

Patrícia Isabel Paulino Simão

# **ENHANCING PALEODEMOGRAPHIC ANALYSIS OF PREHISTORIC POPULATIONS**

*A Geometric Morphometrics approach to Sex Estimation in  
infants using the auricular surface.*



Faro, 2024

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Master in Archaeology

Work carried out under the supervision of

Doctor Ricardo Miguel Godinho.



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**Enhancing Paleodemographic Analysis of Prehistoric Populations. A Geometric Morphometrics approach to sex estimation in infants using the auricular surface.**

*Declaration of work authorship.*

*I declare that I am the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and included in the list of references.*

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*Para o meu avô.*

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## Resumo

A estimativa do sexo biológico é um dos parâmetros mais importantes para o estudo dos comportamentos culturais e das dinâmicas das populações do passado. Permite explorar aspetos destas sociedades, tais como a sua demografia e movimentos migratórios, a sua epidemiologia, ou mesmo as suas práticas funerárias. Contudo, e apesar da sua relevância para estes estudos, a estimativa do sexo biológico é dos parâmetros mais difíceis de realizar, particularmente em vestígios esqueléticos incompletos ou fragmentados. Da mesma forma, geralmente não se efetua para indivíduos não adultos, visto que a sua aplicação é considerada como falível antes do desenvolvimento dos caracteres sexuais secundários.

Nas últimas duas décadas, diversos estudos investigaram a relação entre o dimorfismo sexual esquelético e as diferenças hormonais entre indivíduos não adultos masculinos e femininos. Após o nascimento, ocorre um pico de produção de testosterona nos indivíduos do sexo masculino, enquanto nos indivíduos femininos ocorre um pico de produção de estradiol. Os níveis destas hormonas permanecem elevados até aos 6 a 9 meses de vida, voltando a aumentar no início da puberdade. Considerando estas investigações, o desenvolvimento de metodologias para estimar o sexo de indivíduos não adultos tem-se multiplicado, sobretudo na última década. Assumem particular relevância os resultados promissores de estudos recentes acerca da morfologia da superfície auricular – região anatómica do ílio que articula com o sacro – e de como esta diverge entre os sexos desde idades muito precoces.

É neste âmbito que surge o presente trabalho, que testou o desenvolvimento de um método para estimar o sexo de indivíduos não adultos de contextos arqueológicos, dos 0 aos 6 anos de idade, aplicando Morfometria Geométrica à superfície auricular. O primeiro passo abrangeu o desenvolvimento e a validação da metodologia numa amostra de referência para a qual se sabem o sexo e a idade à morte dos indivíduos. Com esse intuito selecionaram-se 46 ílios (26 masculinos e 20 femininos) pertencentes a indivíduos com menos de 7 anos que fazem parte da Coleção Contemporânea de Esqueletos Identificados do Museu de História Natural de Lisboa (Coleção Luís Lopes). Da seleção excluíram-se os ossos cuja superfície auricular estava fragmentada, incompleta ou mal preservada. Primeiro realizaram-se análises para a amostra total, tendo-se realizado em seguida análises independentes para três grupos etários: < 1 ano, 1,0 a 3,9 anos e 4,0 a 6,9 anos.

Recorrendo a métodos da morfometria geométrica, primeiro foi necessário adquirir imagens tridimensionais (3D) de todos os espécimes amostrados. Em seguida procedeu-se à extração das coordenadas cartesianas de pontos anatómicos da superfície auricular previamente

selecionados (*landmarks* e *sliding semi-landmarks*), de forma a capturar quantitativamente a sua morfologia. Uma vez reunidas as coordenadas, realizaram-se duas análises típicas da morfometria geométrica, a Análise Generalizada de Procrustes (GPA) e a Análise de Componentes Principais (PCA), que foram suplementadas pela visualização das diferenças geométricas através do uso de grelhas de deformação. Por fim foram realizados testes estatísticos (Mann-Whitney e PERMANOVA) para avaliar a significância de diferenças hipotéticas entre os grupos.

A análise morfológica realizada à amostra de referência agregada não revelou diferenças entre os sexos no tamanho na superfície auricular. Quando se dividiu a amostra por grupos etários verificaram-se ligeiras diferenças de tamanho entre os sexos, ainda que estatisticamente não significativas. Nos indivíduos menores de um ano a superfície auricular tinha uma dimensão semelhante em ambos os sexos. Os indivíduos femininos com 1,0 a 3,9 anos apresentavam uma superfície auricular maior do que os indivíduos masculinos na mesma faixa etária. Em oposição, no grupo etário dos 4,0 aos 6,9 anos, a superfície auricular tinha maior dimensão em indivíduos masculinos. No que respeita à geometria da superfície auricular, para a amostra total não se verificaram variações de acordo com o sexo. Nas análises específicas para cada grupo etário, constatou-se que a superfície auricular tinha geometrias divergentes em indivíduos masculinos e femininos menores de um ano. Nos restantes intervalos etários verificou-se a quase total sobreposição dos grupos masculino e feminino, indicando a inexistência de diferenças na geometria da superfície auricular entre os sexos.

Uma vez que se verificaram diferenças entre os sexos na morfologia da superfície auricular, este método foi então aplicado a uma pequena amostra arqueológica de oito indivíduos imaturos. Estes pertencem à Coleção Antropológica do Museu Geológico de Portugal e provém dos concheiros Mesolíticos de Muge. Foram selecionados somente os ílios que tinham a superfície auricular completa e bem preservada. Visto que a técnica desenvolvida tem como pré-requisito a estimativa da idade à morte, este parâmetro foi previamente realizado através do comprimento máximo do íliaco. Contudo, a fragmentação de alguns ossos impediu a aplicação deste método. Em alternativa realizou-se a estimativa da idade à morte com base no tamanho do centroide, comparando os valores dos indivíduos arqueológicos com os dados da amostra de referência. Assim, verificou-se a presença de cinco indivíduos menores de um ano e de três indivíduos entre os 1,0 e os 3,9 anos.

A estimativa do sexo dos indivíduos arqueológicos efetuou-se com base nas análises específicas para os grupos etários. Dos cinco indivíduos com menos de um ano, somente um estava incluído no grupo feminino. Dos outros quatro indivíduos, um estava mais próximo do grupo feminino,

pelo que foi considerado como um possível indivíduo feminino. Os restantes três indivíduos encontravam-se mais próximos do grupo masculino, tendo sido classificados como prováveis indivíduos masculinos. Colocou-se, então, a hipótese de que, dos cinco indivíduos arqueológicos neste intervalo etário, dois possivelmente são femininos (2/5; 40,00%) e os restantes três são, muito possivelmente, indivíduos masculinos (3/5; 60,00%). Ainda que os resultados da amostra de referência não tenham revelado diferenças significativas entre os indivíduos masculinos e femininos dos 1,0 aos 3,9 anos, optou-se por também se tentar estimar o sexo dos indivíduos arqueológicos deste grupo etário. Os resultados foram inconclusivos devido à elevada sobreposição entre os grupos de referência masculino e feminino. Um dos indivíduos arqueológicos encontrava-se incluído no grupo masculino. Quanto aos dois restantes indivíduos, ainda que estivessem próximos do grupo de referência masculino, não foi possível classificá-los categoricamente como tal. Como a maioria dos indivíduos arqueológicos menores de um ano se encontra fora do morfo-espaco dos grupos de referência, os resultados da estimativa do sexo não puderam considerar-se como definitivos. Desta forma, não foi possível explorar, por exemplo, a demografia e proporção sexual da população subadulta exumada dos concheiros de Muge ou a existência de hipotéticas diferenças nas práticas funerárias relacionadas com o sexo.

Os resultados do presente estudo sugerem não só a presença de dimorfismo sexual na superfície auricular em idades muito precoces, mas também que o seu sinal biológico pode ser utilizado para estimar o sexo. No entanto, em indivíduos com idades superiores a um ano, a morfologia da superfície auricular é semelhante para indivíduos masculinos e femininos, sugerindo que o sinal biológico que reflete o dimorfismo sexual se esbate. O pico hormonal neonatal pode estar na origem destes resultados. Por outro lado, a redução das diferenças morfológicas ao nível da superfície auricular observadas em indivíduos masculinos e femininos com mais de um ano poderá ser explicada pela influência das forças biomecânicas associadas à locomoção bípede. A interpretação destes resultados promissores deve realizar-se cautelosamente. Ainda que a amostra de referência utilizada neste estudo seja uma das maiores em qualquer estudo comparável, o seu reduzido tamanho por grupo etário limita as conclusões. Adicionalmente, e ainda que a aplicação da metodologia desenvolvida à amostra arqueológica tenha tido algum sucesso, é necessário averiguar o seu desempenho noutras amostras populacionais. Apesar de os resultados não serem exatamente os mesmos em ambas as amostras analisadas (de referência e arqueológica), possivelmente devido à variação interpopulacional no dimorfismo sexual, existem evidências de que este método pode utilizar-se para estimar o sexo em populações distintas. Finalmente, deve também referir-se que foi avaliada somente uma região anatómica

nos ílios, a superfície auricular. Estudos prévios demonstraram que há outras características fiáveis para a estimativa do sexo, como a chanfradura ciática. A inclusão desta zona do ílio poderia aumentar a discriminação sexual em todos os grupos etários. Outro aspeto a considerar em estudos futuros é a aplicação de técnicas de Inteligência Artificial, como *Deep learning* ou *Machine learning*, uma vez que estudos recentes que aplicaram estas técnicas à estimativa do sexo obtiveram resultados promissores

Concluindo, este estudo demonstrou o potencial da Morfometria Geométrica na estimativa do sexo de subadultos provenientes de contextos arqueológicos. Também mostrou que a superfície auricular pode ser um indicador fiável na estimativa do sexo em indivíduos menores de um ano. Estes resultados devem explorar-se e investigações futuras devem incluir a análise de outras características do ílio, uma amostra de maiores dimensões para testar diferenças interpopulacionais, testes de validação e testes de aplicação noutras amostras arqueológicas e, eventualmente, a utilização de técnicas de Inteligência Artificial.

**Palavras-chave:** Arqueologia; Antropologia Virtual; Vestígios osteológicos subadultos; Ílio; Mesolítico; Período Contemporâneo.

## **Abstract**

Estimating biological sex is an essential tool for studying the dynamics and cultural behaviour of past populations. However, it is not usually performed on non-adult individuals before the onset of puberty, which limits the knowledge that can be gained from immature archaeological skeletal remains. Bearing this in mind, this thesis aimed to develop a three-dimensional Geometric Morphometrics technique to estimate the biological sex of infants aged 0-6 years using the auricular surface – the anatomical region of the ilium that articulates with the sacrum. First, the method was tested and validated in a contemporary sample of 46 individuals (20 females and 26 males) from the Luis Lopes Skeletal Collection (Lisbon). Sex-related morphological differences in the auricular surface were noted in infants under one year, but not in older individuals. The same methodology was then applied to eight infants from two Mesolithic shellmiddens, Cabeço da Arruda and Moita do Sebastião (Muge). Although the results are not conclusive, it was possible to perform sex estimation for individuals under one year. It is tentatively suggested that of the five archaeological infants under one year, two are probably females (2/5; 40.00%) and the remaining three are possibly males (3/5; 60.00%).

This study demonstrates the potential of using 3D GM for sex estimation in archaeological non-adult skeletal remains. It also shows that the auricular surface is a reliable sex indicator in infants under one year of age. In individuals over one year of age, the biological signal reflecting sexual dimorphism appears to fade. These findings should be investigated in more detail and further studies should include the analysis of other iliac features, a larger sample integrating other populations to examine if there are inter-population differences, cross-validation and cross-application testing, and eventually the use of AI techniques.

**Key Words:** Archaeology; Virtual Anthropology; Subadult skeletal remains; Ilium; Mesolithic; Contemporary period.

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## **Abbreviations list**

2D = Two dimensional

3D = Three dimensional

AI = Artificial Intelligence

AS = Auricular Surface

BCE = Before Common Era

BP = Before Present

DNA = Deoxyribonucleic acid

GM = Geometric Morphometrics

GPA = Generalized Procrustes Analysis

LLSC = Luís Lopes Skeletal Collection

LM = Landmarks

MSC = Muge Skeletal Collection

MUHNAC = National Museum of Natural History and Science

PCA = Principal Component Analysis

SLM = Semilandmarks

sSLM = Sliding semilandmarks

TPS = Thin-plate spline

## 1. Introduction

*«We must preserve for the future the status of archaeological human remains as part of the cultural heritage, and as indispensable empirical sources for the reconstruction of human population history through time and space » (Brůžek et al., 2024, pp. 204-205).*

The estimation of biological sex is vital for the study of human population dynamics and cultural behaviour (Curate, 2022). It is particularly relevant for elaborating the biological profile of human remains – description of sex, age, stature and any distinctive features, like evidence of disease or trauma (Rowbotham, 2016; Halcrow & Ward, 2018; Klales, 2020a; Curate, 2022; Brůžek et al., 2024). But what does sex refer to? In bioarchaeology – the study of archaeological human skeletal remains (Halcrow & Ward, 2018) – and forensic anthropology, sex represents morphological and/or genetic traits. Based on their expression in the skeleton, an individual is typically classified as male or female (although sometimes the estimation is inconclusive; Rowbotham, 2016; Klales, 2020a; Curate, 2022).

However, despite its significance for exploring past societies' demographics, funerary behaviour, and epidemiology, biological sex remains one of the most difficult parameters to assess, especially in incomplete or fragmentary remnants (Luna et al., 2017; Klales, 2020a). At the same time, sex estimation is not performed in non-adults, since its application is considered fallible before puberty – e.g. before the development of the secondary sexual characteristics, that become evident in the male/female phenotype (Lewis, 2007; Luna et al., 2017; Halcrow & Ward, 2018; Klales, 2020a; Stull et al., 2020; Boldsen et al., 2022; Curate, 2022).

Archaeological non-adult skeletons are a privileged source of information about past human societies (Lewis, 2007; Mays et al., 2017). From a biocultural perspective (Halcrow & Ward, 2018), they can provide insights into how children's and juvenile's social and physical life were, their age-at-death, their growth and development (Lewis, 2007), and their feeding practices and diet (Mays et al., 2017). Not only can they indicate possible cultural, social and economic issues involved in their diseases and trauma during their short lives (Lewis, 2007), but they can also point out childcare and cultural practices that may have impacted their health and well-being (Mays et al., 2017; Halcrow & Ward, 2018). Funerary behaviours of the communities they grew in can also be explored, like how they were buried, what type of grave goods accompanied them, and, if so, why their graves were on different burial grounds (Lewis, 2007; Halcrow & Ward, 2018). Overall, non-adult skeletons provide important insights into the lives of past

communities (Mays et al., 2017). Still, the picture that can be drawn from them is limited and incomplete because of a missing puzzle piece – the estimation of biological sex.

The development of methodologies for sex estimation in non-adults began in the late 1950s (e.g. Boucher, 1955; 1957). Few studies were undertaken until the 1990s (e.g. Fazekas & Kósa, 1978; Weaver, 1980; Schutkowski, 1987; Hunt, 1990) since the results were not promising. By the end of the 20th century, with more reference skeletal collections available for research, investigations about sex estimation in non-adults re-emerged (e.g. Mittler & Sheridan, 1992; Schutkowski, 1993; Holcomb & Konigsberg, 1995; Lewis, 2007). Sometime later, investigations demonstrated the display of sexual dimorphism at early ages due to a neonatal hormonal surge. While males experience a surge of testosterone a few hours after birth, females have a post-natal peak of oestradiol (Knickmeyer & Baron-Cohen, 2006; Stull & Godde, 2013; Clarkson & Herbinson, 2016; Lanciotti et al., 2018).

So, considering these studies, in the last decade investigations about biological sex estimation in infants multiplied (Curate, 2022), showing promising results (e.g. Olivares & Aguilera, 2016; Estévez et al., 2017). Research has been mainly focused on the skull and the ilium (Stull et al., 2020), but recently several authors have been looking at the morphology of the auricular surface (AS) – the anatomical region that articulates with the sacrum and that is located in the superior part of the *os coxae*, the ilium – and how it diverges between both sexes since early ages (e.g. Luna et al., 2017; Monje Calleja et al, 2020; Luna et al., 2021). Bearing this in mind and what information the estimation of biological sex in non-adults can bring for the study of past populations – for instance, differences in the mortuary behaviour between males and females, mortality rates and related issues, like childcare or infanticide practices, among others (Luna et al., 2017) – the main purpose of the present dissertation is to address the following questions: is it possible to use three dimensional (3D) Geometric Morphometrics (GM) to detect sexual dimorphism in infant skeletons? Is it possible to apply this methodology to archaeological collections? How can this technique contribute to future studies regarding sex-estimation in non-adults?

## **1.1. Aims**

The main purpose of the present study was the development of a new methodology to estimate the biological sex in infants aged between 0 and 6 years coming from archaeological contexts. To achieve this objective, firstly it was necessary to test and validate the technique using a contemporary reference sample with known sex and age-at-death. Therefore, 46 ilia belonging

to individuals in the established age range, from the Luis Lopes Skeletal Collection (LLSC), currently housed at the National Museum of Natural History and Science (MUHNAC), were selected. Applying 3D GM to the AS aimed to verify hypothetical morphological differences between male and female individuals. Iliac with severe post-mortem damage and/or with taphonomic changes on the AS were excluded from the selection.

Afterwards, and using the data from the previous analysis as a reference, the same technique was applied to eight Mesolithic infants of unknown sex. This represents the first attempt, in Portugal, to estimate the biological sex of infants from archaeological contexts using GM. The eight infants' remains originate from two Muge shellmiddens (Salvaterra de Magos, Santarém), Cabeço da Arruda and Moita do Sebastião. They belong to the Muge Skeletal Collection (MSC), which is partially housed at the Geological Museum of Portugal. The examined ilia belong to individuals within the age range of 0-6 years. Poorly preserved bones were not included in the sample.

Through this analysis, the present work also aims to demonstrate how estimating the biological sex of infants can be a valuable tool for investigating past human communities. Due to the lack of information on the biological sex of infants in archaeological contexts, this estimation can be invaluable for understanding and reconstructing the paleodemography of past communities; exploring infant mortality rates and how they changed over time; inquiring about disease incidence based on individual's biological sex; or even studying funerary behaviour regarding infants throughout human history. Hence, this investigation can be a starting point for applying 3D GM to other archaeological collections in the future.

Finally, this work aims to provide a non-destructive, low-cost alternative to estimating biological sex by biomolecular analysis (aDNA or Paleoproteomics). These are currently the most reliable procedures for estimating sex in infant skeletons. However, these high-cost and time-consuming techniques have some limitations. First, it involves the destruction of skeletal material and second, it faces the potential degradation and contamination of DNA or proteomic samples (Wilson & Humphrey, 2017). Therefore, if the efficacy of the method developed in this study is confirmed, 3D GM can be established as an alternative approach to biological sex estimation in infants' skeletal remains from archaeological contexts.

## **1.2. Structure**

This dissertation is divided into five sections. The first is the **State of the Art** (chapter 2), which presents the definition of biological sex estimation and its relevance to the study of past and

present populations. A particular section is dedicated to the relevance of this study for the archaeological non-adults recovered from the Muge shellmiddens. This is followed by a brief historical overview of this parameter, a small summary of current methods for estimating it and a brief explanation of why it is not usually performed in non-adults. Some of the studies carried out on sex estimation in non-adults are then presented, with particular emphasis on those focusing on the ilium. This is followed by a brief description of the ontogeny and development of the *os coxae* and a brief overview of GM. The next section is dedicated to **Materials and Methods** (chapter 3). A brief historical and geographical contextualisation of the skeletal collections is presented, followed by the selection criteria for each of the samples used, and the methods applied in this study. The following section refers to the **Results** (chapter 4). First, the outcomes concerning the morphological analysis of the reference sample are presented. The results of the application test conducted on a small pre-historic sample are also presented. Afterwards, in the **Discussion** section (chapter 5) the achievements and limitations of this research are discussed and considered regarding the studies already conducted in this field of investigation. The discussion considers both the overall and the archaeological results. Lastly, in the **Final Considerations** (chapter 6) a summary of this research's main outcomes and prospects for future studies are presented.

