**ORIGINAL PAPER**

# Osteohistology of the Antarctic penguin *Pygoscelis adeliae* (Aves, Sphenisciformes): definitive evidence of medullary bone

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**Abstract**

Osteohistological studies allow us to obtain valuable information on different aspects related to the bone microstructure, physiology and ecology of organisms. Although the anatomy and morphology of penguin bones are well known, studies in osteohistology are still insufficient. In order to analyze the osteohistological variations between male and female adults of *Pygoscelis adeliae* (Aves, Spheniscidae), histological sections were prepared from various bones including appendicular (humerus, radius, ulna, carpometacarpus, femur, tibiotarsus, tarsometatarsus) and axial (vertebral and sternal ribs) elements. The results indicate that all sections showed compact tissue with reduced or absent medullary cavities and high bone density due to internal tissue compaction. Histologically, we identified three distinct tissue regions based on their bone matrix, vascular channels organization and compactions degree. Our results indicate osteohistological variations between sexes throughout the whole skeleton, including the first definitive evidence of medullary bone in the Sphenisciformes females. While the male specimen exhibited a medullary cavity characterized by an inner circumferential layer, the female displayed a medullary region without an inner circumferential layer, lower bone compaction, presence of medullary bone in all the sections, and greater development of intertrabecular spaces. These results are consistent with previous reports of medullary bone in females from other birds and provided an auxiliary criterion for sex differentiation.

## Introduction

The Sphenisciformes (penguins) are an order of flightless seabirds that exhibit the most extreme specializations for wing-propelled diving (Stonehouse 1975; Simpson 1976). The eighteen extant species are organized into six genera (Winkler et al. 2020) and exclusively distributed following the cold water currents of the southern seas (Martínez 1992). The genus *Pygoscelis* (Wagler 1832) comprises three living species: *Pygoscelis adeliae* (Hombron and Jacquinot 1841), *P. antarctica*, and *P. papua* (Forster 1781), plus the three fossil species *P. tyreei* from New Zealand (Simpson 1972), *P. calderensis* (Acosta Hospitaleche et al. 2006) and *P. grandis* (Walsh and Suárez 2006) from Chile.

Reproductive colonies of *Pygoscelis adeliae* occur in ice-free areas in the Ross Sea, along the west coasts of the Antarctic Peninsula, and on the Scotia Arc islands (Trathan and Ballard 2015). Adélie penguins breed in large colonies, maintain the same reproductive mate for successive years, and exhibit recurrent use of their nests (Trathan and Ballard 2015). The reproductive cycle comprises a migratory stage (April/August), after which they return to the colony for the reproductive period (September/October), spending the following months laying eggs and raising chicks until mid-January (Emmerson et al. 2011). Then begins the period of independence of the juveniles until the first moult in late February and early March (Emmerson et al. 2011). The

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mean age at first reproduction is 4.7-5.0 years for females and 6.2–6.8 years for males (Ainley et al. 1983).

Adult females and males are morphologically similar, although females are usually smaller (Ainley and Emison 1972; Scolaro et al. 1991; Kerry et al. 1992) and males have longer and deeper bills (Polito et al. 2012; Fattorini and Olmastroni 2021). Despite the patterns of both external and skeletal morphological variation established for pygoscelid penguins (Acosta Hospitaleche and Tambussi 2006; Jennings et al. 2016; Fattorini and Olmastroni 2021), bone microstructure studies are still insufficient, not only in *Pygoscelis* (Meister 1962; Wilson and Chin 2014) but also in all penguins (e.g. Chinsamy et al. 1998; de Margerie et al. 2004; Cerda et al. 2015; Ksepka et al. 2015; Garcia Marsà et al. 2020). In this regard, only the microstructure of the femur and tibiotarsus of adult specimens of *Pygoscelis adeliae* have been described (Meister 1962; Wilson and Chin 2014). These contributions mention the presence of high-density bone tissue, with three differentiated concentric regions: a cortical region (outer zone) composed of a circumferential layer with the presence of Volkmann's canals, a perimedullary region (middle zone) with secondary osteons organized in longitudinal vascular channels, and a medullary region (inner zone) composed of spongy tissue with a large number of trabeculae surrounded by lamellar tissue (Meister 1962). Furthermore, the dominance of longitudinal vascular channels and a few other anastomosed channels in the cortex of the tissue have been observed in this species, resulting in a reticular vascular pattern and the absence of growth lines or growth cycles (Wilson and Chin 2014). According to that, it is necessary to extend the analysis to other skeletal elements to contemplate their osteohistological variability comprehensively.

The present contribution aims to improve our knowledge of the bone microstructure and histology of *Pygoscelis adeliae*. Accordingly, osteohistological variations between adult individuals were analyzed from multi-elemental histological sections, performed at the level of the mid-shaft in the humerus, radius, ulna, carpometacarpus, femur, tibiotarsus, tarsometatarsus, and (vertebral and sternal) ribs. A male and a female from the same reproductive colony were compared here.

