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SEX-RELATED VARIATIONS IN THE MORPHOLOGICAL STRUCTURE OF PUMA (*Puma concolor Linnaeus*, 1771) EAR SKIN

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Abstract

The ear tissue of vulnerable felines, such as the puma, holds potential biological material for creating biobanks. However, skin composition can differ significantly between individuals of the of the same species and even between sexes, based on different environments. Therefore, identifying morphological similarities across these populations is crucial for developing accurate protocols. This study aimed to characterize and evaluate the structure and composition of ear skin in both a male and a female puma using histological techniques. Histomorphometric analysis revealed a total thickness of 304.65 µm and 238.95 µm for the male and the female, respectively. The epidermis was notably thinner in the female compared to the male. Specifically, the thickness of the basal, spinous, and corneum layers in the female was 2.62 µm, 10.07 µm, and 3.15 µm, respectively, compared to 3.49 µm, 13.94 µm, and 3.66 µm in the male, respectively. Melanocytes, keratinocytes, and fibroblasts totaled 26, 24, and 50 cells in the male and 21, 25, and 54 cells in the female. Moreover, chondrocytes (male: n=33 and female: n=41) and perichondrium (male=13.99 µm and female=9.05 µm) were observed in the cartilage. These results demonstrate the histomorphometric differences and similarities between a male and a female puma, consistent with observation in other felines. This information is relevant for a targeted approach for establishing biobanks for this species.

Keywords: Biobanks. Conservation strategies. Histomorphometry. Wild felids.

1. Introduction

The puma (*Puma concolor* Linnaeus, 1771) is the fourth largest wild felid and the most widespread native land mammal in the Americas (LaBarge et al. 2022). This species exhibits a remarkable ability to adapt to diverse ecosystems (Culver 2010), with a geographical range extending from the southernmost part of Canada to the southernmost part of South America (Currier 1983). In Brazil, pumas are found in all biomes (Oliveira 1994). Despite this wide distribution, they are currently considered uncommon or rare species in several regions (Laundré and Hernández 2010).

The International Union for the Conservation of Nature (IUCN) assessment through the Red List of Threatened Species classified pumas as Least Concern (LC) in 1996, Near Threatened in 2002, and again as

LC in 2015 (Nielsen et al. 2015). However, there has been a trend towards population decline of the pumas (Nielsen et al. 2015), which is attributed to increasing habitat fragmentation, human retaliation, pre-emptive hunting, and trampling (Azevedo et al. 2013). The decrease in the population of this species has significant implications for biodiversity, considering that carnivore populations are vital to the dynamics of communities, exerting a direct influence on prey density and indirectly on the plant community (Borer et al. 2005). Pumas are considered bioindicators of the environment (Santos et al. 2019) and are important indicators of ecosystem health.

Conservation strategies for pumas include both *ex situ in vivo* methods, which involve transporting of these animals to reserves or zoos (Zanin et al. 2020), and *in vitro* methods, which involve the transporting and storing biological samples to create biobanks (Santos et al. 2021). Ear tissue is particularly valuable owing to its high fibroblast content, which can be cultured *in vitro* (Elliott et al. 2017). Cells obtained from cell cultures can be used in morphological research studies (Mario et al. 2018), nuclear reprogramming for regenerative medicine through the formation of induced pluripotent cells (Echeverry et al. 2020), and in combination with somatic cell nuclear transfer for species multiplication (Moulavi et al. 2017).

Previous studies have explored the impact of tissue cryopreservation on puma skin and analyzed cells recovered from *in vitro* culture (Lira et al. 2021). Moreover, an assessment of morphology and possible adverse effects during different cell passages (1st, 3rd, and 10th passages) has also been conducted (Lira et al. 2022), representing the first steps towards establishing a biobank for the species. Other reproductive strategies such as *in vitro* fertilization (Miller et al. 1989), artificial insemination (Barone et al. 1994), and semen cryopreservation (Deco-Souza et al. 2013) have also been employed.

Understanding skin morphology is crucial for both *in vivo* and *in vitro* conservation methods. Skin composition can vary inter- and intra-specifically, influenced by factors such as sex (Azzi et al. 2005; Praxedes et al. 2019), habitat, environmental conditions, nutritional diet, and stress levels (Araújo et al. 2019)."

To contribute to the formation of biological sample bank and considering the significance of descriptive morphological studies (Borges et al. 2017), this study aimed to morphologically describe the skin of pumas and compare its composition and structure between a male and a female puma. Although the basic structure of the skin is generally similar in the same species, the structural characteristics of its layers can vary between sexes (Azzi et al. 2005). These possible morphological differences in the skin imply the need for more accurate cryopreservation and *in vitro* culture protocols in accordance with these characteristics.