## 2. State of the Art

### 2.1. Sex Estimation

#### 2.1.1. Definition and Archaeological Relevance

*«The anthropological assessment of sex involves the use of skeletal indicators to provide an estimation of whether or not an individual was biologically male or female»*  
(Rowbotham, 2016, p. 261).

The estimation of biological sex plays a vital role in the study of past populations (Blau, 2014; Monge Calleja et al., 2020; Curate, 2022). Not only is it one of the keys to understanding population dynamics and cultural and social behaviour (Bethard & VanSickle, 2020), but it also conditions the estimation of other biological parameters, like age-at-death, stature (Krishan et al., 2016; Ubelaker & DeGaglia, 2017; Klales, 2020a), and ancestry (Blau, 2014; Krishan et al., 2016). Estimating sex usually involves the assessment of skeletal features and based on their size and shape, an individual is classified either as male or as female (although conclusive estimation is not always possible; Rowbotham, 2016, Bethard & VanSickle, 2020; Klales, 2020a; Curate, 2022). The differences in the male and female skeletal form (e.g. sexual dimorphism) vary within and across populations and are impacted by intrinsic (e.g. hormone levels) and extrinsic factors (e.g. geography, nutrition, environment, living conditions) (Wilson & Humphrey, 2017; Ubelaker & DeGaglia, 2017; Klales, 2020a).

Sex estimation is a key element in paleodemographic studies, as it can be used to assess variations in mortality and fertility rates, average life expectancy or differences in the lifespan of male and female individuals (Bethard & VanSickle, 2020; French et al., 2021; McFadden, 2021; Boldsen et al., 2022). By combining age-at-death and archaeological data, such as the location of burial grounds, funerary rituals, or types of grave goods, it is possible to study past societies' mortuary practices (Bethard & VanSickle, 2020; Boldsen et al., 2022) and sex-related burial treatments (Nikita & Chovalopoulou, 2023; Díaz-Navarro et al., 2024). Sex can also provide insights, along with skeletal and dental lesions, about how trauma and disease impacted past communities' lives (Bethard & VanSickle, 2020; French et al., 2021; McFadden, 2021; Boldsen et al., 2022; Díaz-Navarro et al., 2024), and whether male and female individuals were equally susceptible to them (Bethard & VanSickle, 2020; DeWitte & Yaussy, 2020; Dittmar et al., 2021; Nikita & Chovalopoulou, 2023). More recently, sex has also been used to investigate

age-at-weaning and post-weaning feeding practices (French et al., 2021; Velte et al., 2023). Together with age-at-death, sex has likewise been quite useful in archaeological studies regarding population mobility and socioeconomic transitions (McFadden, 2021; Boldsen et al., 2022). Even in human evolution and paleoanthropology, sex estimation has also played an important role in studying, for instance, the evolution of sexual dimorphism in the human species and the impact of adaptation of pelvic morphology to parturition (Bethard & VanSickle, 2020).

An example of the potential of combining biological sex estimation with contextual information from an archaeological site is the study conducted by Díaz-Navarro and co-authors (2023a). They presented a paleodemographic analysis regarding the Chalcolithic burial site of Camino del Molino, located in Caravaca de la Cruz (Murcia, Southeastern Spain). Sex estimation in the adult sample allowed the investigators to assess the sex ratio and differences in life expectancy and morbidity between the sexes. Furthermore, sex information was associated with the spatial distribution of individuals (Figure 2.1) so that radiocarbon samples were representative of the exhumed population. Another study, regarding the same archaeological site, evaluated the extra masticatory use of the dentition, providing evidence of women's specialisation in yarn production (Díaz-Navarro et al., 2023b).

Yet another example is the previous study by Fernández-Crespo and de-la-Rúa (2015), in which they gathered and analysed the demographic data concerning the megalithic monuments of northern Spain. The authors not only identified asymmetries in the sex and age distribution of the assemblages but also assessed potential sex-related differences regarding funerary practices. Interestingly, Cintas-Peña and Herrero-Corral (2020) made a similar approach to the demographic data obtained from the skeletal assemblages from the Iberian Peninsula between the 8th and the 3rd millennia BCE. Regarding funerary practices, Fidalgo and co-authors (2016) found sex-related differences in the type of grave goods in an assemblage from the Bronze Age site of Torre Velha 3, located near Serpa (Beja, Portugal). Ceramics and bronze awls were usually associated with female burials.

Masclans and colleagues (2019) also found that the distribution of stone tools in eight burial sites from the 5th and 4th millennia BCE (northeastern coast of the Iberian Peninsula) depended on the individual's sex. In general, men were buried with more stone tools, which were more often made from exogenous raw materials. Projectiles, frequently associated with hunting activities, were also usually buried with male individuals. Stone tools associated with women tended to be smaller in size. In addition, women were significantly more often associated with bone awls and scrapers, which are commonly associated with, for example, leather and pottery

crafts. Masclans Latorre and co-authors (2021) also investigated sex differences in burial practices at one of the most important Neolithic cemeteries from the Carpathian Basin, Vedrovice (Moravia, Czech Republic). In terms of spatial distribution, male burials were usually clustered along a continuous diagonal axis around the centre of the necropolis. Female burials were scattered throughout the area and their distribution was also age-related, with mature adults near the centre and senile adults and adolescents along the edges. Grave goods (e.g. tools and ornaments) were more often associated with male individuals.

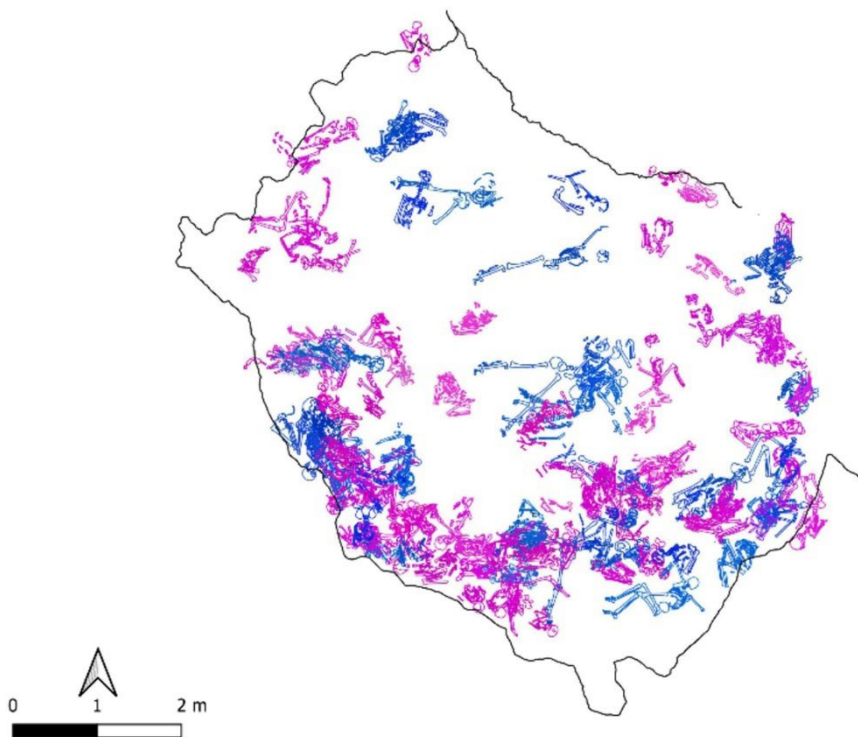


Figure 2.1 – Spatial distribution, according to sex, of the complete and partial human burials recovered from the collective tomb found at Camino del Molino (Caravaca de la Cruz, Murcia, southeastern Spain). The pink colour represents female individuals, while the blue colour refers to males. Image adapted from Díaz-Navarro and collaborators (2024).

Even though sex estimation is an invaluable tool for exploring past societies' demographics, funerary behaviour, and epidemiology, it is usually not carried out on subadult individuals, as it is deemed fallible before the onset of puberty (Lewis, 2007; Luna et al., 2017; Halcrow & Ward, 2018; Klales, 2020a; Stull et al., 2020; Boldsen et al., 2022; Curate, 2022). Non-adult human remains represent an important part of the archaeological record (Luna et al., 2017) and are a privileged source of information regarding past communities' demography, social structure and biocultural dynamics (Lewis, 2007; Mays et al., 2017; Lugli et al., 2020). Through their skeletal remains, it is possible to investigate particular questions about childhood, such as

weaning and post-weaning practices (Mays, 2013; Velte et al., 2023), patterns of growth and indicators of non-specific stress (Mays, 2013; Lugli et al., 2020), or even the incidence of particular pathologies, like deficiency of Vitamin C or D (Mays, 2013). As sex estimation is not carried out for these individuals, many questions remain unanswered. For example, was growth and development similar between males and females? Were the lifeways similar for children and juveniles from both sexes (Lewis, 2007)? Were there sex-related differences in diet and weaning practices (Mays et al., 2017; Velte et al., 2023)? Were mortality rates equal for males and females (Mays, 2013; Lugli et al., 2020) or can they be explained by sex-specific infanticide practices (Smith & Kahila, 1992; Mays, 2013)? Were childcare and cultural practices sex-specific? Were burial practices and funerary treatment the same for male and female non-adults (Mays et al., 2017; Halcrow & Ward, 2018; Rebay-Salisbury et al., 2022)? Although some extrapolations can be made from retrospective studies in adults, non-adults may provide a more complete picture of these issues (Lugli et al., 2020).

#### *2.1.1.1. Muge Non-Adult Burials: Contributions to the Archaeological Study of Mesolithic Communities*

Discovered in the early 1860s, the Muge shellmiddens, located in central Portugal served as burial grounds for the last hunter-gatherers of the Iberian Peninsula (Nogueira et al., 2023; see section 3.1.2.1.). Since then, about 300 individuals have been recovered from four of the 13 shellmiddens – Cabeço da Amoreira, Cabeço da Arruda, Moita do Sebastião, and Cova da Onça (Carvalho, 2009; Bicho et al., 2010; Bicho et al., 2013b; Ferreira et al., 2015; Figueiredo & Gonçalves, 2015; Umbelino et al., 2019) – making this one of the largest known Mesolithic skeletal collections in Europe (Jackes & Lubell, 2012; Nogueira et al., 2023).

Studies by Jackes and Lubell (2012), Figueiredo (2014) and Peyroteo Stjerna (2016) have provided a comprehensive overview of the funerary practices within these communities. Burials seem to have taken place in shallow graves and natural depressions, with no evidence of funerary structure, and a small mound covering the grave after the body was placed (Jackes & Lubell, 2012). Most of the burials correspond to single, double or multiple primary inhumations, in which the body was placed in a supine or lateral position (Carvalho, 2009; Bicho et al., 2010; Nogueira et al., 2023). Variations in the positioning of the arms and legs were observed, as well as in the orientation of the burials (Jackes & Lubell, 2012). Although rare, a few individuals were accompanied by grave goods (e.g. shell beads and perforated shells

from personal ornaments, and lithic tools, among others) (Carvalho, 2009; Bicho et al., 2010; Nogueira et al., 2023).

No grouping of burials by sex or age was observed, except at Moita do Sebastião, where adults and non-adults were buried in separate areas (Figure 2.2), as noted by Jean Roche in his 1972 publication – *Le Gisement Mésolithique de Moita do Sebastião (Muge. Portugal). I – Archéologie*. Denise Ferembach's evaluation (1974) showed that all these individuals had an age-at-death of around or below 3 years. It is important to note that the excavation of Cabeço da Arruda is incomplete, so the possibility of a selective burial area for young children cannot be completely ruled out (Jackes & Meiklejohn, 2008). The burial treatment of adults has been described as similar, regardless of sex (Carvalho, 2009). However, recent findings in Cabeço da Amoreira revealed a male and a female individual with differences in burial practices. The authors concluded that further data is needed to clarify if these differences are related to sex, age or social status (Umbelino et al., 2016).

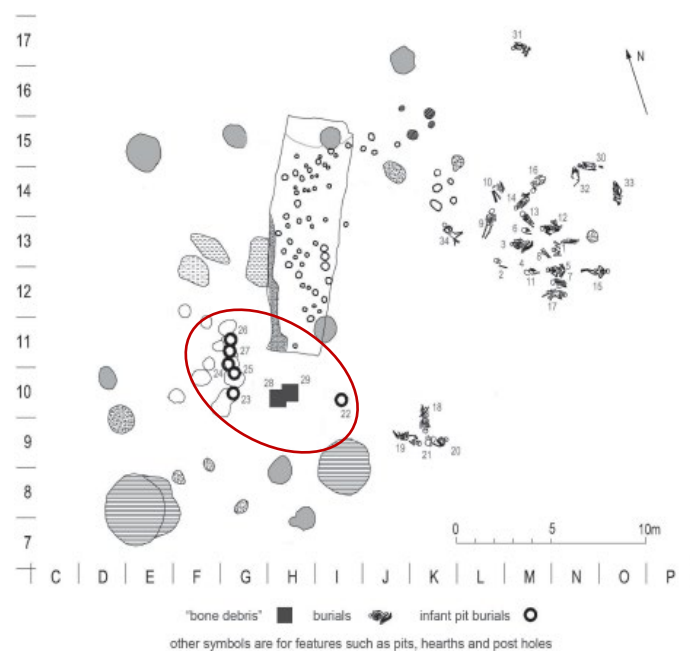


Figure 2.2 – Spatial distribution of human burials by age-at-death in the Moita do Sebastião shellmidden. The non-adults are concentrated in a small part of the site (red circle), separated from the adults. Image adapted from Jackes and co-authors (2014).

For more than a century, anthropological studies have been essential for understanding the cultural behaviour and population dynamics of the communities that lived in the Tagus valley around 8000 years ago (Nogueira et al., 2023). The first studies of skeletal remains were carried out by António Mendes Corrêa in the early 20th century (J. L. Cardoso, 2011; Nogueira et al., 2023). His research focused mainly on the ancestry and variation of cranial morphology

(Corrêa, 1919, 1926, 1936), which was later continued by Alfredo Athayde (1940). Important anthropological research was also carried out in the 1960s and the 1970s by Denise Ferembach (1965, 1969). Her first efforts were aimed at estimating the minimum number of individuals recovered from the sites of Moita do Sebastião (N=168), Cabeço da Arruda (N=113) and Cabeço da Amoreira (N=28 or 29; Ferembach & Roche, 1971). In 1974, she dedicated an entire volume to the study of the Moita do Sebastião assemblage, entitled *Le Gisement Mésolithique de Moita do Sebastião (Muge. Portugal). II – Anthropologie*. In addition to a detailed inventory of each skeleton, Ferembach presented a paleodemographic profile of the skeletal sample.

Over the last four decades, anthropological studies have attempted to reconstruct demographic profiles (e.g. Jackes, 1988; Lubell et al., 1989; Jackes & Lubell, 1999; Cunha & F. A. Cardoso, 2001; Cunha et al, 2003; Jackes & Meiklejohn, 2004, 2008), assess health and disease status (e.g. Frayer, 1987; Lubell et al., 1989; Jackes & Lubell, 1996, 1999; F. A. Cardoso, 2001; Cunha & F. A. Cardoso, 2001; Cunha et al., 2003; Jackes, 2004, 2009; Umbelino et al., 2015, 2019; Godinho et al., 2023a) and even dietary patterns (e.g. Lubell et al., 1994; Jackes & Lubell, 1999; Jackes & Meiklejohn, 2004; Umbelino, 2006; Umbelino et al., 2007, 2015; Peyroteo Stjerna, 2021), mobility (e.g. Price, 2015), morphology (e.g. Meiklejohn & Babb, 2015; Godinho et al., 2022, 2023b) and ancestry (e.g. Jackes et al., 2001; Carvalho et al., 2023) of the Mesolithic populations of Muge (Nogueira et al., 2023). For non-adults in particular, some paleodemographic, paleopathological and dietary information is available.

The study by Cunha and co-workers (2003) indicates that the proportion of non-adults varies between 16.00% (Cova da Onça) and 49.00% (Cabeço da Arruda). On the other hand, Jackes and Meiklejohn (2008) indicate a ratio of adults to sub-adults of around 2:1, between 25.00% and 34.00%. Both studies show that all age cohorts are represented, from neonates to individuals aged 20 years. Jackes and Meiklejohn (2008) point to high infant mortality, especially at younger ages, which allowed the researchers to estimate a fertility rate of four to six children during a woman's reproductive years. Despite these conclusions, the underrepresentation of infants and non-adults in early childhood was noted not only by Cunha and co-authors (2003) but also by Jackes and Meiklejohn (2008). These two researchers urged caution in interpreting these results, as taphonomic changes, excavation and curation methods, osteological analysis techniques, and burial customs and/or cultural practices may contribute to the underrepresentation of non-adult skeletal remains. The low frequency of enamel hypoplasia noted by Cunha and co-authors (2003) suggests minimal growth disturbances during childhood. Finally, concerning diet, an isotopic analysis was recently carried out on the Burial 1 of Cabeço da Amoreira, a non-adult with an age-at-death between 1 and 3 years. The results show a

combination of terrestrial and marine proteins in the diet, with the latter being the predominant component (56.00%). This is in line with the results obtained for the adult individuals of Cabeço da Amoreira, Moita do Sebastião and Cabeço da Arruda (Lubell et al., 1994; Umbelino, 2006; Umbelino et al., 2007, 2015; Peyroteo Stjerna, 2021).

As shown above, the skeletal remains of non-adult individuals have been essential not only for reconstructing the age profile of the Mesolithic communities of Muge (Cunha et al., 2003; Jackes & Meiklejohn, 2008) but also for establishing infant mortality and estimating fertility rates (Jackes & Meiklejohn, 2008). They have also contributed, albeit preliminarily, to confirming that dietary patterns are shared by adults and infants (Umbelino et al., 2015). The apparent absence of growth disorders seems to indicate that these populations had regular access to food resources (Cunha et al., 2003). In terms of funerary behaviour, rituals seem to be similar regardless of age and/or sex, except at Moita do Sebastião, where infants and adults were buried in separate areas of the shellmidden (Roche, 1972; Carvalho, 2009; Jackes & Lubell, 2012).

The non-adult skeletal remains from Muge have provided valuable insights into the demography, social structure and biocultural dynamics of Mesolithic communities (Lewis, 2007; Mays et al., 2017; Lugli et al., 2020). However, the lack of information regarding the biological sex limits our understanding of these non-adult individuals. If this information was available, the age profile of the sample could be analysed alongside the sex ratio. This could reveal whether males or females have higher mortality rates (e.g. Mays, 2013; Lugli et al., 2020) and allow us to explore the reasons for those differences, such as cultural practices like infanticide (e.g. Smith & Kahila, 1992; Mays, 2013) or selective childcare. In addition, knowing the sex of non-adults would allow us to research whether male and female infants are equally susceptible to certain diseases. This information could also help to explore differences in weaning practices or dietary patterns (e.g. Mays, 2013; Mays et al., 2017; Velte et al., 2023), as Umbelino (2006) found for adult females, who consume more plant resources, possibly due to different activity patterns. Differences in burial behaviour between the sexes could also be investigated, in particular the positioning and orientation of the body, as well as the types of grave goods associated with the burial (e.g. Mays et al., 2017; Halcrow & Ward, 2018; Rebay Salisbury et al., 2022). This would be particularly interesting for the Moita do Sebastião shellmidden, where a distinct burial area for infants has been identified (Roche, 1972; Carvalho, 2009; Jackes & Lubell, 2012).