## Materials and methods

The specimens, collected after death by natural causes, come from Bahía Esperanza, Trinidad Peninsula (Antarctica), and are housed in the osteological collection of the Ornithology Section of the Vertebrate Zoology Division of the Museo de La Plata (MLP-O) in Argentina. The examined bones, including the humerus, radius, ulna, carpometacarpus, femur, tibiotarsus, tarsometatarsus, and (vertebral and sternal) ribs, belonged to a male (MLP-O 15,177) and a female (MLP-O 15,137) of *Pygoscelis adeliae* (Fig. 1). During dissection, it was observed that the female was found dead just before laying one egg. Eighteen thin sections were prepared at the Laboratory of Thin Sections of the Instituto de Investigación en Paleobiología y Geología (IIPG). Cross-sections of each specimen were made at the level of the mid-shaft using the protocol proposed by Chinsamy and Raath (1992). The identification of the medullary bone is consistent with the criteria proposed by Canoville et al. (2020); each of their criteria are mentioned in the discussion below. The slides obtained were analyzed with a petrographic microscope (*ZEISS Axio Imager*) under the plane and cross-polarized light. Images of each thin section were captured by a digital camera (*ZEISS Axiocam 105*) and processed with *Adobe Photoshop 2020* and *Adobe Illustrator 2022*.

## Results

### Microanatomy

All the elements analyzed (humeri, radi, ulnae, carpometacarpi, femora, tibiotarsi, tarsometatarsi, vertebral and sternal ribs) show a similar microanatomical pattern for both sexes (Fig. 2). This is characterized by a compact tissue that preserves a reduced (or absent) medullary cavity with some intertrabecular spaces towards the medullary region. In the female (MLP-O 15,137), a greater development of intertrabecular spaces and a lower degree of bone compaction than in the male (MLP-O 15,177) were observed. The sections of the femora, tibiotarsi, tarsometatarsi, vertebral, and sternal ribs of male present a well-defined medullary cavity delimited by an inner circumferential layer, whereas, in the female, a remaining medullary cavity without the development of an inner circumferential layer was observed.

### Histology

Three regions (cortical, perimedullary, and medullary), defined by their bone matrix, organization level of the vascular channels, and compaction degree, are recognized in both specimens. In the cortical region (Figs. 3a, b, e and f, 4a, b, e and f, 5a, b, e and f and 6a, b, e, f, g and h), all sections exhibit an outer circumferential layer (OCL) composed of a thin layer of lamellar tissue, mostly avascular, with lines of arrested growth (LAGs) that vary according to gender and skeletal element. One LAG was observed in the radi, tibiotarsi, and (vertebral and sternal) ribs, but two in the femora and tarsometatarsi of both specimens. However,

**Fig. 1** Examined bones of two specimens of *Pygoscelis adeliae*. (**a-i**) male *Pygoscelis adeliae* (MLP-O 15,177), (**j-r**) female *Pygoscelis adeliae* (MLP-O 15,137). (**a**, **j**) humeri, (**b**, **k**) radi, (**c**, **l**) ulnae, (**d**, **m**) carpometacarpi, (**e**, **n**) vertebral rib, (**f**, **o**) sternal rib, (**g**, **p**) femora, (**h**, **q**) tibiotarsi, (**i**, **r**) tarsometatarsi. (**e**, **f**) right ribs. (**n**, **o**) left ribs. (**b**, **c**, **d**, **k**, **l**, **m**) ventral view. (**a**, **g**, **h**, **i**, **j**, **p**, **q**, **r**) cranial view. (**e**, **f**, **n**, **o**) lateral view. Black line = Cross-sections. Scale 1 cm
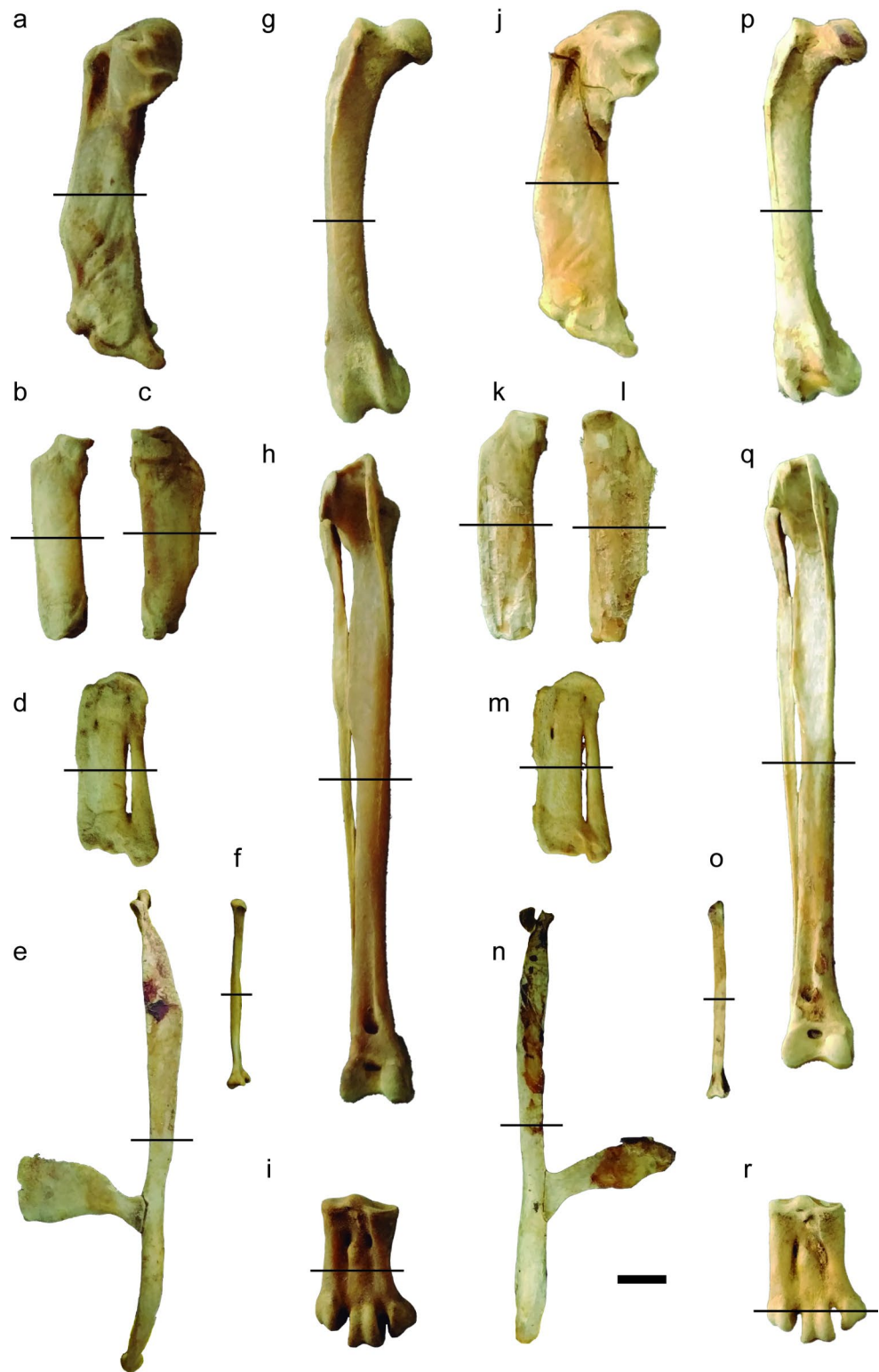
the humeri, ulnae, and carpometacarpi present two LAGs in males and one in females.

