) published his "new observations on bones*." (Ruth, 1953: 420).

INTRODUCTION

Rats have long served as important experimental animal models for understanding human health and disease (Jacob, 1999; Lelovas et al., 2008; Sengupta, 2013). Analyses of their skeletal tissue in palaeontological and archaeological contexts offer insights into spatial and temporal speciation, adaptation, and migration of rats as well as associated fauna (e.g. Dhaliwal, 1962; Rae et al., 2006; Maul et al., 2015; Swift et al., 2018; Veatch et al., 2019). While much is known about the biology, behavior, and anatomy of the common brown (*Rattus norvegicus*) and black (*Rattus rattus*) rat (e.g. Ewer, 1971; Jacob et al., 1995; Aplin et al., 2003), many other rodent species in the Murinae are poorly understood. In Southeast Asia (SEA) alone, the tribe Rattini is estimated to include at least 167 species (Pagès et al., 2010), with new species being continually described (e.g. Louys et al., 2018). The rich genetic and morphological diversity of SEA murines indicates great biological versatility (Hulme-Beaman et al., 2018). Indeed, body size variation within Indonesian rats ranges from small to giant (O'Connor and Aplin, 2007; Van Den Bergh et al., 2009; Locatelli et al., 2012; Turvey et al., 2017; Veatch et al., 2019). Insular rodents experience gigantism under conditions that favor body size enlargement and extended longevity (e.g. island size, limited predation, resource availability; Millien & Damuth, 2004). Therefore, understanding rat bone form and function is vital to a successful reconstruction of their evolution and adaptive radiation in deep time, but also to improving modern experimental study designs that use rat models in bio-medical contexts. While traditional gross anatomy of fossil rat skeletons is well recorded, their micro-anatomical records remain limited.

Histology offers insights into growth and metabolism of extinct animals by reconstructing blood vessel network, as well as different types of bone matrix and osteocyte lacunae distribution, that would have been vital in sustaining and driving bone remodeling and haemodynamics (de Ricqlès, 2011, Miszkiewicz & Mahoney, 2017; Mahoney et al., 2018;

Grüneboom et al., 2019). However, the degree to which cortical bone vascularization differs between species is high (Dominguez & Crowder, 2012; Kolb et al., 2015b; Lafage-Proust et al., 2015). Modern rat cortical limb bone histology is mostly avascular with inner and outer circumferential bone layers, all of which are perforated with osteocyte lacunae (Martiniaková et al., 2006). While adult human cortical bone remodeling results in organization into Haversian systems composed of secondary osteons that house central canals for blood and nutrient supply (Miszkiewicz, 2016; Miszkiewicz & Mahoney, 2019), rats typically do not show secondary osteons in their cortical bone (Lafage-Proust et al., 2015). Rat bone vascularization is predominantly radial, whereby vessels are oriented perpendicularly to the long bone axis (Francillon-Viellot et al., 1990), with a limited number of other longitudinal vessels appearing as circular pores when viewed in transverse thin sections (Martiniaková et al., 2005). Together, a vascular network of joining vessels can be seen three-dimensionally (see µCT reconstruction of rat tibia in Figure 2 in Palacio-Mancheno et al., 2013: 146). This network, however, is not typically a result of, nor would it lead to, Haversian remodeling (Lafage-Proust et al., 2015). Humans, who are large mammals of longer lifespans compared to rats, accrue remodeled bone due to ageing, mineral homeostasis turnover, and mechanical load adaptation amongst other factors (Crowder & Stout, 2011; Miszkiewicz & Mahoney, 2016). They also tend to develop bone intra-cortical porosity with age and hormonal changes as remodeling may become out of balance with osteoclastic activity dominating bone maintenance (Feik et al., 1997).

Why rat bones are organized in this way, and why Haversian remodeling is normally absent in these taxa is not well understood. It is probably due to the relatively short lifespan of a rat, low body mass, and optimal mechanical loads imposed on its skeleton that would not normally require or facilitate Haversian remodeling. This would make the absence of “true” bone remodeling an Order-specific characteristic given how rarely secondary osteons are

reported in rodents (Kolb et al., 2015b; Lafage-Proust et al., 2015; Felder et al., 2017). Therefore, when using rats as experimental models in human health and disease research, Haversian bone remodeling cannot always be used for skeletal adaptation assessments. Rather, trabecular or endo-cortical bone turnover markers in rat bone tissue are usually estimated using fluorescent labeling, differentiating newly formed bone surfaces from earlier events of bone deposition through coupled (remodeling) or uncoupled (modeling) bone resorption and deposition events (e.g. Baron et al., 1984; Erben et al., 1996; Paschalis et al., 2017). However, perhaps not surprisingly, intra-cortical secondary osteon formation has been documented in experimental settings with biomechanical and dietary manipulations to rat skeletal growth (e.g. Ruth, 1953; Miller & Bowman, 2007).