This work aims to fill a significant gap in the study of past human populations by developing a methodology for estimating the biological sex of non-adult archaeological individuals. By

obtaining this data, we will be able to explore and research several important questions, such as those outlined above, and thereby improve our understanding of the cultural and social behaviour of subadults living in the Tagus valley around 8000 years ago.

### 2.1.2. Brief History

The first studies referring to the dissimilarities of the male and female skeletons were published during the late 15th and early 16th centuries. They focused mainly on the pelvic region morphology and its importance for parturition in females. On this matter, of great relevance were the books *Anatomy, or the History of the Human Body* (original title *Historia corporis humani sive anatomice*, 1502), authored by the anatomist Alessandro Benedetti (1445-1525), and *Commentary on the Anatomy of Mondino* (original title *Commentaria, cum Amplissimis Additionibus Super Anatomia Mundini*, 1521), written by the physician Giacomo Berengario da Carpi (1460-1530) (Stolberg, 2003; Rowbotham, 2016; Parent, 2019; Klales et al. 2020a). Some years later Andreas Vesalius (1514-1564), the so-called father of modern anatomy (Zampieri et al., 2016; Klales et al., 2020a; Santos, 2020), published *De Humanis Corporis Fabrica* (1543), where the first illustrations of the human skeleton (Figure 2.3) made from real observations can be found (Zampieri et al., 2016; Klales et al., 2020a).

Even though the innovations in the anatomy field made by Vesalius are widely recognized, it was Felix Platter (1536-1614) who published the first illustration of the female skeleton (Figure 2.3). Besides, in his book *De Corporis Humani Structura et Usu libri III* (1583), one can also find descriptions of 13 sexually dimorphic skeletal features (Stolberg, 2003; Bir et al., 2015; Rowbotham, 2016). Caspar Bauhin (1560-1624), Felix Platters' colleague, improved the anatomical terminology used to describe those features in his two manuscripts, *Anatomica Virilis et Muliebris Historia* (1597) and *Theatrum Anatomicum* (1605). The groundbreaking studies conducted by these two physicians can be considered the first scientific approaches to sex-related skeletal variations (Stolberg, 2003; Rowbotham, 2016; Stöcklin & Vos, 2023).

In the following two hundred years many notable works addressed the skeletal differences between sexes (Klales et al., 2020a). Nevertheless, the knowledge about sexual dimorphism and how it manifests itself in the male and female skeleton was only established in the late 18th century (Rowbotham, 2016). Finally, in the early 1800s, the first rudimentary methods for sex estimation using cranial metrics were developed (Klales et al., 2020a). One example is the study presented by Samuel George Morton (1799-1851). In his 1839 book, *Crania Americana*, he reported how he assembled a collection of human skulls from around the world (The Morton

Collection) and how he took detailed measurements from each one. Even though this was not the main purpose of his research, Morton estimated the sex of the skulls based on the assumption that the male cranium was bigger (Lewis et al., 2011).

Pierre Paul Broca (1824-1880) was the first to record cranial features that could be used for sex assessment (Rowbotham, 2016; Klales et al., 2020a). In his book, *Instructions Craniologiques et Craniométriques*, published in 1875, this anatomist and anthropologist pointed out nine characteristics that differ in the male and female skull (e.g. the supraorbital margin and the glabella). For the postcranial skeleton, Karl Pearson (1857-1836) published in 1915 the pioneer study for the estimation of biological sex – *On the Problem of Sexing Osteometric Material* – using the bicondylar length of the femur (Rowbotham, 2016).

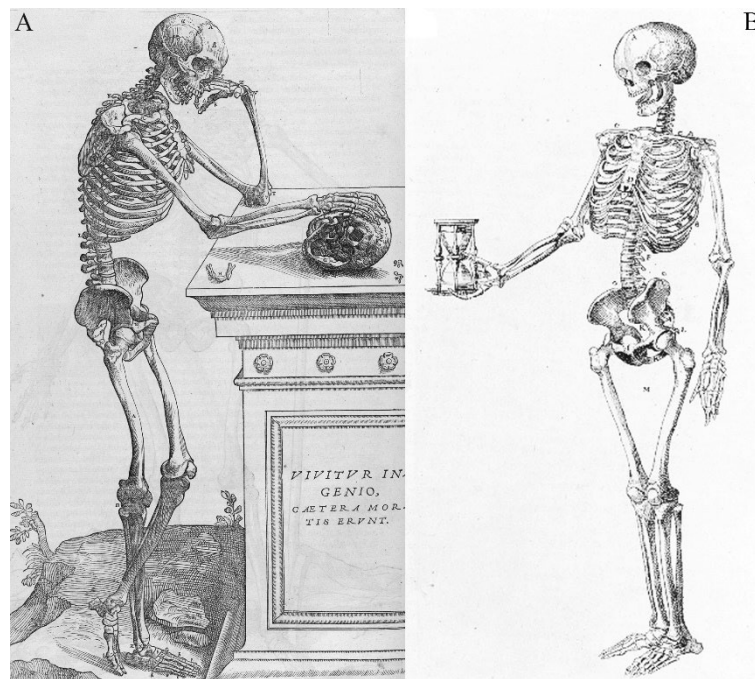


Figure 2.3 – A: Illustration of the human skeleton presented in the second edition of Vesalius's book *De Humanis Corporis Fabrica* (1555). B: Illustration of the female skeleton in Platter's *De Corporis Humani Structura et Usu libri III* (1583).

Curiosity about the dawn of humankind and the diversity of the human species led to the assembly of documented human skeletal collections around the world (Santos, 2020) between the late 1800s and the early 1900s (H. F. Cardoso, 2006a). And so, in the first decades of the 20th century, researchers began to resort to these collections to develop new methods and techniques for biological sex estimation (Klales et al., 2020a; Curate, 2022). Currently, this parameter can be estimated through several approaches (Rowbotham, 2016; Klales, 2020a):

- i. **Morphological methods** – The most common and traditional approach is the visual assessment of morphological skeletal features (Blau, 2014; Rowbotham, 2016; Krishan et al. 2016; Klales, 2020a). Several studies confirmed that the pelvis and cranium are the most dimorphic skeletal regions. Therefore, they are usually favoured for the estimation of biological sex (Blau, 2014; Krishan et al., 2016; Rowbotham, 2016; Cunningham et al., 2017; Verbruggen & Nowlan, 2017; Klales, 2020b).
- ii. **Morphometrical methods** – This includes comparing measurements and indices of specific skeletal elements, preferably the skull and long bones, and/or using statistics (e.g. discriminant functions and regression analysis) to quantitatively examine those differences (Blau, 2014; Krishan et al., 2016; Rowbotham, 2016). Given that sexual dimorphism varies between populations, preference is usually given to population-specific methods (Blau, 2014; Rowbotham, 2016). Sex-related skeletal morphological variations have also been analysed using GM. This approach allows the quantification of shape differences in rigid anatomical structures (Slice, 2005; Krishan et al., 2016; Klales, 2020a; Klales et al., 2020b). This data is then used to assess shape differences and/or similarities between males and females, allowing sex estimation (Klales et al, 2020b).
- iii. **Biomolecular analysis** – Recent techniques have made it possible to extract and analyse DNA from ancient human remains (Blau, 2014). Even though this is a time-consuming, destructive and expensive process, it is currently the most reliable method for sex assessment (Blau, 2014; Rowbotham, 2016; Krishan et al., 2016; Klales, 2020a; Brůžek et al., 2024), reaching almost 100% of accuracy (Klales, 2020a). Latest studies have shown that by using the amelogenin protein in tooth enamel, it is possible to distinguish Y (male) and X (female) isoforms. Given that paleoproteomics may be a minimally invasive method (depending on the sampling technique), it is being used to estimate sex (Stewart et al., 2017; Buonasera et al., 2020; Granja et al., 2023; Mikšik et al., 2023; Brůžek et al., 2024), with some validation studies indicating an accuracy of 100% (Brůžek et al., 2024).

The choice of the appropriate methodology to estimate the sex of unknown archaeological skeletal remains depends on multiple factors: bones available for analysis, their state of preservation (e.g. post-mortem taphonomic damages or pathological alterations), and their age-at-death (Klales, 2020b; Brůžek et al., 2024). Apart from aDNA and paleoproteomic analysis, sex estimation is usually not performed in subadults (Klales, 2020b), since its application is considered fallible before the development of the secondary sexual features in puberty (Blau,

2014; Krishan et al., 2016; Rowbotham, 2016; Cunningham et al., 2017; Luna et al., 2017; Verbruggen & Nowlan, 2017; Stull et al., 2020). On the other hand, several studies have demonstrated that the post-natal hormonal disparities between male and female individuals may result in some display of skeletal sexual dimorphism at early ages (Monge Calleja et al., 2020; Luna et al., 2021).

The neonatal hormonal surge, or 'minipuberty', refers to the surge of testosterone that males experience some hours after birth (Clarkson & Herbinson, 2016; Lanciotti et al., 2018). It also refers to the female surge of oestradiol one week after birth (Knickmeyer & Baron-Cohen, 2006; Lanciotti et al., 2018; Luna et al., 2021). Hormonal levels will remain elevated for around 6 to 9 months, and then they will gradually decrease (Knickmeyer & Baron-Cohen, 2006; Clarkson & Herbinson, 2016; Lanciotti et al., 2018). Between the end of the second year of life and the onset of puberty, there is a period of low hormonal concentration levels, also known as 'prepubertal hiatus' (Cummings & Kavlock, 2004). In light of these studies, numerous researchers have investigated ways to differentiate between non-adult males and females. Studies have mainly focused on the ilium (Krishan et al., 2016; Rowbotham, 2016), as it has been reported to reflect the sex differences found in adulthood (Cunningham et al., 2017; Verbruggen & Nowlan, 2017). It has also been shown that the foetal pelvic girdle is sexually dimorphic from the onset of primary ossification (Kanahashi et al., 2024). Recently, research has likewise focused on the application of GM for sex estimation in subadults (Krishan et al., 2016).

### 2.1.3. Non-adult Studies

Studies of sex estimation in non-adults using iliac features date back to the mid-1950s (Lewis, 2007; Mays, 2013), when Boucher (1995, 1957) presented a technique for differentiating male and female fetuses by the sciatic notch morphometrics (see Table A, Appendix A). Sex-related morphological differences in the dimensions of that trait were also noted by Fazekas and Kosá (1978) some decades later. The first study suggesting the existence of sexual dimorphism in the morphology of the AS was carried out by Weaver (1980). Using a sample of 151 fetuses and infants aged 0-6 months from the Smithsonian Identified Skeletal Collections (Washington), this researcher evaluated the morphometry of two iliac features, the sciatic notch and the AS. No differences were found between males and females for the sciatic notch, but positive results were observed for the AS, particularly for males. A cross-validation test was later carried out by Hunt (1990), but no sex differences in AS morphology were found. Similarly, Mittler and

Sheridan (1992) obtained different results from Weaver when they analysed the AS of mummified Nubian individuals under 19 years of age from the medieval period (6th-15th centuries). Differences were found for both sexes, but only for children over 9 years of age. Accuracy was also higher for males.

In 1993, after successfully estimating the sex of foetuses by the sciatic notch morphometrics (1987), Schutkowski developed a method for estimating the sex of non-adults, by evaluating three mandibular features and four iliac traits in a sample of 61 individuals (0-11 years) from the London Spitalfields Skeleton Collection. The percentage of correct diagnoses for iliac features, namely the sciatic notch and the arc criterion, was high, especially for males. Posteriorly, Sutter (2003), Vlak and colleagues (2008), and Olivares and Aguilera (2016) tested the methodology proposed by Schutkowski, and the results were not consistent. For example, Sutter (2003) found differences between males and females under 16 years old on the sciatic notch and the arch criterion. In contrast, in non-adults under 17 years of age, Vlak and co-authors (2008) found no sex differences in any of the iliac features. These authors suggested that the poor accuracy of these morphological traits may be due to the high variation in the growth patterns of the ilium. As for Olivares and Aguilera (2016), they were able to differentiate male and female foetuses and infants under 7 years of age using the sciatic notch, but not employing the arch criterion.

The first attempt to use GM to estimate sex in subadults was made by Holcomb and Konigsberg (1995). Using photographs of 133 foetal ilia from the Smithsonian Collection, the authors analysed the morphology of the sciatic notch. Although the presence of sexual dimorphism was noted, the authors concluded that it was not sufficient to correctly estimate sex. More than 10 years later, Wilson and colleagues (2008) also used GM to analyse three iliac features. They examined photographs of 25 ilia from individuals under 9 years old belonging to the Spitalfields Collection. They were able to correctly estimate the sex of 84.00% of the specimens based on the morphology of the sciatic notch. The other two criteria, the iliac crest and the AS, did not provide reliable results. A few years later, Wilson and colleagues (2011) conducted a validation test on 126 individuals aged between 0-15 years from two skeletal collections, Lisbon and London. Accuracy levels were lower in the Portuguese sample (62.20% - 89.00%) than in the English sample (67.40% - 93.60%). The low success rate of the AS morphology led the authors to advise against the use of this trait for sex estimation in non-adults. Additionally, they suggested that inter-population variation in sexual dimorphism could explain the distinct outcomes for the two samples.

Based on the positive results of a previous work (García-Mancuso, 2009), García-Mancuso and González (2013) analysed four iliac features and the overall outline of the ilium using GM. They assembled a small sample of 21 infants under 5 months of age from the Lambre Collection, housed at the School of Medical Sciences of the National University of La Plata. Since the results were promising, except for the acetabulum, the authors conducted a new study on a larger sample with a wider age range. For this study, 216 foetuses and non-adults under the age of 17 from four different collections – Lisbon, Coimbra, Granada and La Plata – were examined. The presence of sexual dimorphism was confirmed for the outline of the ilium and the sciatic notch, but the AS showed no differences between the sexes (García-Mancuso et al., 2018). Also of note is the investigation carried out by Estévez and colleagues (2017) on a sample of 71 foetuses from the Granada Skeletal Collection. After photographing and landmarking the sciatic notch and the partial contour of the AS, these researchers identified sex differences in both features. Interestingly, the success rate of the technique was higher when both traits were combined.

Other examples of studies using 3D GM to approach sex estimation in non-adults can be cited (e.g. Mokrane et al., 2013; Wilson et al., 2017). The most outstanding is a study by Garvin and co-workers (2021), in which they evaluated the reliability of the sciatic notch and the iliac crest as sex indicators. A total of 397 modern USA non-adults under 15 years of age were analysed. Poor results led the authors to suggest that these two features should not be used for sex estimation. Nevertheless, in individuals with less than one year, sex-related differences were found. The hypotheses put forward by the researchers were the neonatal hormonal surge and the lack of biomechanical forces associated with bipedal locomotion – usually achieved at 12 to 15 months of age (Cowgill & Johnston, 2018).

In 2017, Luna and colleagues proposed a new methodology that combines the assessment of morphological features with morphometrics analysis of the AS. After selecting 34 individuals aged between 7 and 18 years from the Coimbra Identified Skeletal Collection, the authors were able to correctly classify the sex of 82.00% of the sample. This method was later tested by Monge Calleja and co-authors (2020) on 61 individuals under the age of 19 from the LLSC. To assess the effect of hormonal peaks on sexual dimorphism before and during puberty, the researchers divided the sample into two age groups: 0-12 years and 13-18 years. To assess if the effects of the neonatal hormonal surge could also be reflected in sexual dimorphism, the researchers conducted an independent analysis on individuals with less than 2 years of age. The results obtained demonstrated the reliability of the method regardless of the age group, but the

success rate was higher in individuals under 2 years of age. The authors suggest that this may relate to the neonatal hormonal surge.

Luna and collaborators (2021) re-evaluated this technique, this time on 197 fetuses and infants under 6 years of age from the Lisbon and Granada reference collections. Although the results were more accurate for fetuses, this study confirmed the validity of the method in both males and females, regardless of age cohort. In the same year, Marino and co-authors (2021) carried out another validation test of this method. The selected sample, 127 non-adults aged between 0 and 17 years, belongs to the Identified Skeletal Collection of the University of Bologna. Accuracy was lower in individuals younger than 1 year, which the authors suggest may be due to the high variability of this anatomical region during this period. It was also tentatively suggested that these results, which differ from those obtained by Monge Calleja et al. (2020) and Luna et al. (2021), may also be related to interpopulation variation in the degree of sexual dimorphism.

In conclusion, given the promising results of these studies, the use of the AS – along with other iliac features (e.g. sciatic notch) – as a reliable sex indicator in non-adults should be further investigated.

## **2.2. *Os Coxae* Anatomy and Ontogeny**

The *os coxae*, also known as the innominate – from the Latin expression *in nomen*, meaning 'unnamed' or 'the bone without a name' (White et al., 2011; Cunningham et al., 2017) – is a paired bone that forms part of the pelvic girdle along with the sacrum. This structure has two main functions: the first is to transfer the weight of the head and torso to the legs during locomotion, and the second is to support and protect the internal organs and abdominal viscera (Verbruggen & Nowlan, 2017). It is therefore not surprising that both the adaptation to bipedal locomotion and the biological requirements of parturition have had an enormous impact on the morphology of the innominate (Cunningham et al., 2017). Thus, it is a highly sexually dimorphic bone, providing the most reliable sex estimates (see previous section).

The *os coxae* divides into three parts – the ilium, ischium and pubis – which fuse during adolescence at the triradiate zone in the acetabulum (White et al., 2011; Verbruggen & Nowlan, 2017). The ilium is the superior part of the bone, while the ischium is the inferior-posterior part and the pubis is the inferior-anterior part (Cunningham et al., 2017). In the first three postnatal months, the ilium, ischium and pubis have a remarkably high growth rate, which gradually slows down until puberty (Verbruggen & Nowlan, 2017). Slight changes in the morphology of

the pelvic bones occur up to the age of 5-8 years, around which time fusion begins in the ischiopubic ramus (Cunningham et al., 2017; Verbruggen & Nowlan, 2017). With the onset of puberty, fusion of the triradiate suture and acetabular rim epiphysis begins and consolidation usually ends in mid-puberty (Cunningham et al., 2017; Verbruggen & Nowlan, 2017). Ossification of the epiphyses of the iliac crest, anterior inferior iliac spine and ischial tuberosity begins around 10-13 years of age. Epiphyseal union is usually complete by the middle of the third decade of life (Cunningham et al, 2017).

The innominate has a total of three articulations: one for the femoral head on the acetabulum – forming the hip; the second at the pubic symphysis, connecting the paired pubic bones (which have a cartilage disc in between); and finally, a third for the sacrum – the auricular surface, also known as the sacroiliac joint (Figure 2.4). This ear/'L'-shaped articulation is located on the medial surface of the ilium and consists of two parts, the cranial limb and the caudal limb (White et al, 2011; Vleeming et al, 2012; Cunningham et al, 2017). The cranial limb is the shortest and is oriented dorso-cranially and articulates with the lateral articular surface of the first sacral vertebra; in contrast, the caudal limb, which is larger and oriented dorso-caudally, articulates with the lateral articular surface of the second and sometimes third sacral vertebrae (Vleeming et al., 2012; Cunningham et al., 2017).

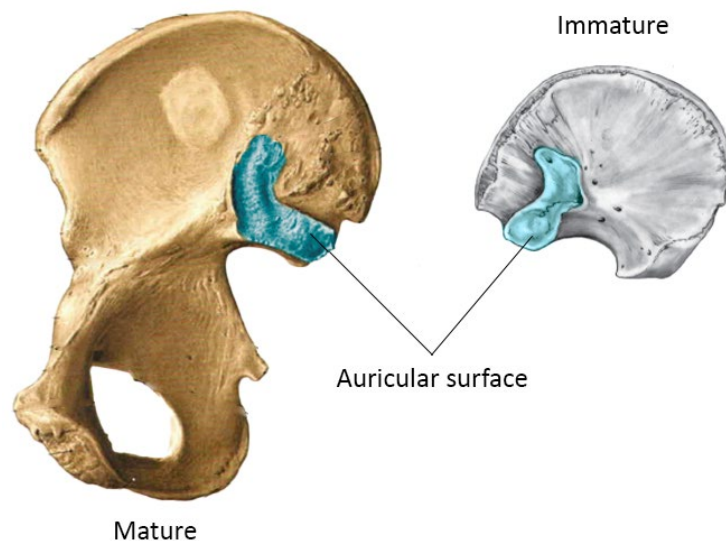


Figure 2.4 – Representation of a mature iliac bone and an immature ilium, with the AS highlighted in blue. Images adapted from Putz and Pabst (2006) and Cunningham and co-authors (2017).