In the perimedullary region (Figs. 3a, b, e and f, 4a, b, e and f, 5a, b, e and f and 6a, b, e, f, g and h), all sections are composed primarily of secondary osteons defined by clear cementing lines arranged mainly in a longitudinal vascular pattern. Nevertheless, some channels with a reticular distribution are observed (except for the vertebral and sternal ribs). In the ribs, the vascular pattern is clearly longitudinal. Osteons, that remodel the primary tissue, present

**Fig. 2** Microanatomical features of *Pygoscelis adeliae*. (**a-i**) male, (**j-r**) female. (**a**, **j**) humeri, (**b**, **k**) radi, (**c**, **l**) ulnae, (**d**, **m**) carpometacarpi, (**e**, **n**) vertebral rib, (**f**, **o**) sternal rib, (**g**, **p**) femora, (**h**, **q**) tibiotarsi, (**i**, **r**) tarsometatarsi. (**a**, **b**, **c**, **d**, **g**, **h**, **i**, **j**, **k**, **l**, **m**, **p**, **q**, **r**) Scale 1 cm. (**e**, **f**, **n**, **o**) Scale 0.5 cm
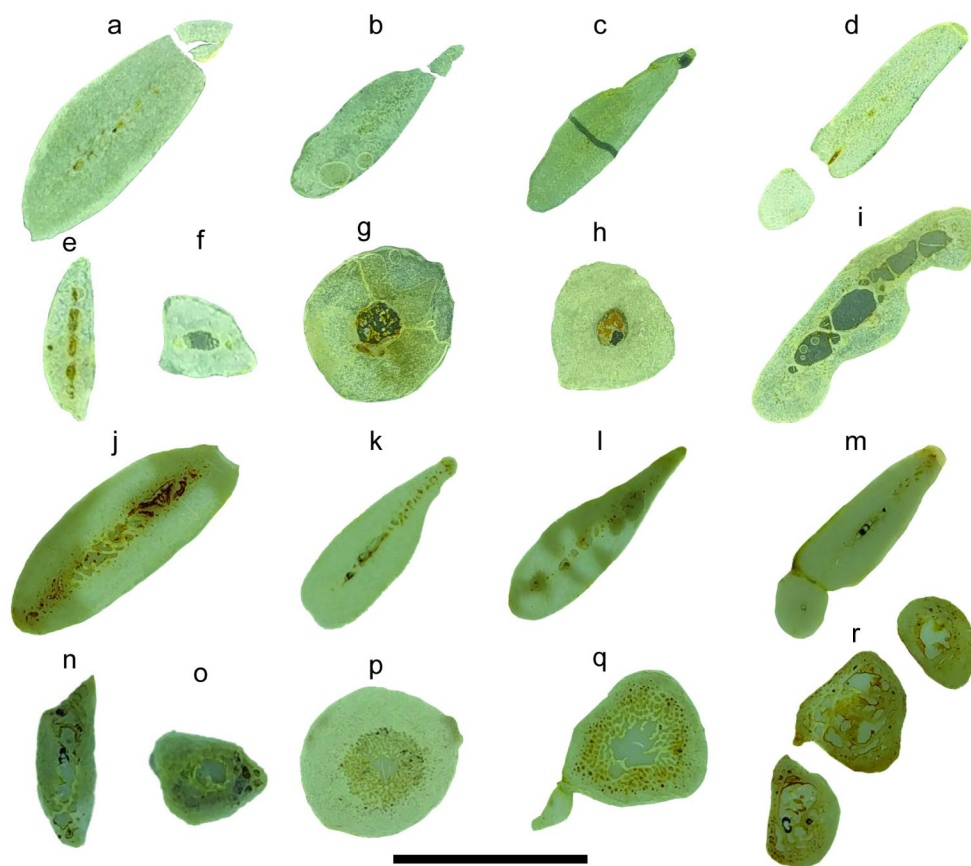
Volkmann's channels. Although the bone matrix cannot be distinguished from the vestiges of the primary tissue under polarized light, remodeling is evidenced by the morphology of the osteocyte lacunae. These lacunae, derived from static osteogenesis, are circular and larger than the lacunae derived from dynamic osteogenesis, which are smaller, discoidal, and oriented according to the distribution of the fibres.