2. Material and Methods

Ethical approval

This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of the Semi-Arid (CEUA/UFERSA, no. 23091.010755/2019-32) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71804-1).

Chemicals, reagents, and media

The chemicals, reagents, and media used were obtained from Sigma-Aldrich, Gibco-BRL, Brazil.

Animals and skin biopsy

Skin biopsies (1–2 cm^2) were collected from the apical ear region of two adult pumas (male=1 and female=1). The samples from the two zoo animals (male from Sargento Prata Municipal Zoo and the female from Ecologic Park Ecopoint) were collected after prior anesthesia with dexmedetomidine hydrochloride 0.08 mg/kg (Dexdormitor®, Zoetis) and mechanical restraint as described previously (Araújo et al. 2019). The samples were collected using pliers washed with 70% alcohol and transported at 4 °C for 4

h to the laboratory in conical tubes containing Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution.

In the laboratory, the samples were trichotomized, fragmented (9.0 mm^3), and washed twice with DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution medium. Finally, four fragments from each animal were used for the analysis.

Histologic processing

To prepare the histology slides, the fragments were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 7 days. After fixation, the fragments underwent a series of steps, including tissue dehydration in increasing concentrations of ethanol (70, 80, 95, and 100%), with each step lasting 30 min. The samples were then diaphanized in xylol (xylol I, xylol II, and xylol III), with each bath lasting 30 min, to eliminate the alcohol present in the tissues.

The fragments were impregnated and embedded in paraffin as described by Praxedes et al. (2019). The resulting paraffin blocks were sectioned in serial sections with a thickness of 5.0 µm. Finally, histological slides were prepared and stained with hematoxylin and eosin (HE).

Hematoxylin-eosin staining process and cell quantification

For morphological and morphometric evaluations, previously prepared slides were stained with HE. The slides were placed in two 10-min baths of xylol, followed by three 3-min baths of alcohol. After staining with HE, the slides underwent five washes in alcohol and three washes in xylol for final diaphanization.

Histological analysis and morphometry were performed using the HE-stained slides. This analysis allowed for the quantification of epidermis, epidermal layers, dermis, cartilage and total skin thickness (in µm), as well as the number of epidermal cells, keratinocytes, Langerhans cells, fibroblasts, and melanocytes. For this assessment, 20 images of each animal were taken at 40 × magnification and quantified using ImageJ software (US National Institutes of Health, Bethesda, Rockville, USA).

Data analysis

Data are expressed as mean ± standard error and were analyzed descriptively. All histological aspects, including skin thickness and cell distribution in the ear skin samples, were also assessed.

3. Results

The morphological characteristics of the ear skin of the male and female pumas are shown in Figure 1A–B. The epidermis of both animals had three distinct layers: basal, spinous, and corneal. The basal layer was composed of a single layer of cuboid-shaped cells. The spinous layer was generally seen as a single layer of cells, and the corneum stratum was seen as a layer of dead, flattened cells, without nuclei. Distinction between the superficial and deep dermis was not evident in the observed dermal layers. Moreover, epidermal cells, melanocytes, keratinocytes, and fibroblasts were observed, including cartilage tissue with identifiable chondrocytes and the perichondrium (Figure 2A–B).

Histomorphometric analyses of the skin revealed a total thickness of 304.65 ± 36.89 and 238.95 ± 42.57 µm for the male and the female, respectively (Figure 1C). The thicknesses of the epidermis and dermis for the male were 21.56 ± 4.95 and 275.39 ± 34.7 µm, respectively, and 15.92 ± 3.15 and 220.65 ± 42.35 µm for the female, respectively. Based on the morphological and structural analyses, the ear skin of the male puma showed greater thickness of its epidermal and dermal layers compared to the female. In the epidermis, a reduction was observed in all layers between male and female: basal layer (male: 3.49 ± 1.1 µm and female: 2.62 ± 0.63 µm); spinous layer (male: 13.94 ± 3.78 µm and female: 10.07 ± 2.5 µm); and corneum layer (male: 3.66 ± 1.08 µm and female: 3.15 ± 0.87 µm) (Figure 1C).

In terms of the quantified cells, similar values were obtained for both the male and the female. The melanocytes, keratinocytes, fibroblasts, and Langerhans cells were counted as 26 ± 5.33, 24 ± 3.53, 50 ± 7.34, and 18 ± 3.88 for the male, respectively, and 31 ± 5.41, 25 ± 4.74, 54 ± 6.92, and 17 ± 3.92, for the female, respectively (Figure 1D). Finally, in the cartilage, the chondrocytes and perichondrium were 33 ± 9.09 and 13.99 ± 2.41 µm for the male, respectively, and 41 ± 6.98 and 9.05 ± 1.84 µm for the female, respectively (Figure 2C–D). In the cartilage, more chondrocytes were observed in the ear skin of the female, while a smaller area of the perichondrium connective tissue which provides nutrition to the cartilage was observed, when compared to males.