### 2.3. Geometric Morphometrics

Morphometrics can be defined as the study and quantification of the morphological variation of an object, organism or structure, through the statistical analysis of traditional measurements, such as length, width or height (Rohlf & Marcus, 1993; Adams et al., 2004; Mitteroecker & Gunz, 2009). Although morphometrics has played an essential role in many fields, including biological anthropology, it has many limitations. To overcome these, alternative approaches have been developed, one of which is GM, a now well-established morphometric method (Rohlf & Marcus, 1993; Slice, 2005). GM gathers a set of computational methods to acquire, process and analyse the geometry of an organism, object or structure (Rohlf & Marcus, 1993; Slice, 2005; Adams et al., 2013). According to Bookstein (1991), GM can be defined as the statistical study of shape variation. Additionally, GM is also a tool for examining the covariation of form with other underlying variables of interest (O'Higgins, 2000). In other words, it aims to examine morphological variance and what explains it (O'Higgins, 2000; Godinho & Gonçalves, 2020). Unlike traditional morphometrics, which quantifies shape through conventional measurements, GM uses Landmark (LM) coordinates to capture morphology, which are represented in a cartesian coordinates system – in two (X and Y) or three dimensions (X, Y and Z; Rohlf & Slice, 1990; Rohlf & Marcus, 1993; Slice, 2005; Mitteroecker & Gunz, 2009; Adams et al., 2013; Bardua et al, 2019; Mitteroecker & Schaefer, 2022). These anatomical landmarks refer to anatomical points that are biologically homologous ('equivalent') between specimens (Rohlf & Marcus; 1993; Slice, 2005; Gunz & Mitteroecker, 2013; Wärmländer et al., 2019). When the analysed specimens lack conventional anatomical landmarks (e.g. the intersection of sutures or the extremes of curvatures), pseudo-landmarks – designated semilandmarks (SLMs) – may be used. These can be placed in curves or surfaces and are especially useful when the structures of interest do not have conventional LMs (e.g. the outline of the orbital rim or the surface of cranial bones). The raw landmark data depends on the coordinate system from which it was extracted. In addition, differences in the location, orientation and size of the specimens affect the coordinates that are used in the analyses. Therefore, such effects of size, location and orientation need to be removed so that the LM configurations representing the specimens can be compared. Thus, the LM configurations are superimposed (most commonly) via Generalized Procrustes Analysis (GPA). This procedure converts the original raw LM data into shape variables (also known as Procrustes coordinates), which are then subjected to standard multivariate statistical analysis (Rohlf & Marcus, 1993; Adams et al., 2013; Mitteroecker & Schaefer, 2022).

Modern GM foundations were laid more than 30 years ago when Fred L. Bookstein published *Principal Warps: Thin-Plate Splines and the Decomposition of Deformations* (1989). The year after, F. James Rohlf and Dennis E. Slice, in their paper *Extensions of the Procrustes Method for the Optimal Superimposition of Landmarks* (1990), presented how the Procrustes method (or GPA) could be applied to LM data. The following year Bookstein authored the book *Morphometric Tools for Landmark Data: Geometry and Biology* (1991), also known as “The Orange Book”, where he introduced the application of several multivariate statistical methods into GM analysis, including the Principal Component Analysis (PCA) (Mitteroecker & Schaefer, 2022). Moreover, he also introduced the use of SLMs (Krishan et al., 2016; Mitteroecker & Schaefer, 2022). The synthesis article published in 1993 by James Rohlf and Leslie Marcus called these innovations *A Revolution in Morphometrics* (Adams et al., 2004; Adams et al., 2013; Mitteroecker & Schaefer, 2022).

Later, mathematics and statistics on the theory of shape were summarised by multiple authors (e.g. Goodall, 1991; Goodall & Mardia, 1993; Bookstein, 1996; Small, 1996; Dryden & Mardia, 1998; Rohlf, 1999; Adams et al., 2004; Mitteroecker & Schaefer, 2022). GM's popularity amongst researchers has grown since then (Krishan et al., 2016) and today it has applications in a wide range of scientific fields (Adams et al., 2013; Mitteroecker & Schaefer, 2022), like biological anthropology (e.g. Coquerelle et al., 2011; Del Bove et al., 2023; Godinho et al., 2023b), human evolution (e.g. Freidline et al., 2012; Rosas et al., 2017; Fournai et al., 2021), biology (e.g. Frost et al., 2003; Gündemir et al., 2023; Martinez-Leiva et al., 2023) and archaeology (e.g. Okumura & Araujo, 2019; Bischoff, 2023; Thulman et al., 2023; Fernández Navarro et al., 2024). It has been applied to the estimation of sex in non-adults since the mid-1990s, as mentioned previously (e.g. Holcomb & Konigsberg, 1995; Vlak et al., 2008; Wilson et al., 2008, 2011, 2017; García-Mancuso & González, 2013; Mokrane et al., 2013; Estévez et al., 2017; García-Mancuso et al., 2018; Garvin et al., 2021).

### 3. Materials and Methods

#### 3.1. Materials

To carry out the present investigation, it was necessary to assemble non-adult skeletal remains from two different collections. For the development and validation of the methodology, 46 individuals were selected from the Lisbon contemporary reference collection – the LLSC (see section 3.1.1.2.; Figure 3.1). The subsequent application test was carried out on a smaller archaeological sample of eight infants from two of the Muge Mesolithic shellmiddens, Cabeço da Arruda and Moita do Sebastião (see section 3.1.2.2.; Figure 3.1).



Figure 3.1 – Map of the Iberian Peninsula, with the location of Lisbon and Muge. Image built from a Google Earth® map using Photoshop 2024 software – Adobe®.

#### 3.1.1. Luis Lopes Skeletal Collection

##### 3.1.1.1. *Historical Background*

The Luis Lopes Skeletal Collection is housed at the Anthropology Laboratory of the MUHNAC, which belongs to the Lisbon University museums network. It was initiated by the anthropologist Luís Alves Lopes (1950-2008) at the beginning of the 1980s to replace the Ferraz de Macedo Collection, which was almost completely destroyed during a fire. So, the MUHNAC – Bocage Museum back then – requested permission from the Lisbon City Council to collect, at local cemeteries, individuals from abandoned graves (e.g. unclaimed by relatives after the

end of the five-year inhumation legal period) that were going to be relocated into collective graves. Until 1991, 1552 skeletons from the graveyards of Alto de S. João, Prazeres, and Benfica, were assembled in the MUHNAC (H. F. Cardoso, 2006a; 2006b; 2014).

Between 2001 and 2004, under the supervision of the anthropologist Hugo Cardoso, another 140 skeletons from the Alto de S. João, Benfica, Olivais, and Lumiar cemeteries were added to this collection. Presently, the LLSC comprises 1692 identified skeletons, belonging to individuals of both sexes, with age ranges from 0 to 98 years, that were born between 1805 and 1972 and that died between 1880 and 1975. The biographic information available for every individual usually includes the name, sex, age, place of birth, marital status, occupation, address, place, and cause of death. Besides the identified skeletons, the LLSC also includes the remains of 75 unidentified individuals (H. F. Cardoso, 2006a; 2006b; 2014).

Since its assemblage, the LLSC has been the focus of multiple investigations seeking to develop new methodologies for biological sex and age-at-death estimation. Its great scientific value is vastly demonstrated by the number of publications issued between 2007 and 2020: more than 30 master's and doctorate dissertations and over 100 scientific articles (Garcia, 2020).

#### *3.1.1.2. Sample*

According to Baccino and collaborators (2013), the terms 'subadult', 'non-adult' and 'immature' refer to individuals until 20 years of age. In this category, we can include foetuses (under nine foetal months), newborns, infants (0-6 years), children (7-12 years) and adolescents (13-20 years). The infant category was chosen as the focus of the present investigation.

Forty-six individuals, from 0 to 6 years of age – 26 males (56.52%) and 20 females (43.38%) – from the LLSC were thus selected (Figure 3.2). Individuals for whom the ilium was absent, or present but with severe post-mortem damage, were excluded from the selection. Preference was given to the left ilium, but the right ilium was used when the left was not available.

### 3.1.2. Muge Archaeological Skeletal Collection

#### *3.1.2.1. Historical Background*

The Muge shellmiddens are one of the most important Mesolithic sites in Western Europe (van der Schrieck et al., 2007; Abrunhosa & Gonçalves, 2023). Regionally known as *concheiros*, these open-air sites are, in fact, artificial mounds, resulting from the successive accumulation of molluscs' shells. Usually surpassing 2500 m<sup>2</sup> of area, the shellmiddens present an intricate stratigraphy, where one can find structures of a housing nature, like hearths, pits or postholes.

At the same time, they were also used as burial grounds by the last hunter-gatherers of the Iberian Peninsula between 8200 and 6100 cal BP (Bicho et al., 2010; Bicho et al., 2013a; Umbelino et al., 2019; Bicho et al., 2021; Nogueira et al., 2023).

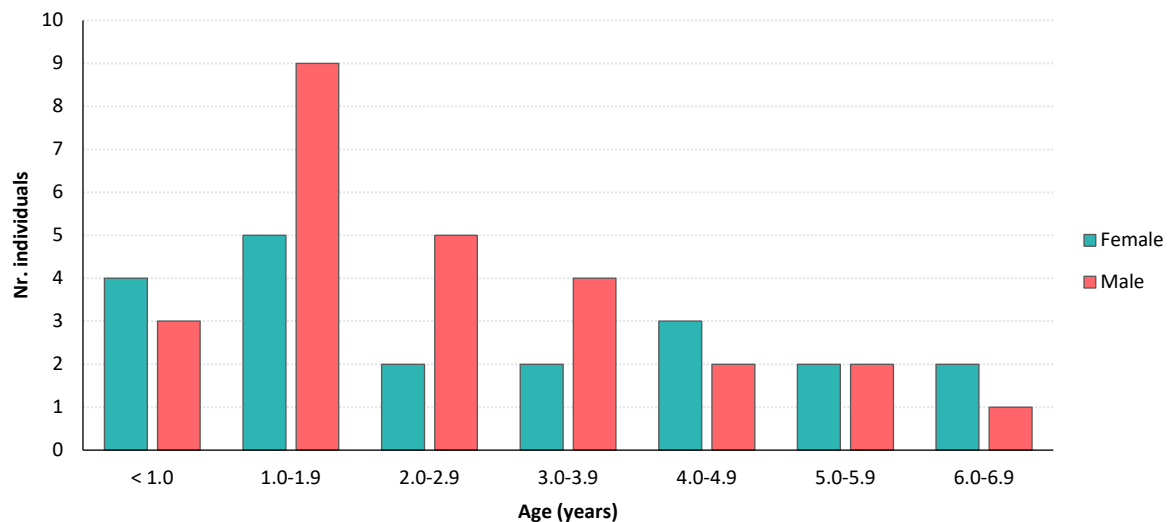


Figure 3.2 – Composition, by sex and by age categories, of the sample of the LLSC analysed in the present investigation.

This Mesolithic complex is located in the central part of Portugal, in the region of the lower Tagus valley and the vicinities of Muge and Salvaterra de Magos towns (Santarém district). Thirteen shellmiddens were detected on the shores of the streams of Magos, Muge and Fonte da Moça, all river Tejo tributaries (Figure 3.3). The first to be discovered was Quinta da Sardinha (also known as Arneiro-do-Roquete). During a field survey for the elaboration of the Geological Chart of Portugal, in 1863, the geologist and archaeologist Carlos Ribeiro identified, in the Magos stream, the Arneiro-do-Roquete shellmidden and, in the Muge stream, the Cabeço da Arruda shellmidden (J. L. Cardoso & Rolão, 1999/2000; Umbelino, 2006; Umbelino et al., 2019; Bicho et al., 2021; J. L. Cardoso, 2023; Abrunhosa & Gonçalves, 2023). In the following year, another field survey would reveal the presence of three more shellmiddens in the Muge stream, Cabeço da Amoreira, Moita do Sebastião (also designated as Fonte da Burra), and Fonte do Padre Pedro (J. L. Cardoso & Rolão, 1999/2000).

Since then, there have been several excavations at the Muge shellmiddens. By the end of the 19th century, archaeological campaigns had been conducted at Cabeço da Arruda and Moita do Sebastião, first by Carlos Ribeiro and, after he died in 1882, by Francisco de Paula e Oliveira (J. L. Cardoso & Rolão, 1999/2000; Umbelino, 2006; Umbelino et al., 2019; J. L. Cardoso,

2023). This researcher also began the excavation of Cabeço da Amoreira in the mid-1880s (J. L. Cardoso & Rolão, 1999/2000; Cunha & F. A. Cardoso, 2001). Some decades later, the human remains recovered during these interventions were studied by António Mendes Corrêa. His growing interest in the collections of Muge led him to conduct four excavation seasons between 1930 and 1937, at Cabeço da Amoreira and Cabeço da Arruda (J. L. Cardoso & Rolão, 1999/2000; Umbelino, 2006; Bicho et al., 2013b; Umbelino et al., 2019; Bicho et al., 2021; Abrunhosa & Gonçalves, 2023).

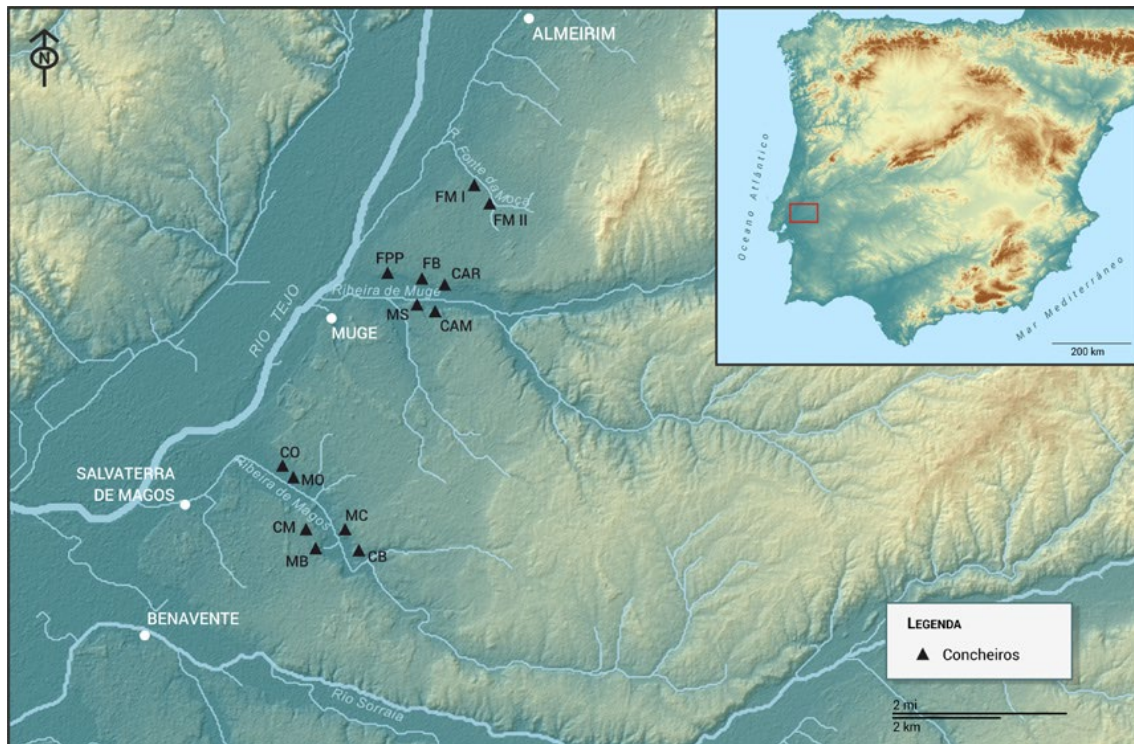


Figure 3.3 – Location of the Vale do Tejo shellmiddens. Magos stream: CO – Cova da Onça; MO – Monte dos Ossos; MC – Magos de Cima; CB – Cabeço da Barragem, CM – Cabeço dos Morros; MB – Magos de Baixo. Muge stream: FPP – Fonte do Padre Pedro; FB – Flor da Beira; CAR – Cabeço da Arruda; MS – Moita do Sebastião; CAM – Cabeço da Amoreira. Fonte da Moça stream: FMI – Fonte da Moça I; FMII – Fonte da Moça II (in Bicho et al., 2021: p. 61).

After a gap of more than one decade, the shellmiddens excavations resumed in the 1950s under the guidance of Jean Roche and Octávio da Veiga Ferreira. Between 1952 and 1965 they conducted a set of archaeological campaigns at Moita do Sebastião, Cabeço da Amoreira and Cabeço da Arruda (J. L. Cardoso & Rolão, 1999/2000; Cunha & F. A. Cardoso, 2001; Umbelino, 2006; Bicho et al., 2013b; Umbelino et al., 2019). Almost twenty years later, José Rolão, under the guidance of Manuel Farinha dos Santos, led a series of surveys at the Fonte da Moça I, Fonte da Moça II and Cabeço dos Morros shellmiddens (Rolão & Roksandic, 2007). At the turn of the millennium, José Rolão restarted excavations at Cabeço da Amoreira and

Cabeço da Arruda (Cunha & F. A. Cardoso, 2001; Umbelino, 2006; Rolão & Roksandic, 2007; Ferreira et al., 2015; Figueiredo & Gonçalves, 2015; Umbelino et al., 2019; Bicho et al., 2021). A team from the Algarve University, first led by Nuno Bicho and after by Célia Gonçalves, resumed excavations at Cabeço da Amoreira shellmidden in 2008. Since then, this site has been continuously studied (Figueiredo & Gonçalves, 2015; Umbelino et al., 2019; Bicho et al., 2021). As one might expect, the archaeological assets retrieved from the Muge shellmiddens during the last 160 years comprise numerous lithic tools, an impressive collection of faunal remains and uncountable adornments made mainly from shells, among others (van der Schrieck et al., 2007; Bicho et al., 2021). As for the human remains, they were recovered from four shellmiddens: Cabeço da Amoreira, Cabeço da Arruda, Moita do Sebastião and Cova da Onça (Ferreira et al., 2015). At least 263 individuals are accounted for (Nogueira et al., 2023), but some estimations point to more than 300 (Bicho et al., 2010; Bicho et al., 2013b; Figueiredo & Gonçalves, 2015; Umbelino et al., 2019). The collection includes complete skeletons in several states of preservation, as well as some isolated bones. The burial practices include mostly primary depositions, single or multiple, in which the corpse was positioned in supine or side decubitus. Although rare, a few individuals were accompanied by grave goods (e.g. shell beads) (Bicho et al., 2010; Nogueira et al., 2023).

The majority of the MSC is currently housed at the Geological Museum in Lisbon and the Museum of Natural History of the Sciences Faculty of Porto University. The skeletons recovered during the 20th century excavations are housed at the Department of Life Sciences of Coimbra University (Umbelino et al., 2019; Nogueira et al., 2023).

#### *3.1.2.2. Sample*

The human remains recovered from the Muge shellmiddens during the 19th and mid-20th centuries are housed at the Geological Museum and belong to the Pre-Historic Archaeology collection. During a visit to that institution, a total of eight infants (0-6 years), seven from Cabeço da Arruda (87.50%) and one from Moita do Sebastião (12.50%), were selected for the present investigation (Figure 3.4). Individuals for which the ilium was absent, or present but severely damaged, were excluded from the selection. Preference was given to the right ilium since it had greater representation (7/8; 87.50%). Parts of the Muge skeletal collection were commingled (Jackes & Meiklejohn, 2004), and so, to guarantee that the same individual was not sampled twice, the left ilium was used only when the right bone was unavailable (e.g. severely damaged).