In the medullary region (Figs. 3c, d, g and h, 4c, d, g and h, 5c, d, g and h and 6c, d, e, f, g and h), the osteohistological differences between the male and the female are strongly marked. A secondarily compacted trabecular tissue, which retains some irregular intertrabecular spaces surrounded by lamellar tissue, is observed. The male is characterized by the lower development of intertrabecular spaces and consequently a greater degree of bone compaction. In the particular cases of the femur, tibiotarsi, tarsometatasi, and ribs, a medullary cavity well-defined by an inner circumferential layer was observed in the male. On the other hand, greater development of intertrabecular spaces and a medullary space, which is not delimited by an inner circumferential layer, is observed in the female. Likewise, all the female sections have medullary bone surrounding the intertrabecular spaces of the tissue, and in the cavities of the medullary

region. Finally, no Sharpey's fibres were observed in any of these sections.

## Discussion

### Sex differences

The results exposed above show that the microanatomy of all the elements analyzed presents a compact tissue at the level of the mid-shaft with some differences between the male and female. In the male, a medullary cavity defined by an inner circumferential layer was observed. In the female, a medullary space without the development of an inner circumferential layer, with a lower degree of bone compaction, presence of medullary tissue, and large intertrabecular spaces were found. In both, bone compaction appears as the result of secondary compaction of the trabecular tissue, but in the male, the thickening of the inner circumferential layer is added. This general pattern is considered to be the result of osteosclerosis, in which reduced tissue cavities produce a compact and heavy bone structure. Osteosclerosis has been widely recognized in penguins (de Ricqlès and de Buffrénil 2001; Ksepka et al. 2008; Ksepka et al. 2015; Cerda et al. 2015). The tissue compaction, and the thickening of the

**Fig. 3** Histological features of the humerus and radius. (**a**, **c**) male humerus, (**b**, **d**) female humerus, (**e**, **g**) male radius, (**f**, **h**) female radius, left with normal transmitted light and right with polarized light. (**a**, **b**, **e**, **f**) cortical and perimedullary region, (**c**, **d**, **g**, **h**) medullary region. (is) intertrabecular space, (lt) lamellar tissue, (mb) medullary bone, (so) secondary osteon, (vc) vascular canal and (yellow triangle) LAGs. Scale 200 μm
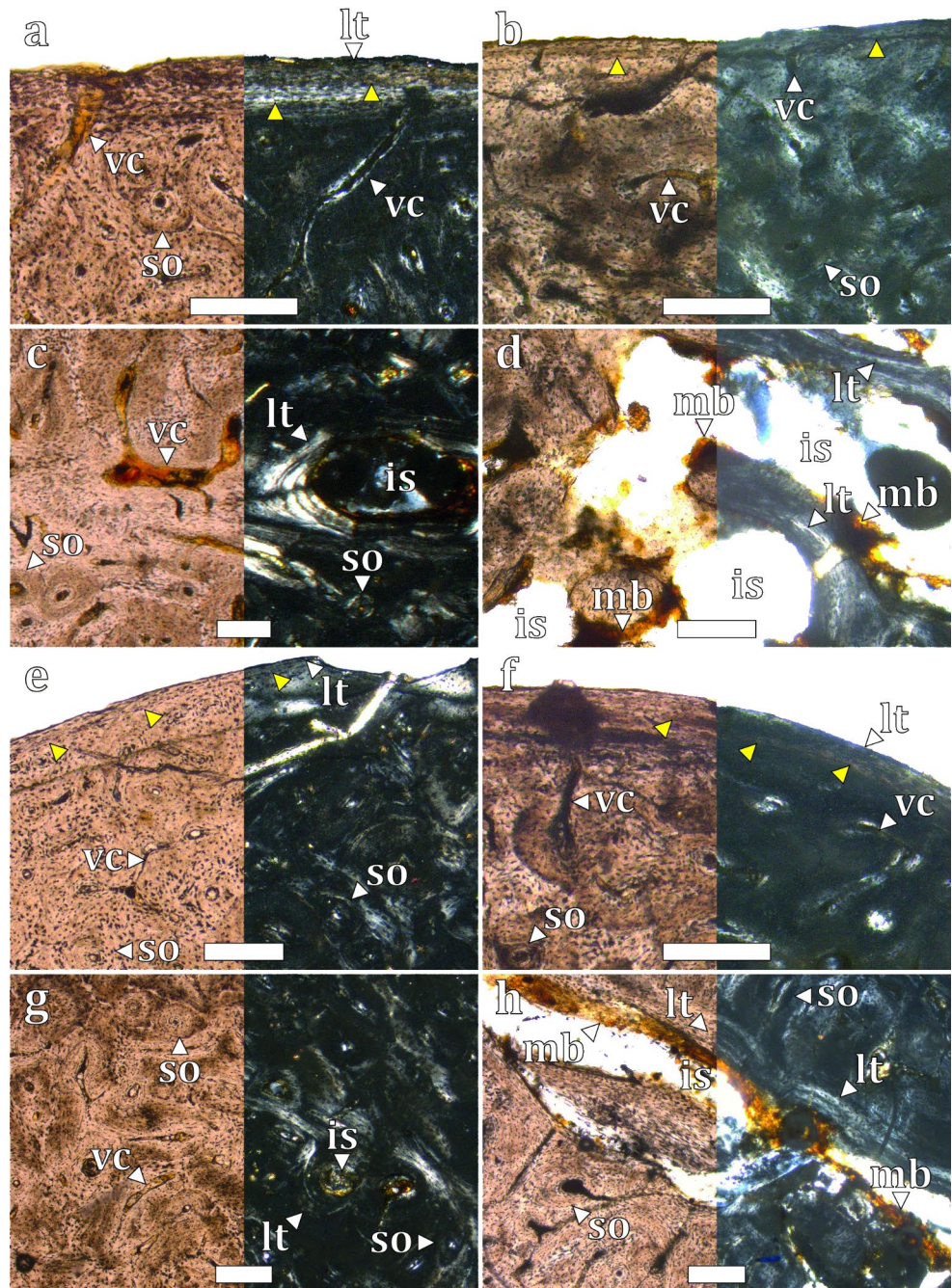