Ruth's classic (1953) examination of lactating female rats that were fed a calcium free diet reported the development of increased cortical porosity in response to accelerated mineral depletion. In an attempt to heal and remodel bone compromised during lactation, osteons with concentric lamellae surrounding canals formed following a reintroduction of normal diet. Multiple more recent studies have confirmed the relationship of bone remodeling to calcium variation in lactating female rats, highlights that it may serve a key role in restoring bone health associated with calcium loss (Bowman et al., 2002; Miller & Bowman, 2007; Ross & Sumner, 2017). This is further supported by ongoing research indicating that female bone microarchitecture (both trabecular and cortical) adapts in a protective way to the negative effects of reproduction and oestrogen loss (de Bakker et al., 2018a; 2018b).

Diet aside, mechanically loaded rat model research, where cyclical strain induces fatigue-initiated bone remodeling, also highlights the need for localized bone renewal in response to stress and strain (Bentolila et al., 1998). That mechanically driven bone micro-damage can be "fixed" by targeted bone remodeling is now well established (see Mori & Burr, 1993; Martin, 2002), and how the interaction between diet, hormones, and mechanical loading

influences bone adaptation at the microstructural level understood (Robling et al., 2006). Thus, the potential for behavior and health reconstruction in deep time is vast (Chinsamy-Turan, 2005; Kolb et al., 2015a).

Rat bone remodeling questions extend to the relationship with body size. Mammals heavier than 2 kilograms show secondary osteons in their cortical bone (Currey, 2002; Felder et al., 2017). In other mammals, for example the dwarfed Pleistocene *Candiacervus* from Crete and the Irish giant deer *Megaloceros giganteus,* skeletal growth rates and associated life histories appear to change (accelerate or slow down) depending on body size and island conditions (Kolb et al., 2015a). Ideally, this should be investigated in giant forms of rats from island populations. Kolb and colleagues (2015b) reported cortical femur bone histology in the Late Miocene giant *Mikrotia magna* from Gargano island in Italy and demonstrated secondary osteons amongst parallel-fibred bone matrix (see Figure 7 in Kolb et al., 2015b: 23). According to the island rule, insular gigantism, under favorable conditions that include limited predation, can extended longevity and facilitate an increase in body size (Miller et al., 2000; Michaux et al., 2002; Raia & Meiri, 2006). This increase would explain the need for cortical bone remodeling. Here, we report new data and evidence for the formation of secondary osteons in an insular giant rat cortical bone tissue. We provide the first bone histology record for an extinct form of a giant rat from Timor island in SEA.

MATERIALS AND METHODS

The focus of this study is a femur from one extinct giant rat, but we also include a femur from a fossil rat (a likely extinct and undescribed endemic; see Aplin and Helgen 2010) of similar dimensions and morphology to *Rattus rattus.* The associated thin sections (giant specimen ID: TDS 0-30 #4, small specimen ID: TDD 1 #11) can be accessed at School of

Archaeology and Anthropology at the Australian National University in Canberra, Australia. This study design facilitates comparisons of bone histology between two vastly different sized taxa, at the same time allowing us to control for environmental context, geochronological age, and taphonomic factors. Both specimens were estimated as adult due to their fused epiphyses. However, we do note the unavoidable taphonomic fragmentation in some distal and proximal locations of the femora (Figure 1), and heterochrony of epiphyseal plate fusion in mammals (Geiger et al., 2014). To further sustain our assessment of the "adult" age in the small rat femur, we observed the same bone microscopic organization as in mature Wistar rat femur cortical bone histology (Martiniaková et al. 2005: 46; see also Sengupta, 2013 for discussion about laboratory rat lifespan). The presence of secondary bone in the giant femur would also indicate maturity (Singh & Gunberg, 1971).

The specimens date to a minimum of ca. 5–18 ka and derive from fossil deposits of Matja Kuru TD on Timor Island (Louys et al., 2017). Definitive identification of species or sex of the postcranial elements of Timor fossil rats is not possible due to lack of articulated and/or associated diagnostic material, lack of recoverable aDNA, and the undescribed nomenclatural status of all but two of the endemic rats in Timor (Aplin & Helgen, 2010). Nevertheless, these specimens were found in an assemblage whose terrestrial mammal component consists only endemic rat species, both giant and normal-sized, as judged by dental remains recovered from sieved material.