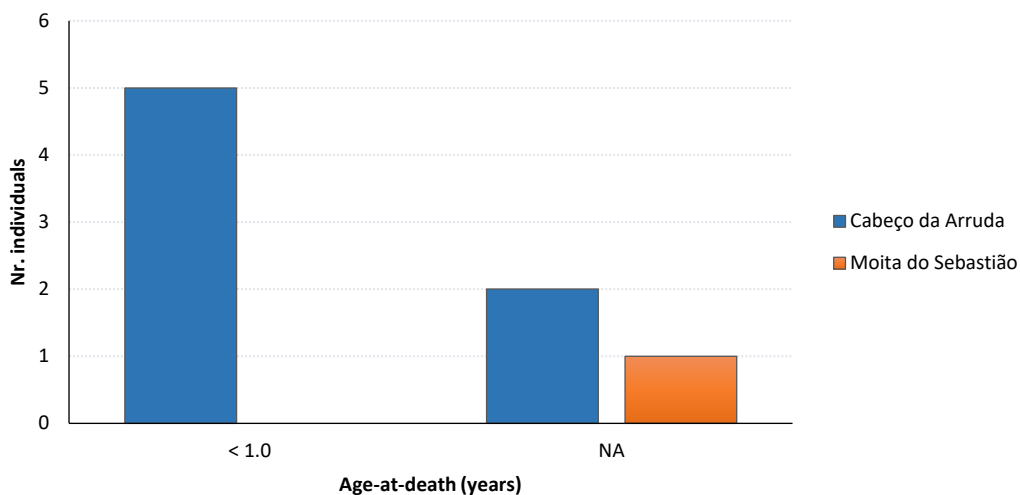


Figure 3.4 – Composition, by age categories, of the MSC sample analysed in the present investigation. NA stands for non-applicable (e.g. unobservable).

### 3.2. Methods

Following the GM methodology (Rohlf & Slice, 1990; Bookstein, 1991; Zelditch *et al.*, 2012), the present analysis was divided into three main steps: acquisition of 3D surfaces, landmarking and data collection, and morphological analysis. Before the morphological analysis, an intra-observer error test was also conducted on a small subset of the reference sample.

#### 3.2.1. Acquisition of 3D surfaces

The first step of this approach involved acquiring 3D models of all the sampled specimens. The task was performed using a portable structured-light 3D surface scanner – EinScan Pro 2X Plus model (Shinning 3D®). All the specimens were previously photographed, and the maximum iliac length and maximum iliac width were collected using a sliding calliper, according to the specifications indicated by Fazekas and Kósa (1978). After calibrating the device, the surface scanner was positioned on a tripod in front of an automated turntable in a room with controlled lighting. One by one, each ilium was placed in the turntable and scanned. The 3D models were produced by employing the appropriate software for the scanner, the EXScan Pro (Shinning 3D®) (Figure 3.5). The LLSC sample was digitized at the Anthropology Laboratory of the MUHNAC in May 2023, while the MSC sample was digitized at the Geological Museum in June 2024.

During this process, an inventory was elaborated, one for the LLSC sample and another for the MSC sample, using Microsoft® software Excel. The LLSC spreadsheet gathered the following

information: specimen identification, sex and age-at-death, the bone that was scanned and its laterality, the state of preservation of the AS, the model of the surface scanner and the image acquisition date. As for the MSC spreadsheet, it was used to collect the following information: specimen identification, age-at-death of the individual, the box and shelf where it can be found, archaeological site of provenance, the bone that was scanned and its laterality, state of preservation of the AS, the model of the surface scanner and the date of the images acquisition.

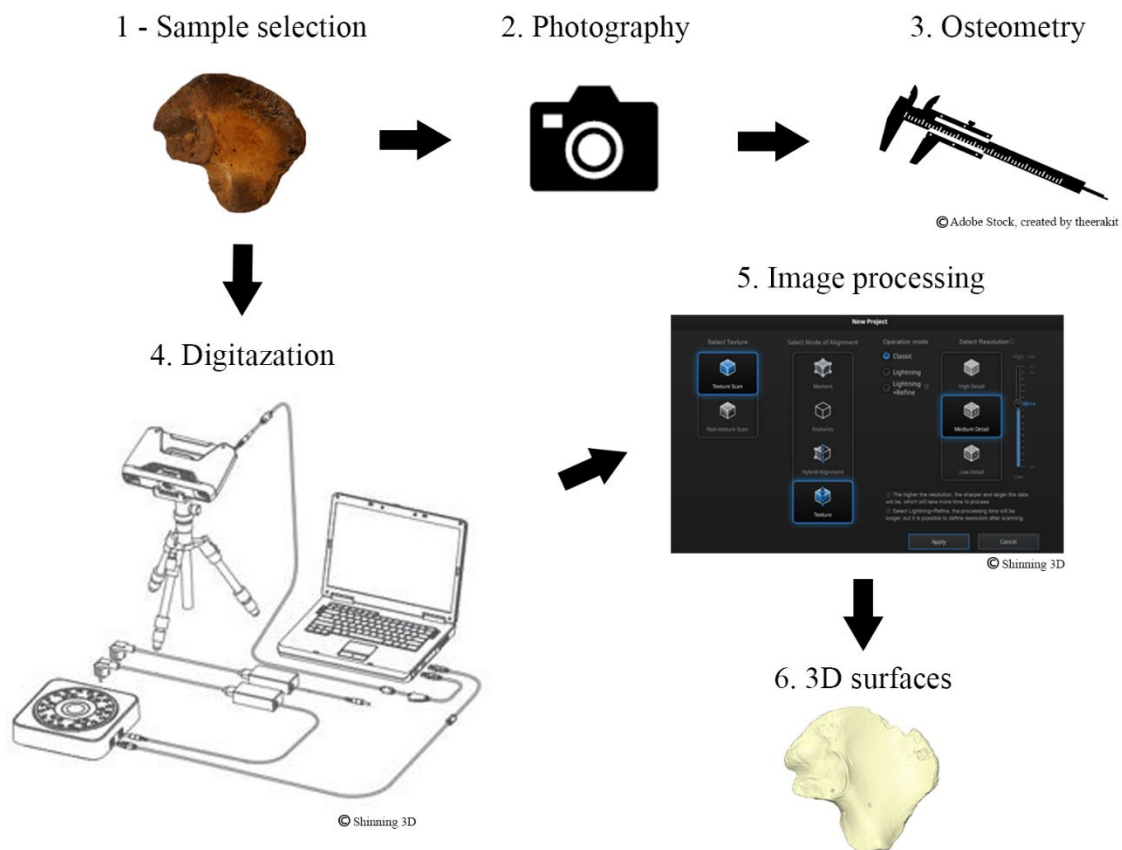


Figure 3.5 – Process of the sampled specimens 3D images acquisition (image built using Photoshop 2024 software – Adobe®; ilia presented belongs to the LLSC; image of sliding calliper acquired in Adobe Stock; images of 3D surface scanner acquired in User manual of Einscan Pro 2X/2X Plus/EP and EXScan Pro Software V3.3, Shinning 3D®, 2019).

### 3.2.2. Landmarking and data collection

The second task of this analysis was the collection of landmark coordinates (X, Y and Z) from the 3D images of the ilia to capture the morphology of the AS (Zelditch et al., 2012). Using Viewbox, version 4.1.2.1 (dHAL Software®, 2020) – first a high-resolution 3D representation (or 3D surface) of a left ilium was selected as a model to build a template. After loading the image into the software, the bone was positioned in the medial view, with the iliac crest facing

upward and the greater sciatic notch facing downward. Next, the LMs were placed on the AS on homologous anatomical landmarks (Rohlf & Marcus, 1993; Gunz et al., 2005; Mitteroecker & Gunz, 2009; Gunz & Mitteroecker, 2013; Bardua et al., 2019; Mitteroecker & Schaefer, 2022). Since this area of the ilium does not have any discrete features (Type I landmarks), its extremes of curvature were used as reference points (Type II landmarks) (Bookstein, 1991; Gunz et al., 2005; Slice, 2005; Bardua et al., 2019). Based on previous studies that employed GM to analyse the AS (e.g. Anastasiou & Chamberlain, 2013; Estévez et al., 2017; Rmoutilová et al., 2017; García-Mancuso et al., 2018; Robertson et al., 2019), four LMs were selected and placed manually, in clockwise order, as follows (Figure 3.6):

**LM1** = Medial point of maximum projection.

**LM2** = Point of maximum flexion at the intersection between the cranial and the caudal limb.

**LM3** = Superior point of maximum projection.

**LM4** = Inferior point of maximum projection at the intersection between the cranial and the caudal limb.

Due to the reduced number of conventional LMs, the analysis included the use of sliding semilandmarks (sSLM) – or Type III landmarks – along the outline of the AS (Bookstein, 1991; Bookstein, 1997; Rohlf & Marcus, 1993; Gunz et al., 2005; Gunz & Mitteroecker, 2013; Bardua et al., 2019). First, four curves were added manually to the template. Each curve started and ended in a traditional LM. The number of SML for each curve was determined based on two criteria: the mean length of that curve – quantified in Viewbox – in three different specimens, and the spacing between points – around 3 mm. To ensure the points were equidistant, they were automatically resampled for even spacing. Hence, a total of 22 SLMs were added to the template and divided as follows (Figure 3.6):

**C1** = Curve between LM1 and LM2, with six SLMs.

**C2** = Curve between LM2 and LM3, with two SLMs.

**C3** = Curve between LM3 and LM4, with six SLMs.

**C4** = Curve between LM4 and LM1, with eight SLMs.

The template was finished after setting the landmarking sequence of the 26 points – four conventional LMs and 22 SLMs. Using it as a landmarking protocol, the LMs and the curve SLMs were then placed in each specimen following the same order. The SLMs were automatically placed equidistantly along the curves and their position was adjusted (slid) according to the reference specimen. This process was achieved by minimizing the bending energy (e.g. minimum value of energy) necessary to match the target specimen landmark

configuration to the reference specimen landmark configuration. The procedure guarantees the geometrical correspondence of the SLMs in every AS of the sample, making their positioning comparable (Bookstein, 2005; Gunz et al., 2005; Mitteroecker & Gunz, 2009; Gunz and Mitteroecker, 2013; Bardua et al., 2019). Finally, all the specimen information and landmark coordinates were collected in a *morphologika* file (commonly used in GM) as a standard format text (.txt) file (O'Higgins & Jones, 1998, 2006). Keeping in mind that the reference specimen used as a template was a left ilium, and that all specimens must have the same arrangement, it was ensured that the sets of coordinates collected from right specimens were reflected to the opposite side (Zelditch et al., 2012).

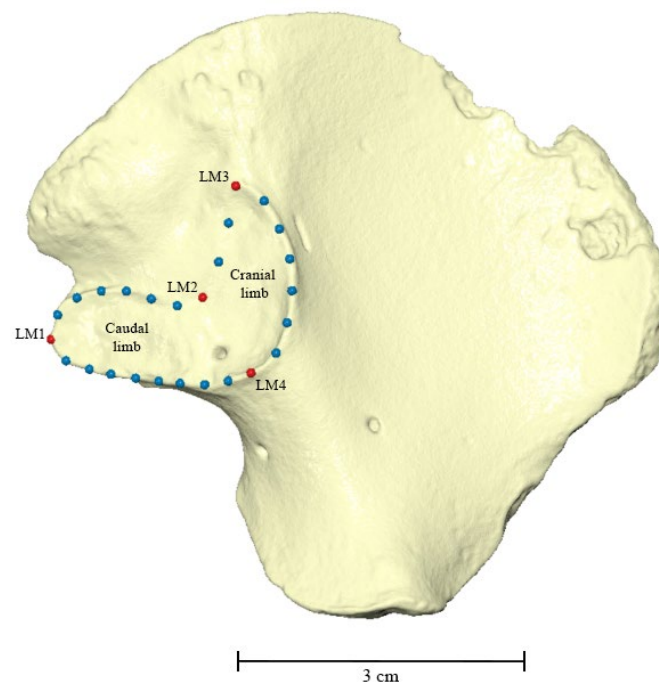


Figure 3.6 – 3D image of the left ilium used as the model for the Viewbox template. The conventional LMs (4) are the red dots, as indicated in the image. The blue dots are the sSLMs (22), which were distributed equidistantly between the four curves disposed along the outline of the AS.

### 3.2.3. Morphological Analysis

The morphological analysis was first conducted for the LLSC sample (n= 46). Based on their evaluation, a second morphological analysis was performed on both samples (n=54), reference and archaeological (n=8). The Geomorph package, version 4.0.8, (Adams & Otárola-Castillo, 2013), developed for standard GM analyses of 2D and 3D landmark data in the open-source software R (version 4.4.0, released on 24-04-2024 and available on <https://www.r-project.org>), was used for all data analysis. First, the *morphologika* file was imported into R and a GPA was

performed, which executes the superimposition of the landmark configuration of all specimens (Figure 3.7). By removing size, orientation and location as variables, the raw coordinates were converted into Procrustes shape coordinates (Slice, 2005; Mitteroecker & Gunz, 2009; Zelditch et al., 2012; Gunz & Mitteroecker, 2013; Mitteroecker & Schaefer, 2022). The standard GM size measure used for landmark configurations is the centroid size (Mitteroecker & Gunz, 2009; Manthey & Ousley, 2020; Mitteroecker & Schaefer, 2022). This variable was, therefore, used to compare the size of the AS across specimens.

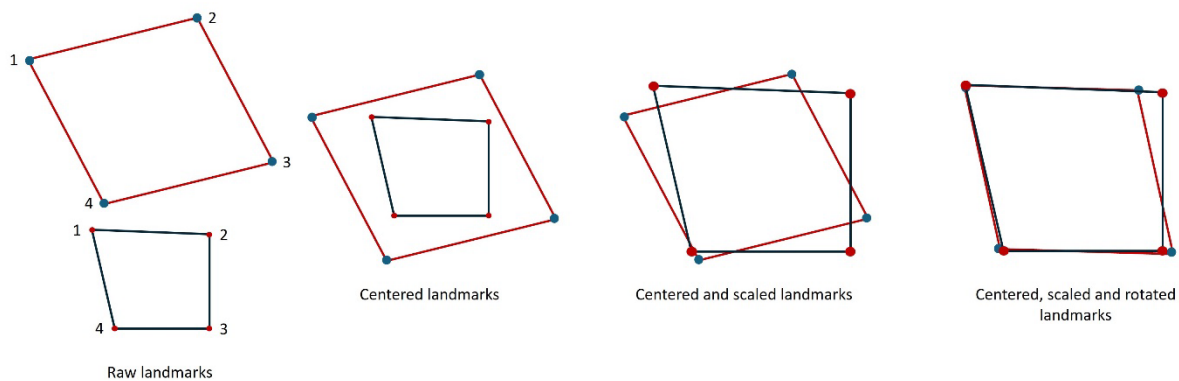


Figure 3.7 – Superimposition of two specimens, so that the homologous LM match as closely as possible (Rohlf & Marcus, 1993). The three steps of the GPA are represented (Mitteroecker & Schaefer, 2022): translation of specimens to the same centroid (1), scaling of the specimens to the same centroid size (2) and rotation of the specimens to minimise the difference regarding the sample average (3) (adapted from Mitteroecker & Gunz, 2009: 239).

To examine shape (e.g. geometric properties of an object) variance in the AS, a PCA was performed based on the shape coordinates, which reduced dimensionality. The resulting Principal Components (PC) scores represent the dataset variation (Mitteroecker & Gunz, 2009), with the first Principal Component accounting for the greatest amount of variability (Manthey & Ousley, 2020). Afterwards, shape variance associated with each relevant PC was visualized using thin-plate splines (TPS) deformation grids (Bookstein, 1991; Rohlf & Marcus, 1993).

Finally, statistical tests were performed to test for hypothetical differences between groups. Potential AS size differences related to sexual dimorphism were examined using Mann-Whitney tests. To investigate possible sex-related differences in AS shape, a PERMANOVA (Permutational Multivariate Analysis of Variance) was conducted using all the PC scores that explain  $\approx 94\%$  to  $98\%$  (depending on the cases) of shape variance. When necessary, the Mann-Whitney test was also used to compare scores of specific PCs to assess differences between male and female AS morphology.

These analyses were carried out firstly for the whole sample, combining all age cohorts, and afterwards independently by age group. Thus, GPA superimposition and all subsequent analyses were performed for the entire sample and then independently for each age cohort. This procedure ensures that hypothetical shape differences in each of the age groups are not obscured by examining all age cohorts together. To that end, specimens were re-arranged by age groups according to the following criteria: < 1.0 years, which corresponds to the neonatal surge; 1.0-3.9 years and 4.0-6.9 years, corresponding to age intervals of three years, the latter of which coincides with the approximate age of the eruption of the first permanent tooth (AlQahtani et al., 2010; Figure 3.8).

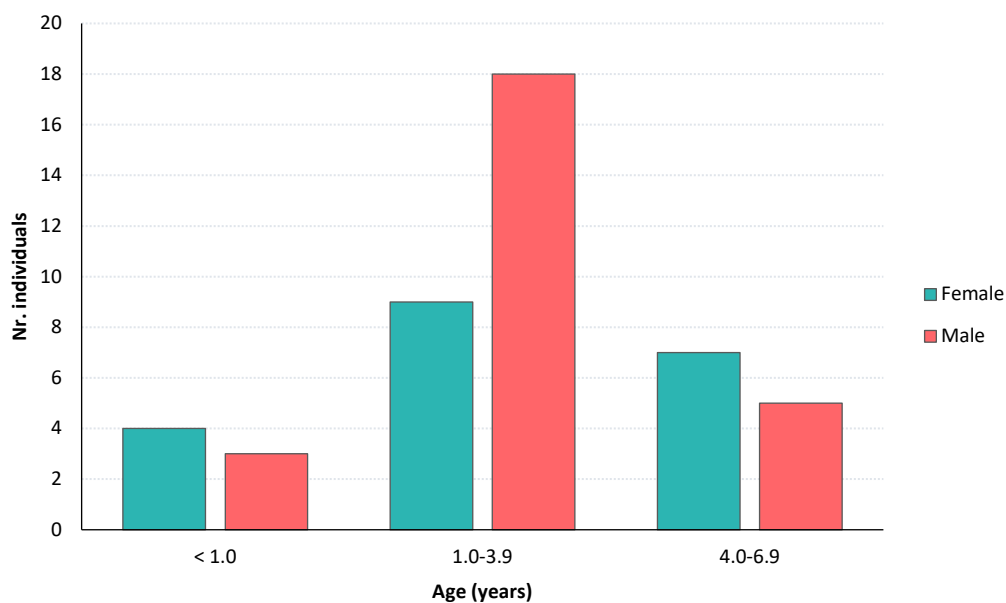


Figure 3.8 – Number of individuals of the reference sample here analysed, grouped by sex and by age categories.

#### 3.2.4. Intraobserver error test

To evaluate the level of variation introduced by the observer during data collection (i.e. landmarking), a test for measurement error – involving the repeated measurement of the same specimen (Menéndez, 2017) – was conducted on a subset of the reference sample. Seven specimens were chosen according to two criteria: having a clear outline of the AS and being left-sided, to match the Viewbox template model. The same set of LMs was acquired from each ilium three times independently, with each observation being performed at least two days apart. These were all collected in a *morphologika* file, which was afterwards imported into R. A GPA was conducted on the raw landmarks collected, and pairwise Procrustes distances, which

represent shape difference between paired sets of shape landmarks configurations (Mitteroecker & Gunz, 2009; Mitteroecker et al., 2013), were analysed to determine if the intraobserver error is negligible. Following previous studies (e.g. Rosas et al., 2017; Fernández-Navarro et al., 2024), intraobserver error is acceptable if the largest Procrustes distance among the repeated measures (intra-individual distances) is lower than the smallest Procrustes distance among all other individuals (inter-individual distances).