circumferential layer, have already been defined as specific adaptations to the aquatic environment. It reduces buoyancy, preserves more energy during diving, and improves resistance to bending or torsional loads (de Ricqlès and de Buffrénil 2001; Habib and Ruff 2008; Houssaye 2009; Habib 2010; Houssaye 2013).

The difference in the degree of compaction between the male and the female is due to the osteoclastic action that will result in the development of the medullary bone, which serves as a temporary calcium reservoir during egg laying (Bloom et al. 1941; Dacke et al. 1993; Bailleul et al. 2019;

Canoville et al. 2019, 2020). Medullary bone was found in the intertrabecular spaces and the cavities of the medullary region (criterion #1, and criterion#5 only in ribs). Likewise, medullary bone was identified in the appendicular and axial bones (criterion #7) of the female, reinforcing the concept of a complete skeletal distribution (Bailleul et al. 2019; Canoville et al. 2019, 2020). Besides, no bone pathologies were detected (criterion #4). Although medullary bone is used as a marker of reproductive activity or maturation (Bailleul et al. 2019), it is unknown whether it takes place before or after somatic maturation. However, in birds it usually

**Fig. 4** Histological features of the ulna and carpometacarpus. (**a**, **c**) male ulna, (**b**, **d**) female ulna, (**e**, **g**) male carpometacarpus, (**f**, **h**) female carpometacarpus, left with normal transmitted light and right with polarized light. (**a**, **b**, **e**, **f**) cortical and perimedullary region, (**c**, **d**, **g**, **h**) medullary region. (is) intertrabecular space, (lt) lamellar tissue, (mb) medullary bone, (so) secondary osteon, (vc) vascular canal and (yellow triangle) LAGs. Scale 200 μm
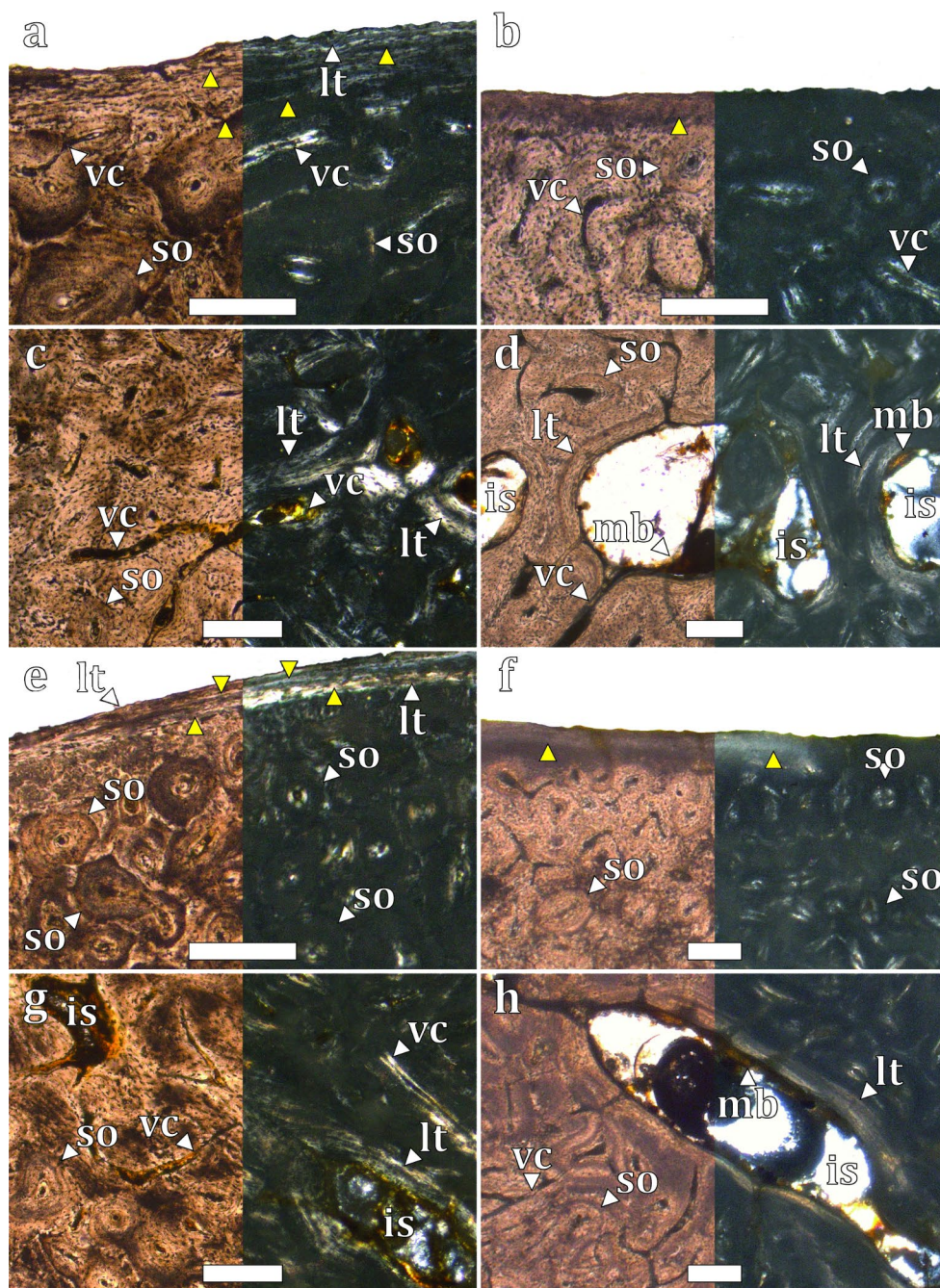
occurs after somatic maturation has been reached (Padian and Woodward 2021).