We recorded femoral midshaft diameter in the medio-lateral (M-L) and cranial-caudal (C-C) planes to represent size differences. Sections of approximately 100-150 μm thickness were produced following standard methods for palaeontological material (Chinsamy-Turan, 2005). Femora were embedded in Buehler® epoxy resin, cut on a low speed saw with a diamond blade, attached to glass slides, ground, polished, dehydrated, cleared, and cover slipped. The sections were imaged under transmitted light using an Olympus BX53 microscope

with an attached DP74 camera at 4x magnification first, and a range of 10x – 60x objective magnification second, using transmitted and linearly polarized light. Incomplete preservation of the micro-anatomy was apparent in both sections due to taphonomic factors, bio-degradation, and fossilization of the specimens. However, we were able to identify bone regions that clearly show microstructures that are of interest. These include osteocyte lacunae that would have housed osteocytes in live tissue, vascularity pattern of cortical bone, as well as presence of secondary osteons.

By definition, a true secondary osteon is separated from the remaining bone matrix by a distinct cement line that indicates closing and reversal of the Bone Multicellular Unit cutting cone, and new bone deposition during remodeling (Suzuki et al., 2000; Miszkiewicz & Mahoney, 2019). A primary osteon, on the other hand, does not feature a cement line, and is usually detected by a vascular canal embedded within primary bone matrix (Locke, 2014). In humans, primary osteons are usually found in young and immature bone and are gradually replaced with secondary osteons as we age (Pitfield et al., 2017). Previous studies of bone histology in rats have reported difficulty in identifying "true" Haversian remodeling, but presence of localized secondary osteons has been confirmed in experimental cases (see discussion by Martiniaková et al., 2011: 9). For the sake of clarity, and to make the description of the giant rat femur histology methodologically comparable to other mammals, we refer to secondary osteons where cement lines and concentric lamellae enveloping a vessel can be seen in transversely viewed histology. This follows descriptions established by Ruth (1953) and Enlow and Brown (1958).

RESULTS

The giant femur measured 6.15 mm in M-L and 4.87 mm in C-C midshaft diameter, whereas the smaller rat's femur midshaft was 2.33 mm and 1.98 mm wide in the M-L and C-C aspects respectively. By extension, the giant femoral size is likely to have been associated with a larger body mass and skeletal frame (Damuth et al., 1990), well above a typical 150 - 300 g weight reported for common rats (e.g. Bromage et al., 2009; Felder et al., 2017; Garg et al., 2018). At the histological level, both samples exhibited circumferential bone periosteally and endosteally, and multiple osteocyte lacunae scattered throughout the entirety of bone (Fig. 1, 2). We note exceptional preservation of osteocyte lacunae in the small fossil rat's bone, with multiple delicate canaliculi protruding out of some of the lacunae (Fig. 2). The smaller rat femur shows expected primary bone with radial canals running perpendicularly to the bone axis, as well as a largely uniform avascular structure (Fig. 1). When compared to the giant rat's bone, no evidence for Haversian remodeling can be observed in the smaller sample. However, remodeled bone in the form of secondary osteons and compact vascularity through its cortex is evident in the giant form (Fig. 1, 3). Individual secondary osteons can be seen amongst other tightly packed and organized longitudinal vascular canals. These appear mostly next to the periosteum and endosteum and are longitudinal rather than radial in orientation. However, some radial canals can also be identified next to the endosteum (Fig. 1D), forming a localized reticular vascularization-like pattern of bone when considering together with the predominating cortical transverse vascularity expression of longitudinal canals. Both specimens showed compact bone only, and neither specimen displays visible lines of arrested growth.

DISCUSSION AND CONCLUSION

This short study evaluated femur bone vascularity in an extinct giant rat from Timor. It appears that rat cortical bone can undergo Haversian remodeling, and is organized in a highly vascularized manner, in taxa demonstrating insular giant body mass. This can be explained by principles of bone haemodynamics and physiology that consider calcium metabolism and adaptation to mechanical contexts amongst other intrinsic and extrinsic factors (Robling et al., 2006; Ross & Sumner, 2017; Grüneboom et al., 2019). Changes in bone growth dynamics inferred from histology have been shown to relate to body size and life history (Kolb et al., 2015a), and these can be seen in the case of extinct giant rats from Timor island as well. While in modern rats bone remodeling is usually absent, where secondary osteons have been noted as present, they are usually in the central portion of the intra-cortex enveloped by relatively thick periosteal and endosteal layers that are almost completely avascular (Martiniaková et al., 2011). Secondary osteons reported from the giant *Mikrotia magna* recovered from Gagrano (Italy) (Kolb et al., 2015b: 23) were also located intra-cortically, but appeared more irregular and oblique in their shape when compared to the organized vascularity reported in our Timorese giant rat. Kolb and colleagues (2015b) also note radial vessels in the more periosteal and endosteal cortex, which may be similar to the endosteally localized radial and longitudinal combination of canals in the Timorese giant rat in our study. Isolated secondary osteons have been previously noted in *Phoberomys pattersoni* - giant caviomorph rodent from Trinidad (Geiger et al., 2013), though no evidence for several generations of Haversian remodeling was observed. The presence of secondary osteons from the most inner (i.e. lining the medullary cavity surface) endosteum to the most outer periosteum in our giant sample is indicative of remodeling and a highly increased vascularity case in a murid. However, we did not identify