#### 3.2.5. Age-at-death estimation of the archaeological specimens

The age-at-death of infants from the MSC sample was initially estimated using the maximum iliac length method proposed by Molleson & Cox (1993) and referred to by Cunningham and collaborators (2017; see Figure 3.4). However, some of the ilia were fragmented and this method could not be applied. As age-at-death was a prerequisite of the morphological analysis, an alternative approach was carried out. Using the centroid size of the AS of the LLSC sample as a reference, the age-at-death of all archaeological specimens was estimated. The results were then checked with those obtained using the conventional morphometric technique above mentioned.

## 4. Results

### 4.1. Intraobserver Error

When analysing the Procrustes distances of the three landmark sets from the seven specimens selected for the test (see Table B, Appendix A), the highest intra-specimen Procrustes distance value is 0.0386005, while the lowest inter-specimen Procrustes distance value is 0.0757551. Therefore, the intraobserver error is acceptable.

### 4.2. Luis Lopes Skeletal Collection

#### 4.2.1. Overall sample

Differences in AS size, as assessed using centroid size, were not observed between males and females in the reference sample (when lumping all age cohorts), as shown in Figure 4.1. Consistently, the Mann-Whitney test shows no statistically significant differences between male and female AS size ( $p=0.3814$ ).

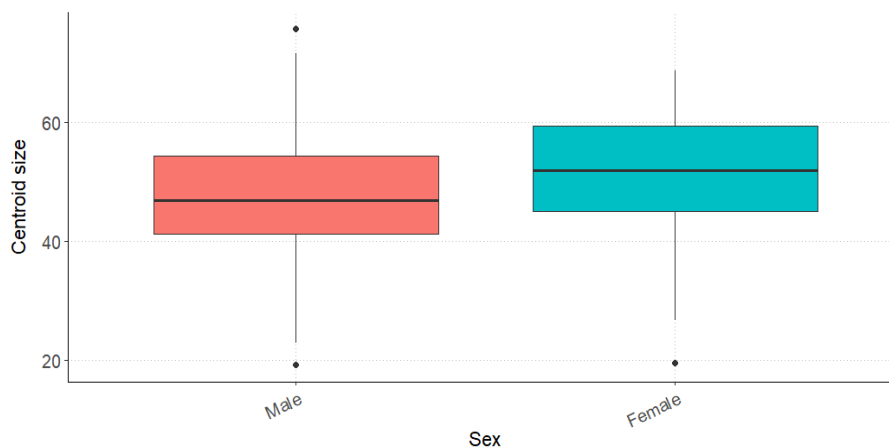


Figure 4.1 – AS size of the 46 specimens in the LLSC sample, grouped by sex. No size differences were found between male and female individuals.

Regarding shape, the PCA conducted on the entire reference sample did not reveal any differences between the two groups. Upon examination of the distribution of the 46 individuals along PC1 (explaining 24.06% of the total variance) and PC2 (explaining 18.35% of the total variance), a significant overlap between males and females was noted (Figure 4.2). A PERMANOVA was performed on the first 13 PC scores, representing about 95% of the total variance (see Figure D, Appendix A), to assess potential shape variances between the groups. The analysis showed no statistically significant differences between males and females ( $p=0.7202$ ).

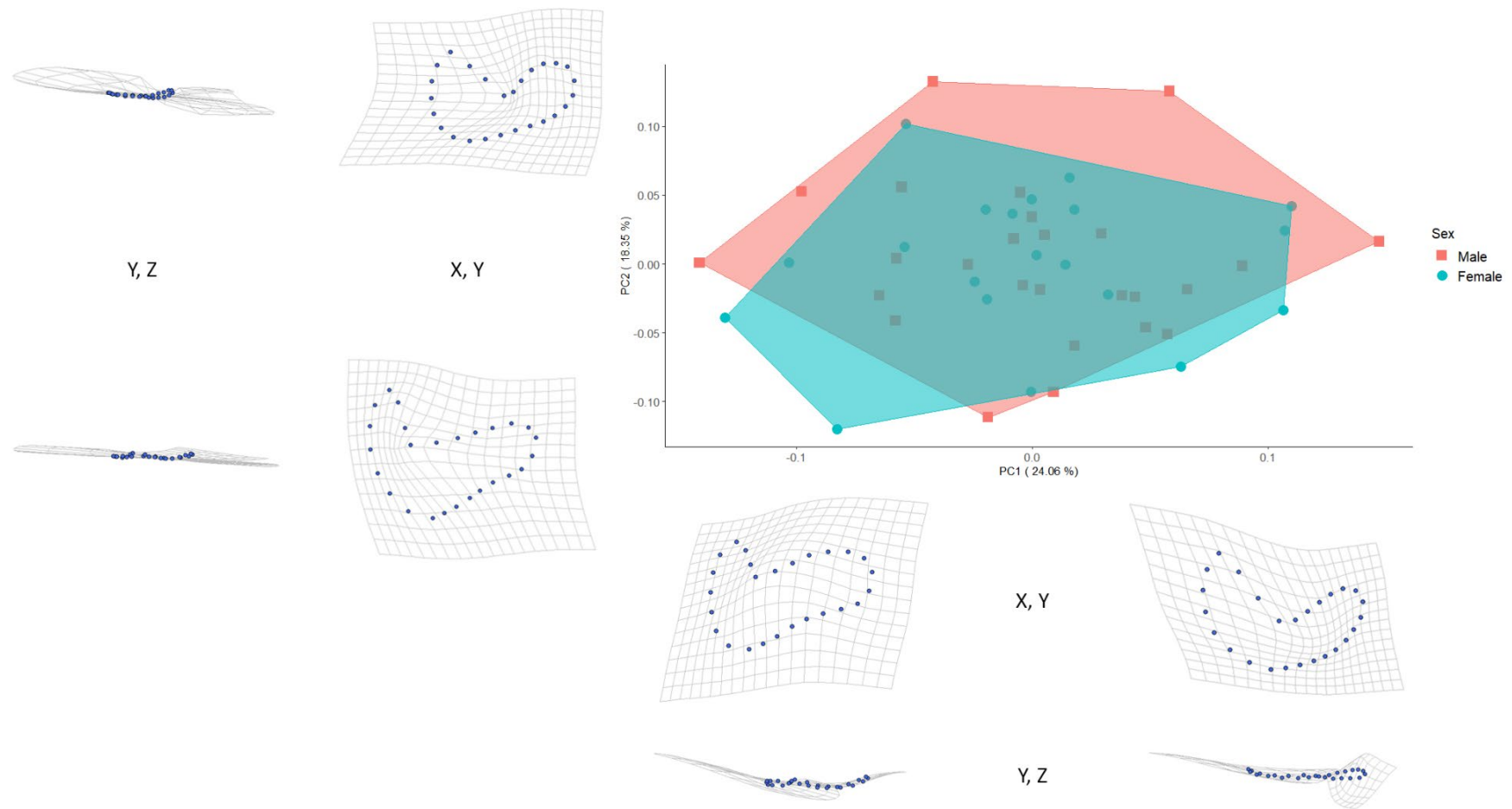


Figure 4.2 – PCA of the AS shape of the LLSC sample, with individuals grouped by sex. The data shows a substantial overlap between the male and female groups, indicating no discernible morphological differences between them. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes, and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes, and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times.

Warping of the AS morphology using deformation grids along PC1 (Figure 4.2), shows that specimens closer to the minimum PC1 scores are L-shaped, with a shorter cranial limb and a longer caudal limb. On the other hand, specimens closer to the maximum PC1 scores exhibit a U-shaped AS and nearly equal limb sizes. Specimens near the minimum PC2 scores also have an L-shaped AS, but the apex region is wider, and the cranial limb is smaller and narrower compared to specimens near minimum PC1. Finally, the specimens' AS closer to the maximum PC2 scores is U-shaped, resembling the AS of specimens near the maximum PC1. The difference is that specimens closer to the maximum PC2 have shorter limbs.

Given that no significant morphological differences in the AS were found between male and female individuals, the reference sample was then divided by sex into age categories of one year (see Figure 3.2) because lumping of all age cohorts could obscure age-specific morphological differences. As shown in Figure 4.3, the AS size gradually increases with age. The median centroid size is higher for female individuals than for male individuals up to 3.0-3.9 years, but this trend reverses for older age groups. However, the number of individuals per group varies and is relatively small. For example, the age group with the highest number of specimens is 1.0-1.9 years with five females and nine males. On the other hand, the age category with the fewest individuals is 6.0-6.9 years, with only one male and two female individuals. For this reason, the reference sample was rearranged into different age groups to increase the group size.

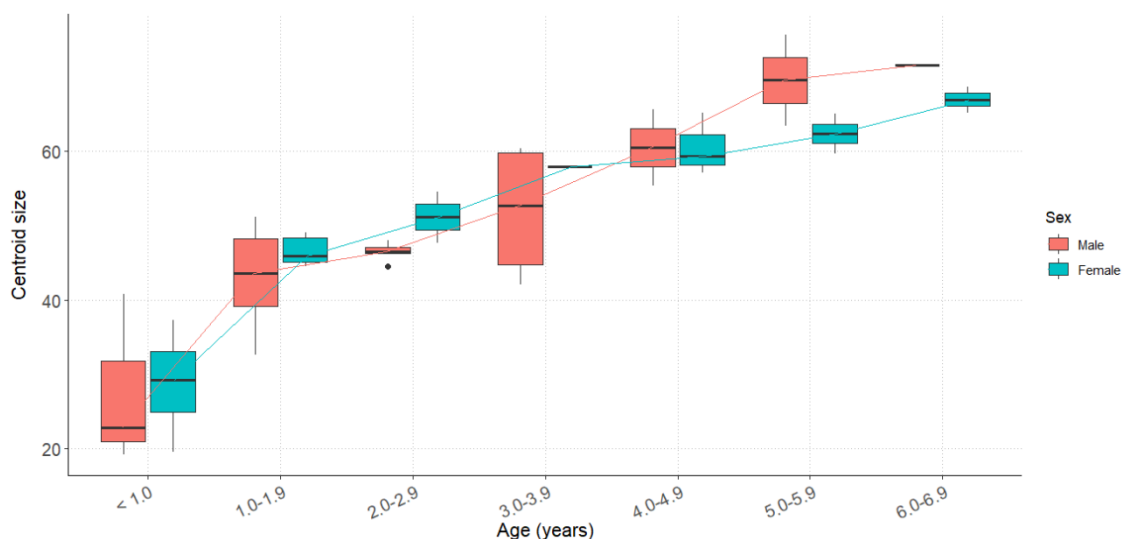


Figure 4.3 – AS size of the specimens from the LLSC sample, grouped by sex and age. Size increases gradually with age and some differences between male and female individuals are observed. The lines connect the medians of the different age categories, by sex.

When examining the size of the AS according to the selected age groups, differences between both sexes are observed (Figure 4.4). For infants under one year, both males and females have similar AS sizes. Females aged 1.0-3.9 years have a larger AS than males of the same age. In contrast, in the 4.0-6.9 age group, the AS size is larger in males than in females (see Figure A, Appendix A). Results of statistical testing for sex differences in each of the age groups are reported below.

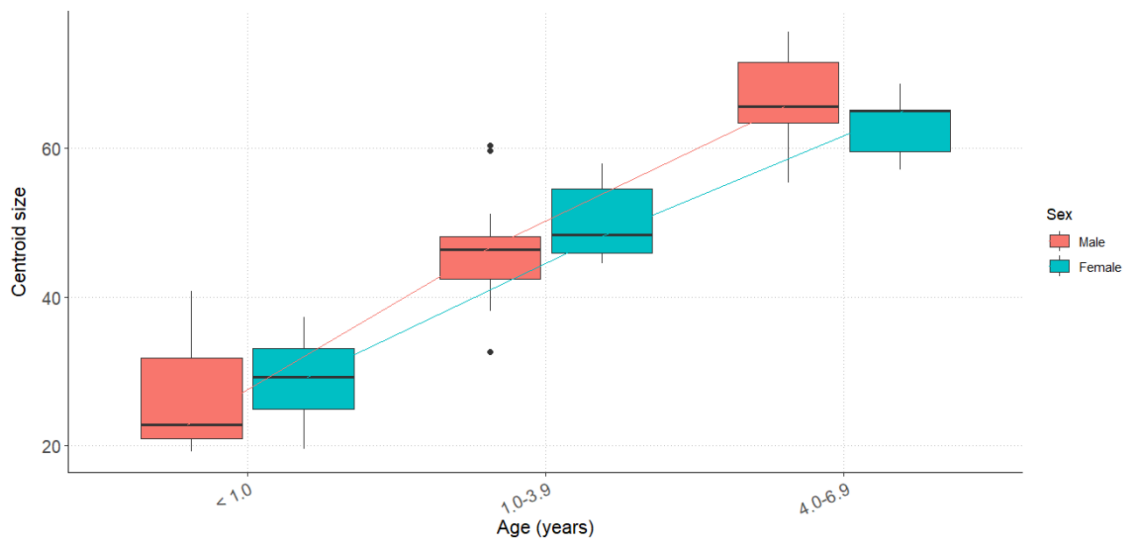


Figure 4.4 – AS size of the specimens from the LLSC sample, grouped by sex and age categories. Size increases gradually with age. Some differences between male and female individuals are observed for the 1.0-3.9 and 4.0-6.9 age categories. The lines connect the medians of the different age categories, by sex.

Regarding shape, there is an apparent overlap of the different groups along PC1 (explains 24.06% of variance) and PC2 (explains 18.35% of variance), despite being grouped by sex and age cohort, as can be observed in Figure 4.5. Yet, when plotting each age group separately (Figure 4.6), some group differences become clear. Specifically, differences in AS shape were found between male and female infants under one year, with a complete separation of both groups. No significant differences in AS morphology between male and female individuals in the other age groups emerged (see Figures B and C, Appendix A).

#### 4.2.2. Age cohort-specific samples

##### 4.2.2.1. *Individuals under one year*

The following step of the present work was to subdivide the reference sample and to conduct separate analyses for each age group to ensure potential shape differences are not obscured by lumping all age groups together.

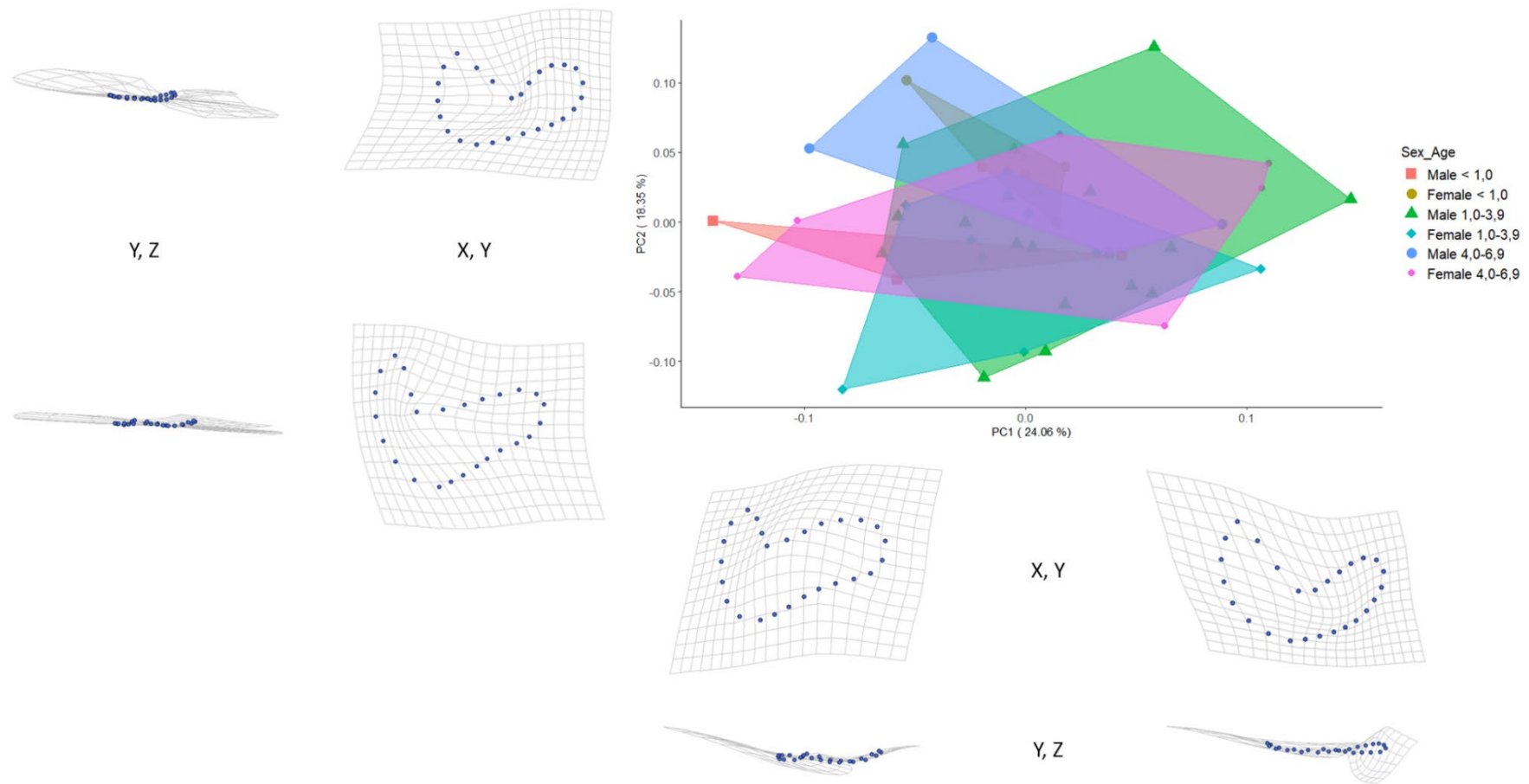


Figure 4.5 – PCA of the AS shape of the LLSC sample, with individuals grouped by sex and by age groups. The data shows a substantial overlap between the groups. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times.



In infants under one year, no size differences in the AS were found between male and female individuals (Figure 4.7). A Mann-Whitney test was carried out on the median values of the centroid size for both sexes and the results do not show statistically significant differences between the groups ( $p= 0.8597$ ).

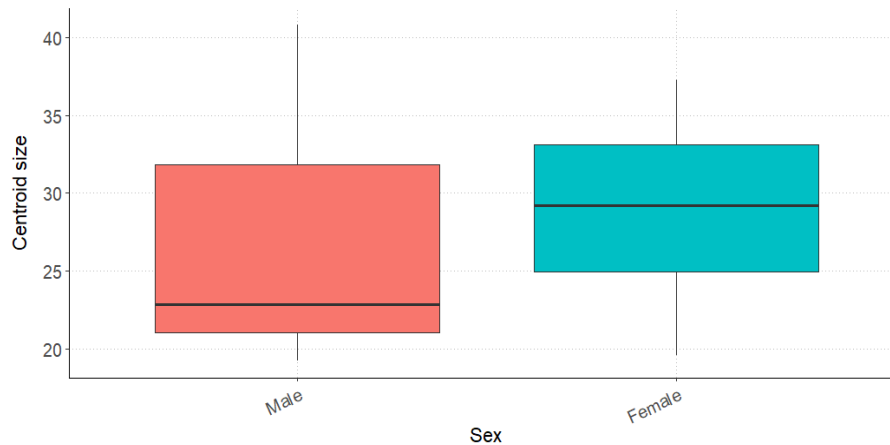


Figure 4.7 – AS size, as assessed using centroid size, of the seven infants under one year, grouped by sex. No size differences were found between male and female individuals.