In future research, it is still necessary to evaluate the histology of female penguins outside the oviposition period to observe if the development of an ICL occurs and then disappears by the osteoclastic intervention (during the egg laying) or if it never developed. Under this criterion, the absence of an ICL in adult females outside the oviposition period could be used as a tool for sexual differentiation in *Pygoscelis adeliae* and probably in penguins in general,

including fossil species, in which the sources of morphological variations are in many cases poorly misunderstood.

The objective differentiation between females and males through the osteohistological examination of a single bone could solve some classic taxonomic problems that concern some fossil species which are based on isolated bones. For example, it is possible that remains referred to different species of the same genus, like *Anthropornis grandis* and *A. nordenskjoeldi*, or *Palaeeudyptes gunnari* and *P. arctowski*, correspond in fact to females and males of the same species. Successive (qualitative, morphometric, morpho-geometric)

**Fig. 5** Histological features of the femur and tibiotarsus. (**a**, **c**) male femur, (**b**, **d**) female femur, (**e**, **g**) male tibiotarsus, (**f**, **h**) female tibiotarsus, left with normal transmitted light and right with polarized light. (**a**, **b**, **e**, **f**) cortical and perimedullary region, (**c**, **d**, **g**, **h**) medullary region. (eb) erosion bay, (icl) inner circumferential layer, (is) intertrabecular space, (lt) lamellar tissue, (mb) medullary bone, (mc) medullary cavity, (so) secondary osteon, (vc) vascular canal and (yellow triangle) LAGs. Scale 200 µm
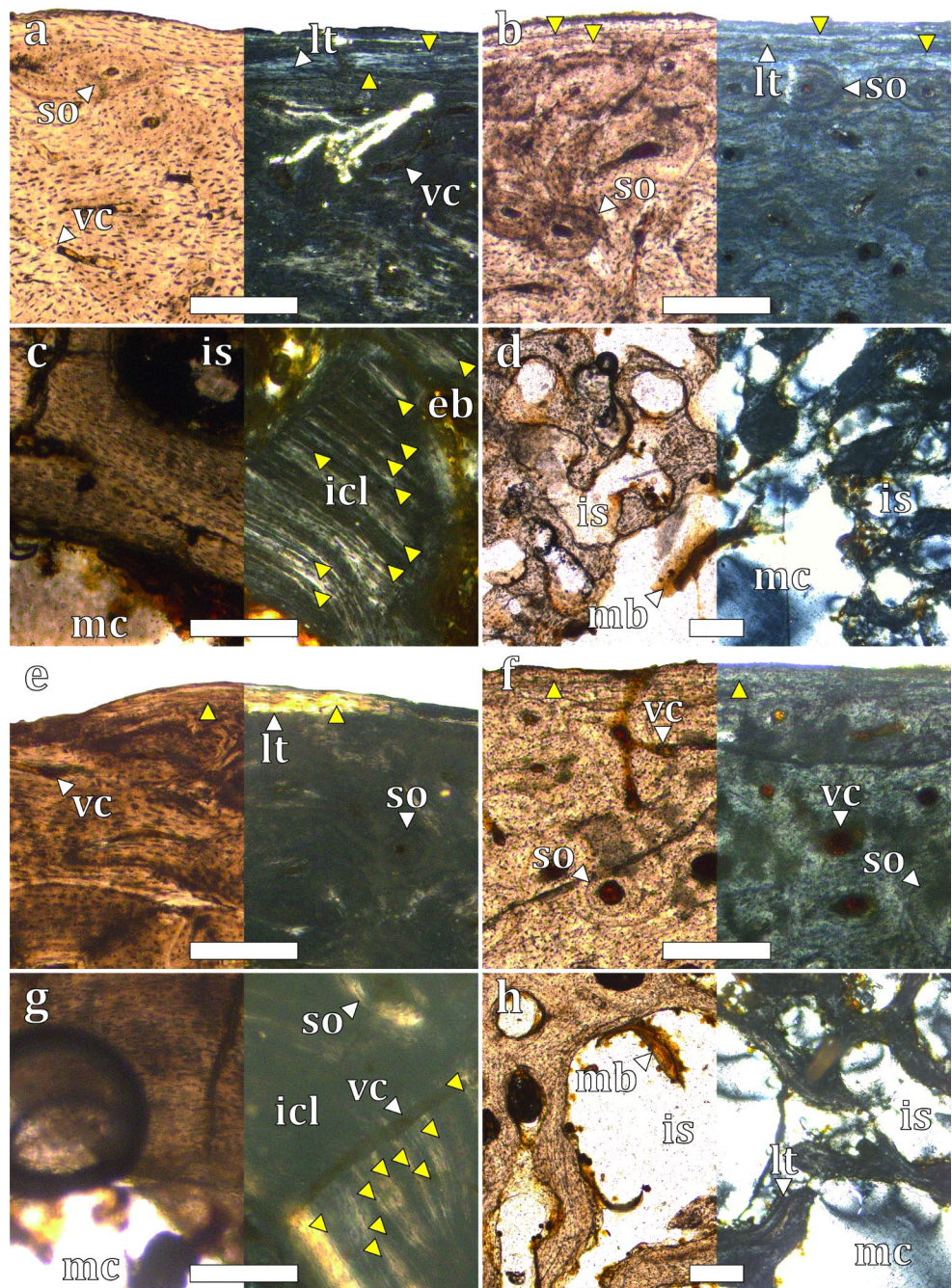