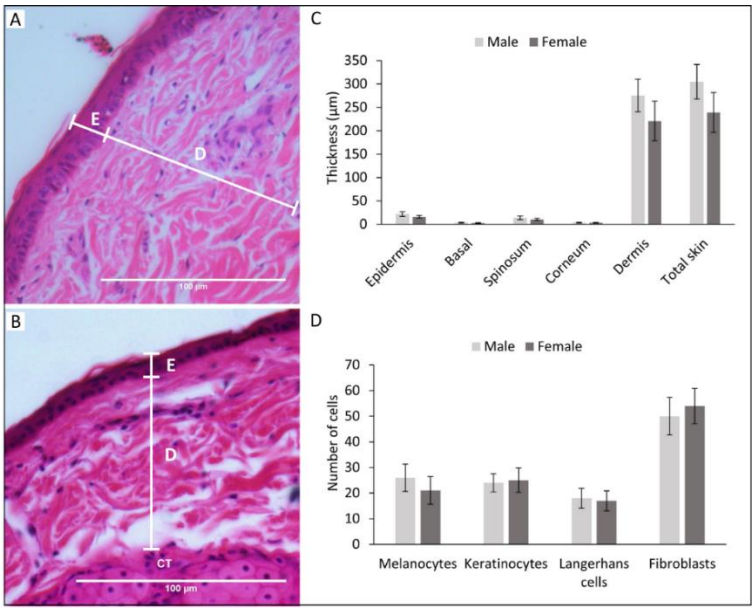

Figure 1. Histological images of the ear skin of a male and a female puma. **A -** Male puma ear skin. **B -** Female puma ear skin. **C -** Thickness of puma ear skin layers. **D -** Number of cells evaluated in the epidermis and dermis of the male and the female pumas. Epidermis (E). Dermis (D). Cartilage (CT).

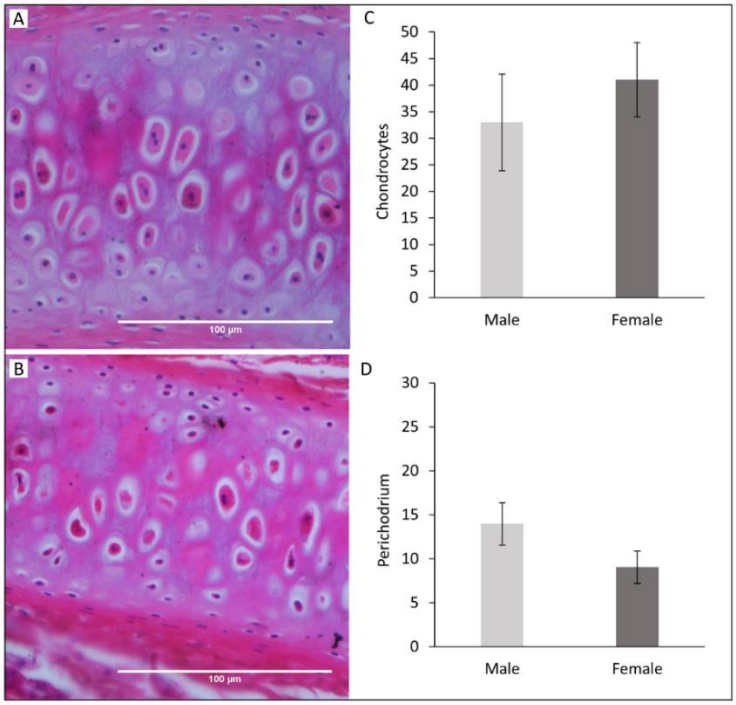

Figure 2. Histological images of the cartilage of the ear skin of male and female pumas. **A -** Male puma ear cartilage. **B -** Female puma ear cartilage. **C -** Number of chondrocytes quantified in the cartilage. **D -** Thickness of the perichondrium observed in the cartilage.

4. Discussion

This study evaluated and compared the morphological composition and structure of the ear tissue of a male versus a female puma. This information is crucial for the conservation of this species, as it contributes to the formation of somatic banks (Leon-Quinto et al. 2009; Guan et al. 2010), which is one of the methodologies that can be applied to the conservation of the species.

In wild felines and other mammals, the skin architecture consists of an external epithelial portion, the epidermis, which includes the corneum, lucid, granular, spinous, and basal layers (Praxedes et al. 2019). Regardless of sex, puma skin shows a structure and pattern of skin architecture similar to the general architecture of mammals, which is divided into the epidermis and dermis (Praxedes et al. 2020; Viana et al. 2023).