In terms of shape, there is an even distribution of the individuals along PC1 (explaining 34,89% of the variance), but along PC2 (explaining 26,77% of the variance) there is a separation between groups (Figure 4.8). Male individuals are located along the PC2 negative scores, while female individuals are mostly distributed along the PC2 positive scores. A PERMANOVA was performed on the first five PC scores, which account for approximately 98% of the total variance (see Figure E, Appendix A), to assess potential shape variations between the two groups. Results do not show statistically significant differences in the AS morphology ( $p= 0.2286$ ). Due to the reduced size of this age group, and since differences in PC2 were noted between male and female individuals, a Mann-Whitney test was also performed on the same PC scores (Figure 4.9). Even though there are no statistically significant differences between groups, the p-value for PC2 borders statistical significance ( $p = 0.057$ ).

Concerning shape (Figure 4.8), specimens closer to the minimum PC1 scores have a V-shaped AS with similarly sized cranial and caudal limbs. On the other hand, the AS of specimens closer to the maximum PC1 scores is L-shaped, with a longer caudal limb and a smaller cranial limb. The AS of specimens closer to minimum PC2 scores is also L-shaped, but the caudal limb is slenderer, and the apex region is wider. Finally, specimens closer to the maximum PC2 scores have a U-shaped AS, with a longer cranial limb.

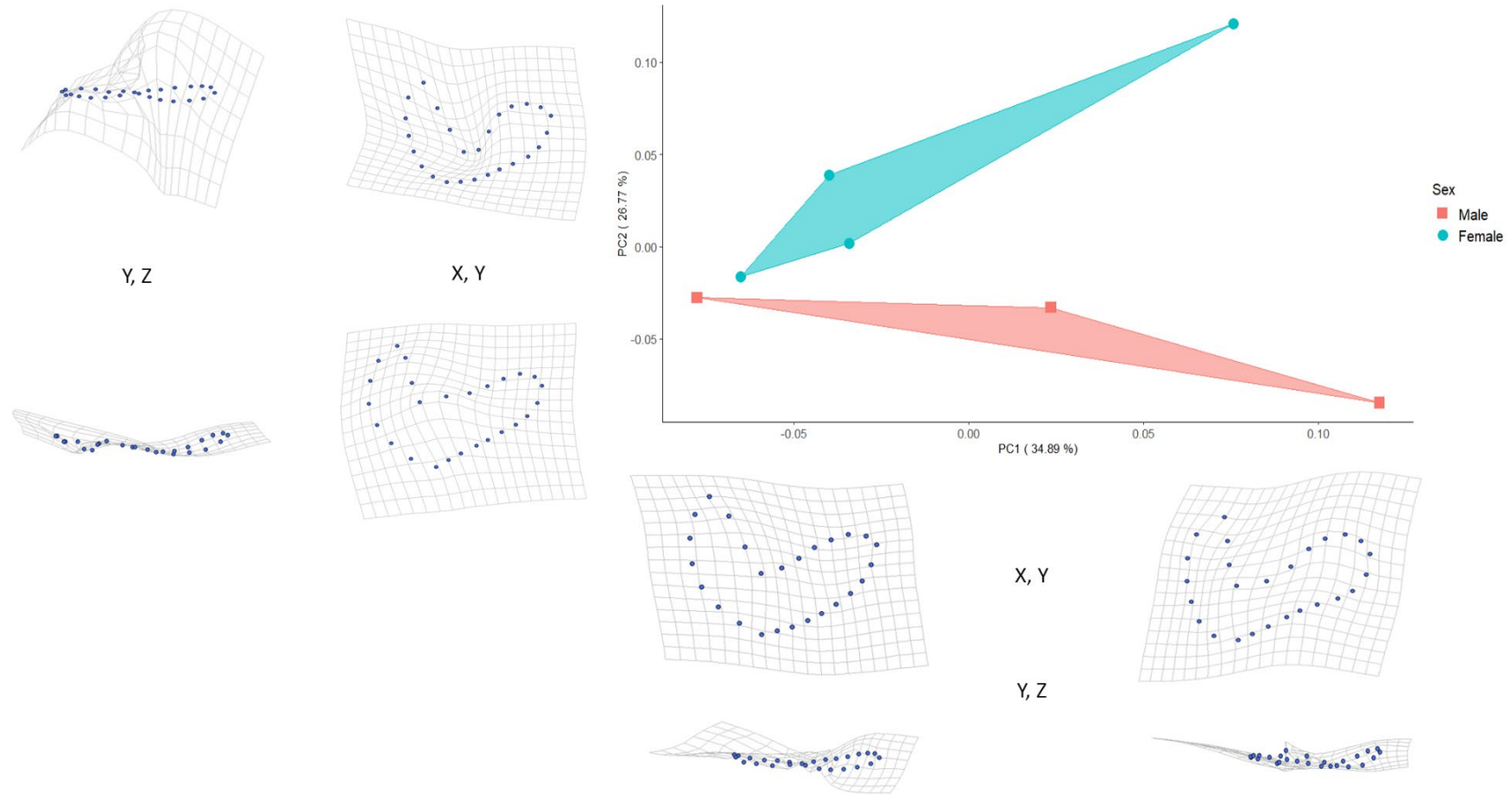


Figure 4.8 – PCA of the AS shape of the infants from the reference sample under one year, grouped by sex. The data shows no overlap between males and females along PC2, indicating differences in the AS morphology. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times.

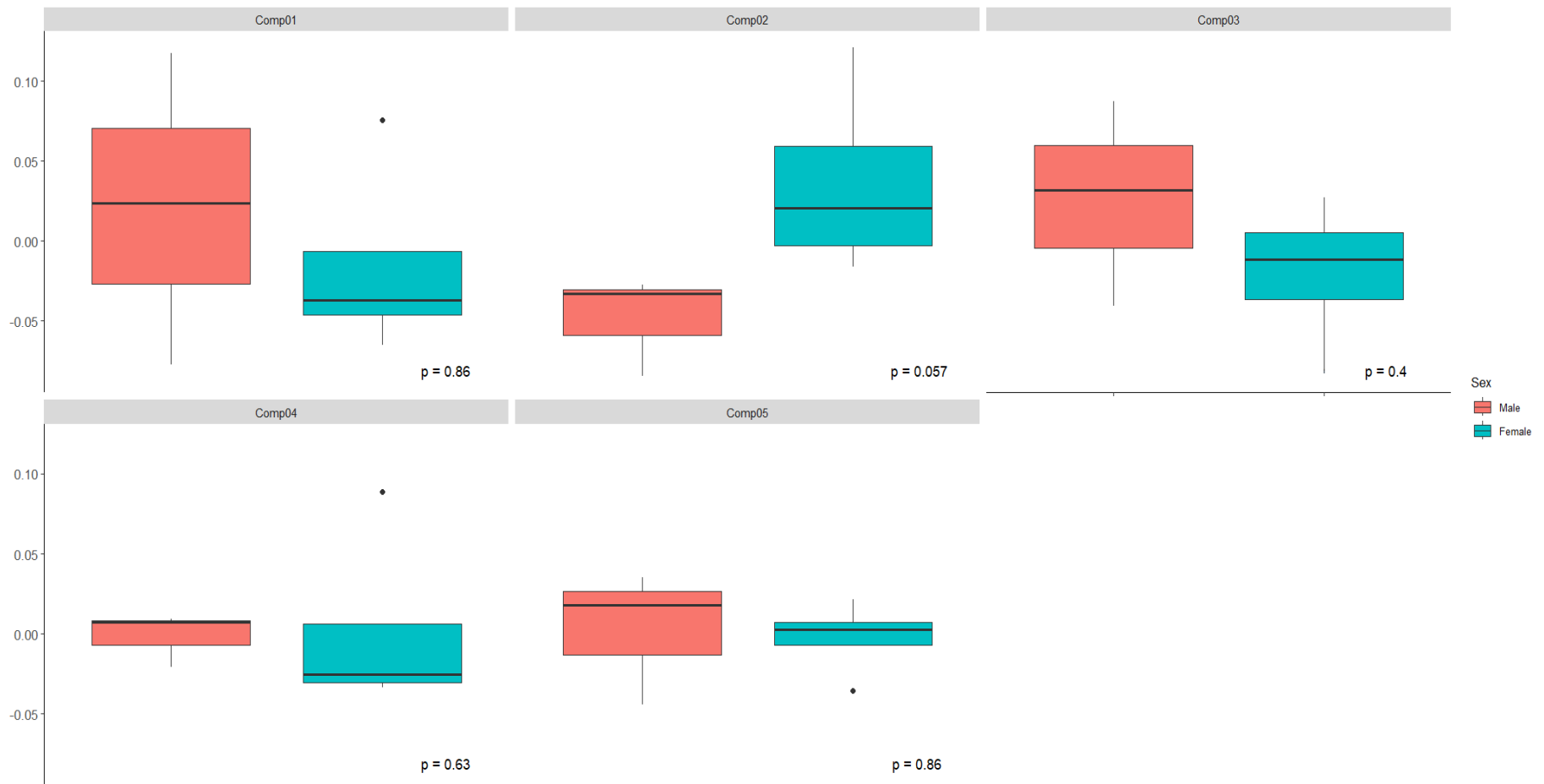


Figure 4.9 – Results of the Mann-Whitney test performed on the first five PC scores that explain approximately 98% of the total variance of the AS shape of infants under 1 year, grouped by sex. Note that there is overlap between the two groups, except for PC2. The p-value for this PC score is very close to being statistically significant ( $p = 0.057$ ).

#### 4.2.2.2. *Individuals with 1.0-3.9 years*

For individuals aged between 1.0 and 3.9 years, the AS of females is slightly larger than that of males, even though some overlap between both groups was noted (Figure 4.10). The Mann-Whitney test conducted on the median centroid size did not find statistically significant differences between male and female individuals ( $p=0.1572$ ).

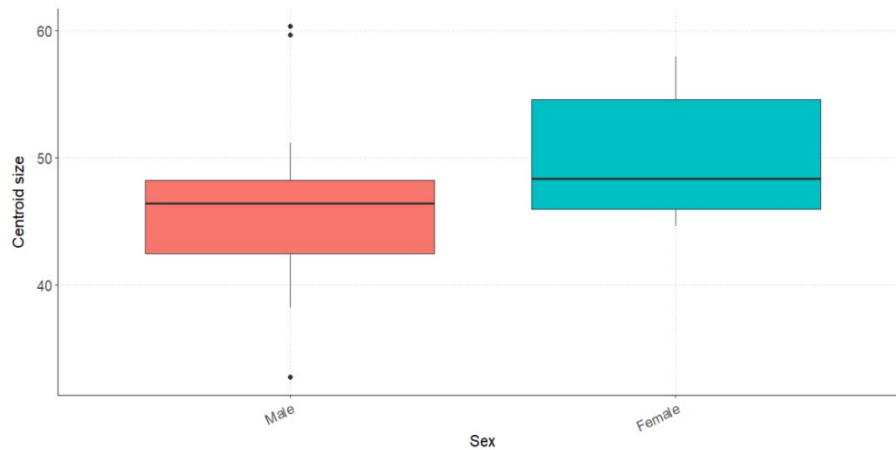


Figure 4.10 – AS size, as assessed using centroid size, of the 27 infants aged 1.0-3.9 years, grouped by sex. Some size differences were found between male and female individuals.

Regarding shape, the PCA performed for this age group does not indicate differences in the AS morphology between male and female individuals. As shown in Figure 4.11, the female group is almost completely overlapped with the male group along PC1 (explaining 24,25% of variance) and PC2 (explaining 18,29% of variance). Even though there seems to be more variability among the male individuals, caution is advised because they are twice the number of female individuals. The PERMANOVA conducted on the first 11 PC scores accounting for approximately 95% of the total variance (see Figure F, Appendix A) did not find statistically significant differences between the groups ( $p=0.5653$ ).

Upon examination of the AS shape (Figure 4.11), it is evident that specimens closest to the minimum PC1 scores are L-shaped, with a shorter cranial limb and a longer caudal limb. As for specimens closer to the maximum PC1 scores, they have a U-shaped AS, a broader apex region, and nearly equal limb sizes, but the cranial limb is much wider than the caudal limb. Specimens near the minimum PC2 scores exhibit a somewhat V-shaped AS, with a short cranial limb and a long caudal limb. Finally, the AS of the specimens closer to the maximum PC2 scores is U-shaped, with the caudal limb longer than the cranial limb and a wider apex region.

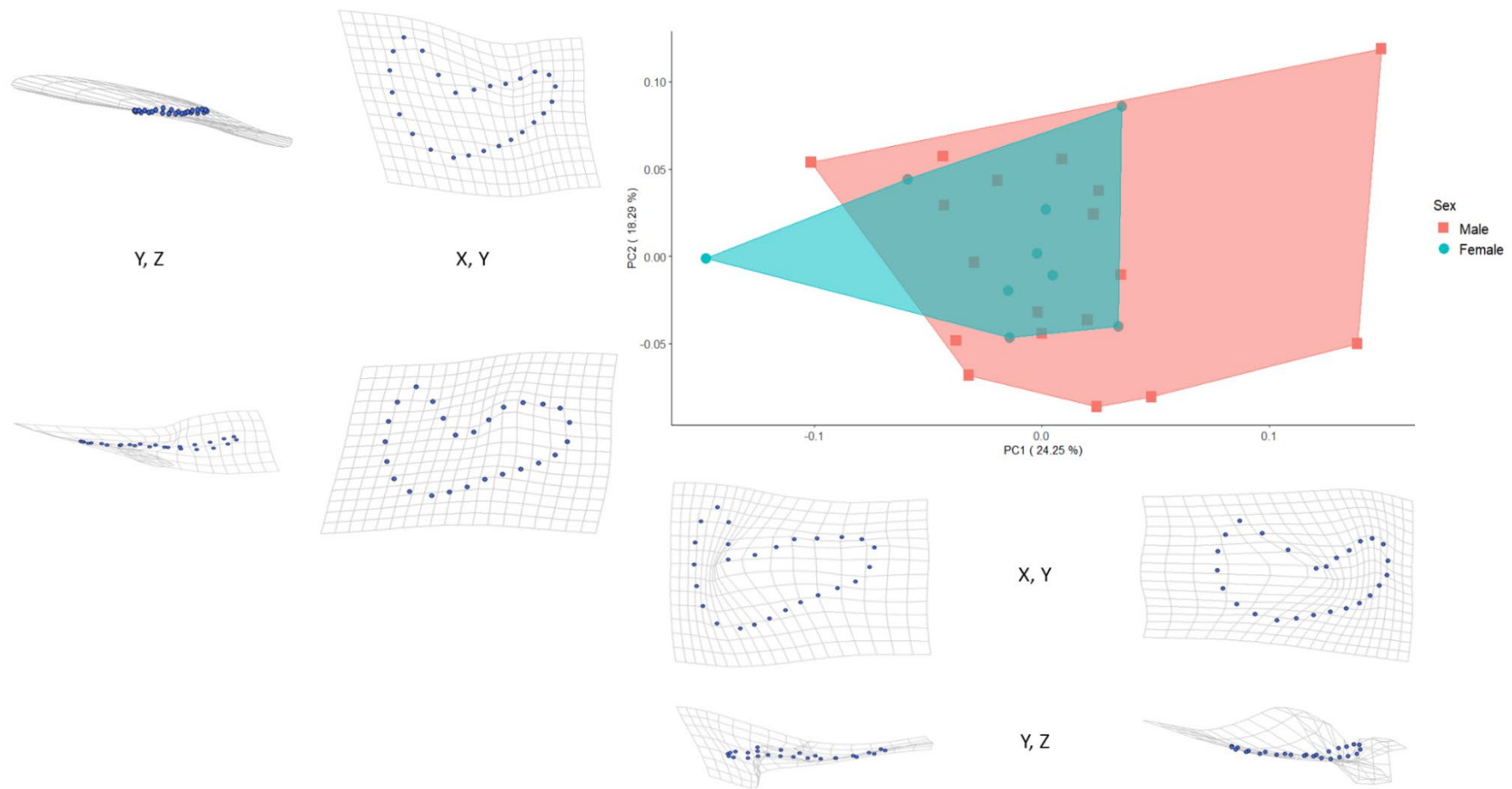


Figure 4.11 – PCA of the AS shape of the infants from the reference sample aged between 1.0 and 3.9 years grouped by sex. The data indicates a nearly complete overlap of the female and male groups, suggesting no differences in AS morphology. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes, and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes, and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times.

#### 4.2.2.3. *Individuals with 4.0-6.9 years*

Finally, in infants aged between 4.0 and 6.9 years, there is a slight overlap between the male and female groups regarding AS size, but male specimens have a slightly larger AS (Figure 4.12). A Mann-Whitney test does not indicate statistically significant differences in the median centroid size of both groups ( $p= 0.4168$ ).

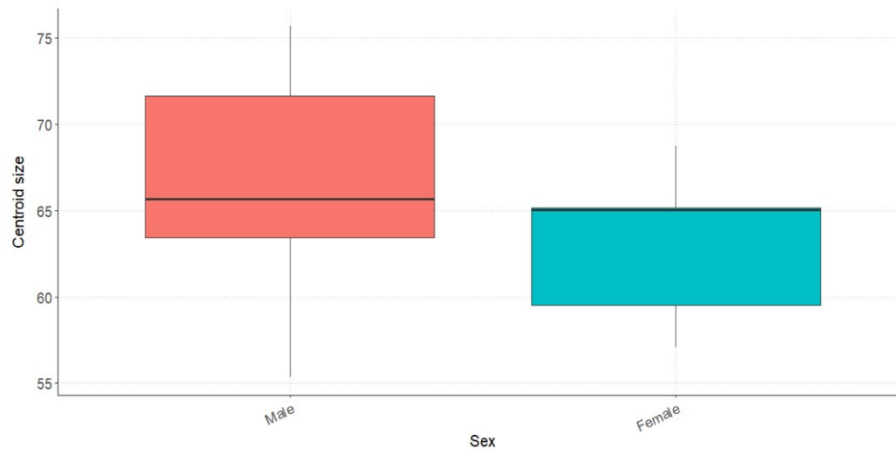


Figure 4.12 – AS size, as assessed using centroid size, of the 12 infants aged 4.0-6.9 years, grouped by sex. Slight non-significant size differences were found between male and female individuals.

Concerning shape, looking at the distribution of the individuals along PC1 (which explains 44.69% of the total variance) and PC2 (which explains 19.79% of the total variance), there were some differences between the male and female groups, especially in the PC2 scores (Figure 4.13). But even though some differences were identified, there is still a high overlap of the groups, indicating no significant dissimilarities in AS morphology. To assess potential shape variances between the groups, a PERMANOVA was conducted on the first seven PC scores, representing about 94% of the total variance (see Figure G, Appendix A). The analysis does not indicate statistically significant differences between male and female individuals ( $p= 0.8269$ ). Looking at the deformation grids shown in Figure 4.13, it is clear that specimens closest to the minimum PC1 scores have a U-shaped AS, with both limbs nearly the same size. On the other hand, the AS of individuals closest to maximum PC1 scores is L-shaped, with a small cranial limb and a long caudal limb. Individuals near the minimum PC2 scores present an L-shaped AS, very similar to individuals near the maximum PC1 scores. Lastly, specimens closest to the maximum PC2 scores have a V-shaped AS, with both limbs almost the same size.