analyses have dealt with this issue, without reaching a completely satisfactory result, which allows postulating without further doubts that the remains a single species or two different species in both cases (a further discussion can be found in Acosta Hospitaleche and Reguero 2010, 2014; Jadwiszczak and Acosta Hospitaleche 2013). We think this is an interesting point to analyze in the near future.

## Outer circumferential layer in *Pygocelis adeliae*

Cortical LAGs are rare in extinct and extant groups of birds (e.g. Amprino and Godina 1947; Padian et al. 2001; Turvey et al. 2005; Castanet 2006; Bourdon et al. 2009; Canoville et al. 2022). Castanet (2006) was the first to report the presence of LAGs in penguins, based on the description of the femur of a juvenile *Aptenodytes patagonicus*. These growth marks were interpreted as the result of the long periods of fasting that this species endures (Castanet 2006) or as the reflection of the seasonal environment stress (Tütken et al. 2004).

**Fig. 6** Histological features of the tarsometatarsus, vertebral and sternal ribs. (**a**, **c**) male tarsometatarsus, (**b**, **d**) female tarsometatarsus, (**e**) male vertebral rib, (**f**) female vertebral rib, (**g**) male sternal rib, (**h**) female sternal rib, left with normal transmitted light and right with polarized light. (**a**, **b**, **e**, **f**, **g**, **h**) cortical and perimedullary region, (**c**, **d**, **e**, **f**, **g**, **h**) medullary region. (icl) inner circumferential layer, (is) intertrabecular space, (lt) lamellar tissue, (mb) medullary bone, (mc) medullary cavity, (so) secondary osteon, (vc) vascular canal and (yellow triangle) LAGs. Scale 200 μm
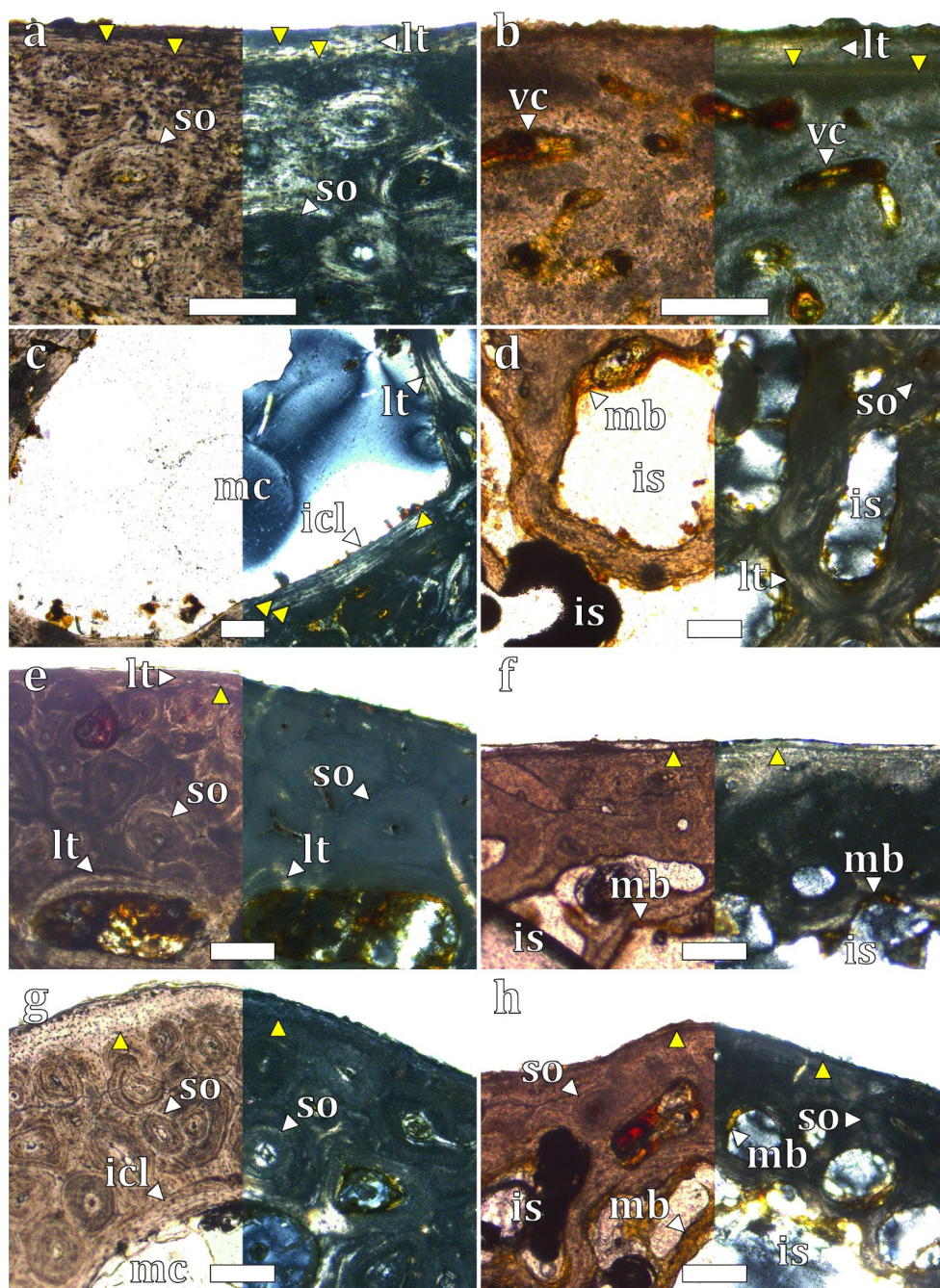
Conversely, individuals that develop an azonal bone without interruption of LAGs and with less isotopic variability can be interpreted as migratory organisms (Tütken et al. 2004). To test these, Wilson and Chin (2014) analyzed the osteohistology of pygoscelid penguins contemplating migratory (*Pygocelis adeliae*, *P. antarctica*) and non-migratory (*P. papua*) species, identifying an outer circumferential layer (OCL) and the absence of LAGs in the bone tissue of the three species. Based on that, the absence of growth marks would indicate that the migratory habit is not recorded in the bone tissue in the form of LAGs or zones (Wilson and Chin 2014). Likewise, the presence of an OCL would indicate skeletal maturity within the first year, which is usual in most modern birds (Padian and Woodward 2021), except for the kiwi (Bourdon et al. 2009). In the present work LAGs were identified but occur at the cortical margin within an avascular lamellar matrix. Therefore, this tissue has been interpreted as an OCL. This evidence, added to the bone remodeling degree, we interpret as an indicator of somatic maturity of both individuals.