fragmentary secondary osteons suggesting several generations of remodeling, and it was not the entirety of the section that showed secondary osteons.

Intra-cortical remodeling can be induced both in dietary and biomechanical settings, but periosteal (or sub-periosteal) bone formation can also take place in response to mechanical stimuli (Brown et al., 1990; Robling et al.. 2006; Pivonka et al., 2018). While the giant size of our Timorese rat does not necessarily imply increased strain associated with rigorous mechanical load (Biewener, 1989), it probably indicates greater experience of weight-bearing on its limbs (Tommerup et al., 1993; Mosley & Lanyon, 1998). To that end, a giant rat's femur may require remodeling to accommodate increasing body size as its primary bone becomes gradually replaced by secondary bone. Access to more samples will help clarify this in future research. While we do not attempt to quantify body mass in our samples, the formation of secondary osteons in other animals of body mass heavier than 2 kilograms (Currey 2002; Felder et al., 2017), support evidence for remodeling in giant rat bone histology.

Gigantism experienced by insular mammals can also lead to extended lifespans (e.g. Michaux et al., 2002). Thus, giant rat bone tissue would have had enough time to remodel and accumulate osteons, as seen in ageing bone tissue in other animals (Ortner, 1975). Given that gigantism also may occur due to access to favorable resources and limited predation on islands (Millien & Damuth, 2004), increased availability of nutritious diet may have encouraged giant bone remodeling strengthening rat bone quality and quantity. Results from experiments on lactating rats (Ruth, 1953; Miller & Bowman, 2007) subjected to dietary manipulation provides some support to this interpretation.

The effects of sexual dimorphism and species-specific bone histology could also affect our results. Given the large effect of rat lactation and reproduction on bone micro-architecture in the female sex (de Bakker et al., 2018b), future research investigating giant rat bone

histology in fossils should aim to account for sexual dimorphism. However, our results constitute the first micro-anatomical record of remodeled cortical bone in a fossil giant rat in SEA. Our results mirror those reported for the remodeled intra-cortex in a Late Miocene island murid *Mikrotia magna* recovered from Gagrano (Italy) (Kolb et al., 2015b). Our research further contributes to the presently available bone histology data in extinct giant murids.

The small sample size examined here does not allow us to evaluate intra-specific variability in giant murids. Nevertheless, this initial finding is important to several disciplines that examine rat bone tissue. Primarily, where macro-anatomical examination is limited in paleontological and archaeological contexts, histological observations of rat fossil material have the potential to aid the reconstruction of extinct rodent ecology and adaptation. Secondarily, modern experimental research design using rat bone in the context of diet and mechanics may benefit from our data by incorporating bone dimensions into microstructural research in an effort to account for any patterns observed in histology, as has been shown for humans previously (Miszkiewicz & Mahoney, 2019). Finally, as extant giant rats are still found across SEA and elsewhere, our results contribute new insights into skeletal adaptation and life history of these animals.

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FIGURE CAPTION

Figure 1. Summary comparisons of bone histology in a small and giant fossil rat (late Quaternary, Timor) midshaft femur (top: intact femora positioned in cranial view). The patches of darker matter and cracks visible in the images are an unavoidable result of taphonomic processes. Regions of interest shown are the least affected by bio-degradation and are located as follows: A – caudal region of periosteal bone, B – lateral region of endosteal bone, C – bone on the medio-cranial aspect, D, E – caudal region of endosteal bone, and F – all of cortical bone (endosteal border top, periosteal border bottom) of the cranial region of bone.

Figure 2. Localized exceptional preservation of osteocyte lacunae with protruding canaliculi in the midshaft femur cortical bone of the small fossil rat from late Quaternary Timor.

Figure 3. Sub-periosteal region of femur midshaft cortical bone in the giant rat sample showing secondary osteons (white arrow, bottom image) and an organized transverse expression of longitudinal canals.