The thickness of the epidermis can vary depending on the location on the animal's body (Isola et al. 2013). In regions of the body with thin skin, such as the ear, there are usually three to four layers of epidermis (Affolter and Moore 1994). The data obtained in our study corroborate this, as only three layers of the epidermis were observed in both animals. The total epidermal thickness of the male puma (21.56 µm) was similar to the data found in another felid study on jaguars (*Panthera onca*), by Praxedes et al. (2020), which found that the epidermal thickness of jaguar coats ranged only between 29.3 and 32.6 µm. In contrast, female pumas had lower epidermal thickness (15.92 µm), which was similar to that found in other mammals (range: 10.0 to 45.0 µm) (Meyer and Neurand 1987). This variation may be associated with the hormonal and functional differences between males and females, which can influence skin thickness and affect cell production and extracellular matrix organization (Thornton 2002; Hall and Phillips 2005).

Furthermore, the epidermis represents an initial barrier to the penetration of substances into the skin, with the corneum being the first and most important layer owing to its lipid matrix (Lane 2013; Moser et al. 2001). The thickness of this layer in the male and female pumas ranged 3.66–3.15 µm, similar to that observed in the same species by Lira et al. (2021) and thinner than that of jaguars (Praxedes et al. 2019). There were no marked variations in thickness when compared to other wild mammals (Borges et al. 2017).

Additionally, the dermis of the male puma (275.39 µm) was thicker than the dermis of the female puma (220.65 µm) and the dermis of jaguars (242.0 µm) (Praxedes et al. 2019). However, both values were lower than those found for the neck region of *Cunniculus paca* (3,120.90 µm; Isola et al. 2013), the thoracic region of the rhesus monkey and pigs (1,457.2 µm and 3,848.2 µm, respectively) (Grabau et al. 1995). These variations in skin thickness may be related to the different regions analyzed and to factors specific to the species and habitat in which they live (Grabau et al. 1995; Salehi et al. 2013). Additionally, the dermis in males tends to be thicker than that in females because of higher collagen production stimulated by testosterone (Markova et al. 2004; Haag et al. 2012).

The number of melanocytes, which are responsible for melanin synthesis (Khavkin and Ellis 2011), showed only slight variations, with values ranging between 26 and 21 for the male and the female pumas, respectively. This supports the suggestion that the male puma exhibits greater activity in melanin production (Khavkin and Ellis 2011), possibly owing to genetic factors, hormonal influences (including the interaction between keratinocytes and melanocytes in the production of hormones that regulate melanin synthesis) or greater exposure to sunlight (Sulaimon and Kitchell 2003; Cichorek et al. 2013). In addition, the values observed were similar to those previously reported for the same species, both before and after cryopreservation, with values of 29 and 30, respectively (Lira et al. 2021). Notably, these values were higher than those observed in yellow (7) and black (11.3) jaguars (Praxedes et al. 2020).

Finally, the keratinocytes and fibroblasts make up the epidermis (Souza et al. 2009). Considering the basal layer as the place where the keratinocyte formation process takes place and considering that the basal layer was thinner in the female (2.62 ± 0.63 µm) compared to the male (3.49 ± 1.1 µm), a lower production of these cells could be anticipated. However, there was no variation in the number of keratinocytes in the male (24 ± 3.53) and female (25 ± 4.74). These values were similar to values previously identified for *P. concolor*, both before (25 ± 2.9) and after cryopreservation (24 ± 2.9) in the study by Lira et al. (2021). Furthermore, for fibroblasts, which are cells of great interest for biotechnological applications, the differences between the sexes were not marked, with values found for males (50 ± 7.34) and females (54 ± 6.92) showing minor variation. Similar values were obtained before and after cryopreservation (52 ±

5.1 and 53 ± 3.4, respectively) for the ear tissue of *P. concolor* (Lira et al. 2021). Considering that fibroblasts are fundamental to the synthesis of collagen and maintenance of the extracellular matrix (Esteves and Brandão 2022), the similarity observed between males and females indicates that both sexes can repair and maintain skin integrity. This uniformity is beneficial for conservation strategies such as the formation of tissue biobanks, where consistency in cellular responses is essential to ensure the effectiveness of treatments.

5. Conclusions

Histological analysis of the skin of a male and a female puma revealed differences in tissue architecture, as evidenced by variations in the thickness of the epidermis and its layers and the dermis. However, some similarities were identified, including in the number of evaluated cells. This information corroborates the hypothesis that sex can influence the histological characteristics of animal tissues, even when they belong to the same species. Therefore, there is a need to establish specific protocols to make the formation of species-specific biobanks viable.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Ethics Approval: This study was approved by the Ethics Committee of Animal Use of the Federal Rural University of the Semi-Arid (CEUA/UFERSA, no. 23091.010755/2019-32) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71804-1).

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