## Vascular patterns

Certain microstructural characteristics are related to habits. Thus, for example, flying birds display high degrees of vascularity with a reticular organization, a thin but compact periosteum, low bone density, and absence (or scant presence) of lines of arrested growth (Hall 1991; Chinsamy 1995). These features reflect the high metabolic rates of the group and its biomechanical properties to take flight. However, when we analyze in detail the microstructure of some flightless birds, such as *Hesperornis* and *Patagopteryx* (but not the dodo and many Ratites), the structural patterns change radically in several ways: it lacks the typical triple-layer structure generally associated with adult modern birds (with an ICL and OCL of the bone surrounding a central tissue of fibrolamellar bone), vascular canals are longitudinally oriented (with some circumferential and radial arrangements) and, distinctive LAGs are developed (e.g. Houde 1987; Chinsamy et al. 1998).

In the individuals analyzed here, the vascular pattern is predominantly longitudinal; however, in the perimedullary region, vascular channels with a reticular distribution pattern are observed. This pattern could be interpreted as a retention of the vascular pattern of their flying ancestors (Raikow et al. 1988) or secondarily caused by bone compaction and remodeling. To test this hypothesis, it is necessary to study specimens in different ontogenetic stages.

## Conclusions

Sexual differences and variations in microstructure and histology along the skeleton were found in the penguin *Pygoscelis adeliae*. The osteosclerotic condition found in humeri, radi, ulnae, carpometacarpi, femora, tibiotarsi, tarsometatarsi, and (vertebral and sternal) ribs are less marked in the female than in the male, as a result of the reabsorption of the osteological tissues. In the male, a medullary cavity defined by an inner circumferential layer was observed, while in the female, a medullary space without an inner circumferential layer was observed. However, the male and female exhibit a reduced (or null) medullary cavity and a high density due to the internal compaction of the tissues.

As expected, the medullary bone was found only in the medullary cavity and in the intertrabecular spaces of the female. This tissue was found surrounding the medullary spaces of the humeri, radi, ulnae, carpometacarpi, femora, tibiotarsi, tarsometatarsi, and (vertebral and sternal) ribs, and no evidence of osteopathologies was detected. Medullary bone was recognized in the females of other birds, but the precise morphology in each bone of the skeleton, and the comparison with the homologous elements in the male,

represent a novelty result. Thus, we can certainly confirm that the presence of the medullary bone can be used as a tool for the differentiation of females from males in *Pygoscelis adeliae* and probably in other extant and extinct penguin species. Here we present the first report of medullary tissue in Sphenisciformes.

## Declarations

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