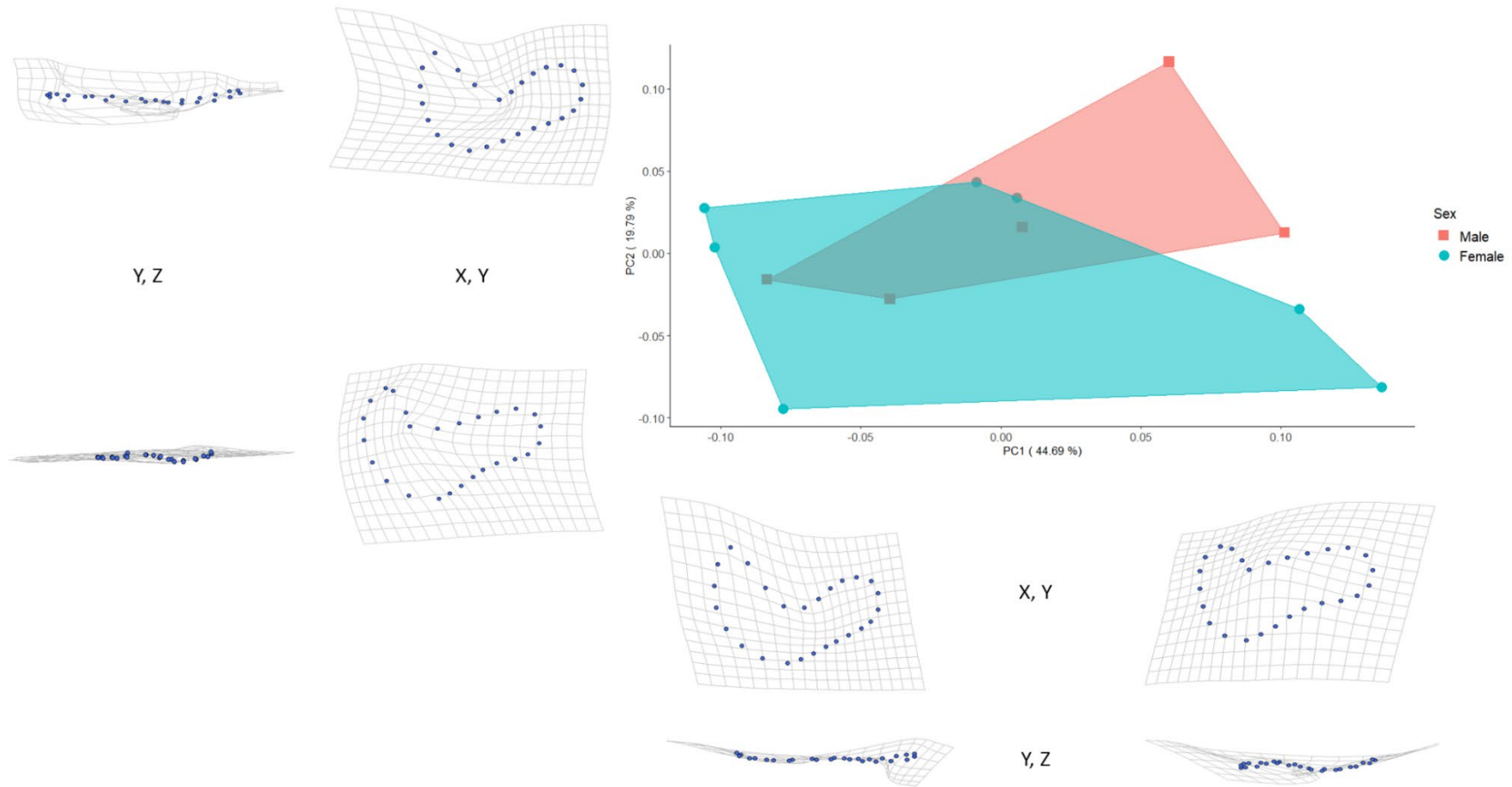


Figure 4.13 – PCA of the AS shape of the individuals aged between 4.0 and 6.9 years grouped by sex. The data indicates a high overlap of the female and male groups, although slight differences along the PC2 scores were identified. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes, and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes, and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times.

### **4.3. Muge Archaeological Skeletal Collection**

#### **4.3.1. Age-at-death**

Using the maximum iliac length, it was possible to estimate the age-at-death for five of the eight (5/8; 62.50%) individuals from the archaeological sample. All are under one year of age-at-death (see Figure 3.4). Since data concerning the size of the AS was available, the centroid size of the LLSC sample was used as a reference to estimate the age-at-death of the remaining three individuals (3/8; 37.50%). As shown in Figure 4.14, these archaeological specimens (numbers 1, 2 and 3) were allocated to the 1.0-3.9 age group. Likewise, the age-at-death of the infants under one year was also confirmed (numbers 4, 5, 6, 7 and 8). Hence, the archaeological sample here analysed is composed of five infants under one year and three infants aged between 1.0 and 3.9 years (Figure 4.15).

#### **4.3.2. Morphological Analysis**

Sex estimation was based on age group specific analysis alone because the previous results show that lumping male and female age cohorts reveals no sex differences in AS morphology (see section 4.2.1.).

Of the five archaeological infants under one year of age-at-death (Figure 4.16), one falls within the range of the female group alone, so it is fair to assume that it is most likely a female individual. The remaining four individuals fall outside the range of the reference sample. Nevertheless, based on group proximity and the clear sex separation of the reference sample, the three specimens closest to the minimum PC2 scores may be tentatively hypothesised males, while the individual closest to the maximum PC2 scores may be a hypothesised female.

Although the results of the reference sample do not reveal significant differences between males and females in the 1.0-3.9 age group, there were some (statistically non-significant) differences between the groups in PC1 and PC2 – areas in which there was no overlap. Thus, a tentative sex estimation analysis of the three archaeological individuals was undertaken (Figure 4.17). Only one is within the male group range alone. The remaining two individuals fall outside the range of both groups of the reference sample. One is located very close to the male group, near the minimum PC1 scores. The other is located near the minimum PC1 and positive PC2 scores. Based on these results and the overlap between the reference sample's male and female groups, it is not possible to reliably estimate the sex of the archaeological individuals belonging to this age category. Nevertheless, the one that occupies the exclusive morphospace of the males

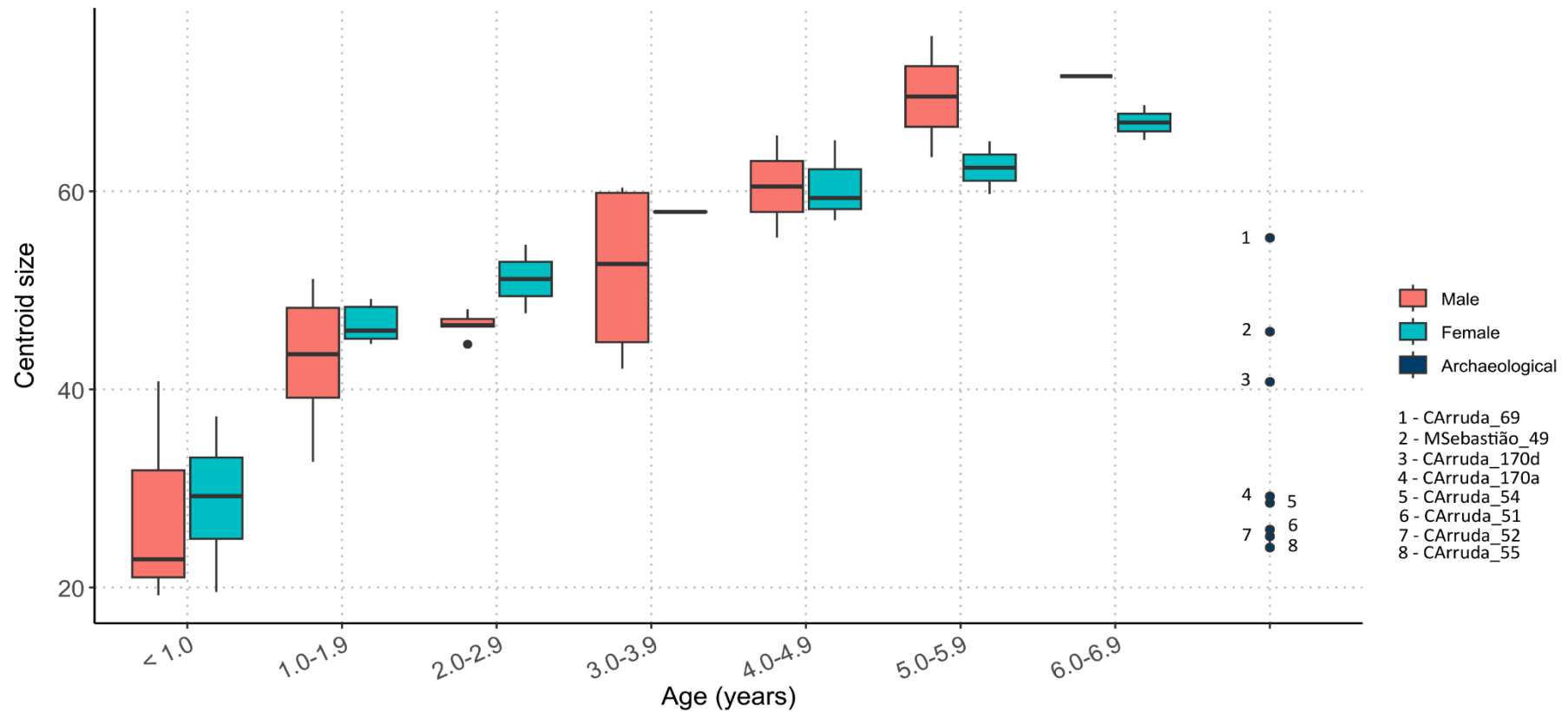


Figure 4.14 – AS size, as assessed using centroid size, of the males and females of the reference sample, grouped by sex and age group. The archaeological specimens are shown on the right side of the plot, each represented by a numbered dot. Numbers 1, 2, and 3 correspond to individuals with AS centroid sizes matching the reference sample age groups 3.0-3.9, 2.0-2.9 and 1.0-1.9, respectively. Numbers 4 to 8 represent individuals whose age-at-death is under one year, based on their maximum iliac length. The centroid size of the AS of these individuals falls within the centroid size range of specimens in the reference sample of the same age.

appears more likely to be a male. The remaining two seem to be, as well, closer to the exclusively male morphospace.

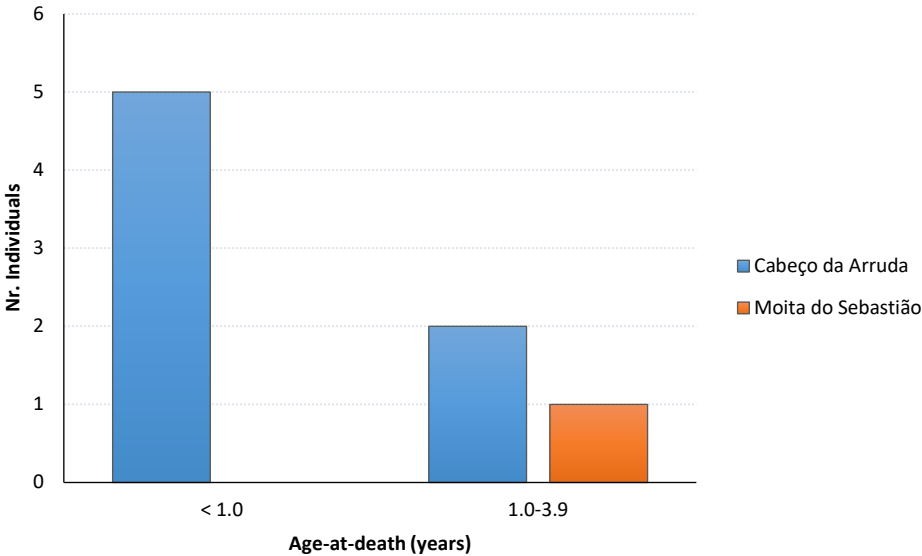


Figure 4.15 – Age-at-death of the archaeological sample, as assessed using the AS centroid size of the reference sample.

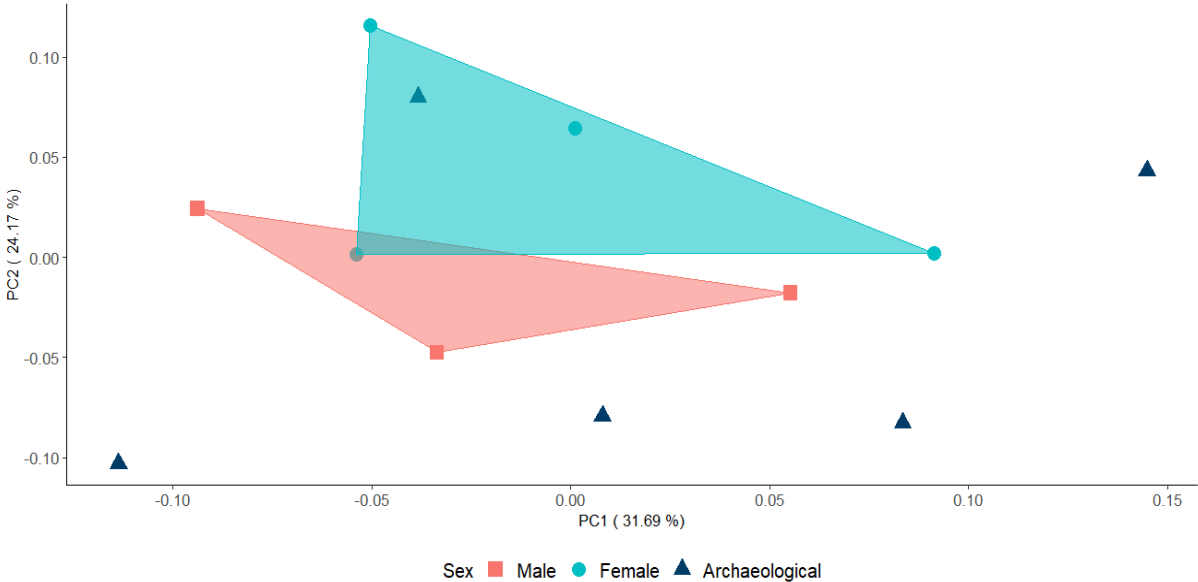


Figure 4.16 – PCA of the AS shape of individuals under one year of age. Individuals belonging to the reference sample are grouped by sex, while the archaeological specimens are apart. The data shows an almost complete separation of the male and female groups. Only one of the archaeological specimens falls in the female group range. The remaining four individuals are outside of the range of the female and male reference groups.

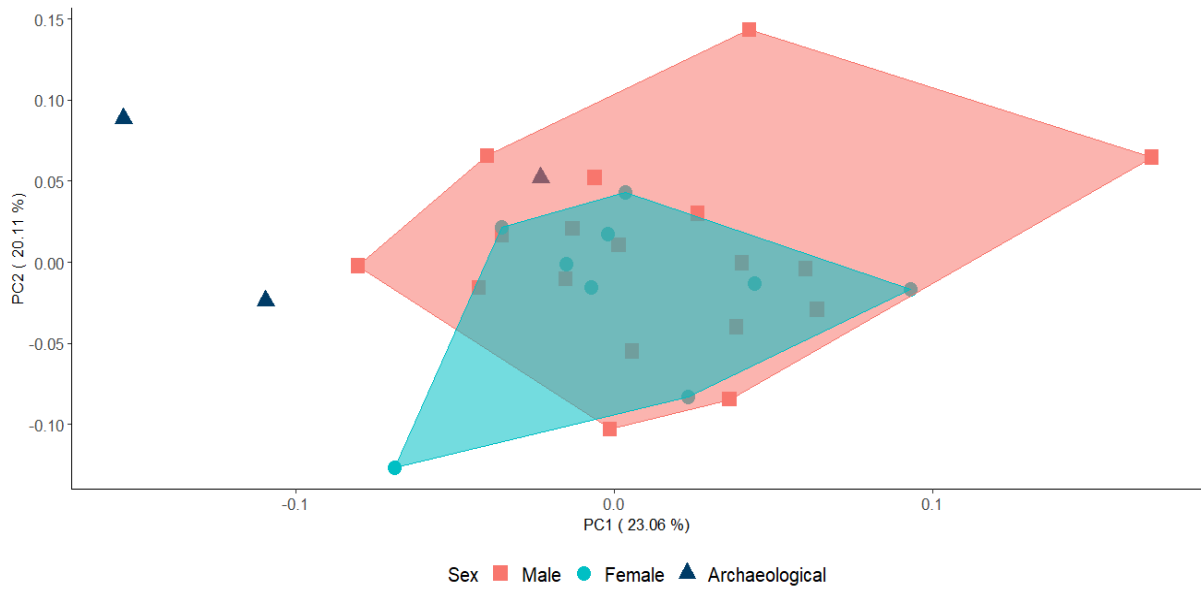


Figure 4.17 – PCA of the AS shape of individuals aged between 1.0 and 3.9 years. Individuals belonging to the reference sample are grouped by sex, while the archaeological specimens are apart. The data shows an almost complete overlap of the male and female groups. Only one of the archaeological specimens falls in the male group range. The remaining two individuals are outside of the range of the female and male reference groups.

## **5. Discussion**

In the past decades, numerous studies have suggested that the AS is a dependable indicator of sex in non-adults (e.g. Weaver, 1980; Mittler & Sheridan, 1992; Wilson et al., 2008; García-Mancuso & González, 2013; Estévez et al., 2017; Luna et al., 2017, 2021; Monge Calleja et al., 2020; Marino et al., 2021). Considering recent investigations' promising results, the present work aimed to develop a method for sex estimation in infants from archaeological contexts by applying 3D GM to the AS. Overall, results indicate the presence of sexual dimorphism in infants under one year, but no such differences were found in older individuals. It is tentatively suggested that this method has the potential to differentiate between male and female infants younger than one year. Nevertheless, the small sample size per age group constrains the results and conclusions.

### **5.1. Main Outcomes: Significance and Interpretation**

The first part of the present study was developing and implementing the sex estimation method in a reference sample of 46 infants with known age and sex. Two variables were analysed for the reference sample, size and shape. Regarding size, looking at the sample grouped by sex and age categories of one year (see Figure 4.3), a gradual increase in AS size is noted, but the growth rate is different for males and females. AS size seems to be comparable for individuals of both sexes under 2 years of age, but from 2 to 4 years, AS size is larger for females, suggesting a higher growth rate for this sex during this period. In contrast, male AS size is larger from 5 to 6 years of age, suggesting an increased growth rate of this anatomical region for this sex during this period.

The analysis of the AS size by larger age groups replicated these results, with individuals under one year having similar AS size (see Figures 4.4 and 4.7), females from 1.0 to 3.9 years presenting a larger AS than males the same age (see Figures 4.4 and 4.10), and this tendency reversing for individuals within the 4.0 to 6.9 age range (see Figures 4.4 and 4.12). However, despite these slight distinctions in the AS size between sexes, the statistical tests performed do not indicate statistically significant differences. Nevertheless, caution is advised when examining the AS size results for the 1.0-3.9 age group. Females are less represented than males – 33.33% and 66.67%, respectively – and, therefore, there may be some sex bias influencing results for this age group (see Albanese et al., 2005; Milner & Boldsen, 2012; Luna et al., 2017; Monge Calleja et al., 2020).

Differences in the AS size between male and female infants are not unprecedented. Wilson and collaborators (2015) have compared the degree of sexual dimorphism in three features of the ilium, in non-adults under 17 years old originating from the Skeletal Collections of London (St. Bride's Church and Christ Church Spitalfields) and Lisbon (LLSC). Results have shown slight differences in the AS between pre- and post-adolescence male and female individuals, but they do not seem to be correlated with sexual dimorphism. These differences become more evident, in the Lisbon sample, around 5 years of age, when there is a larger increase in male AS size relative to the female AS size.

In terms of AS shape, for the overall reference sample no significant differences were observed between males and females (see Figure 4.2). When the sample was analysed according to age groups, males and females under one year showed dissimilarities in the AS shape (see Figure 4.8). Observing the first two principal components, which together account for 61.66% of the total variation, a clear separation of both groups is noted, with males located near the second component minimum scores and females positioned along positive scores of PC2. No statistically significant differences were reported in the first PC scores accounting for about 98% of the total variance. However, when those scores were examined individually (see Figure 4.9), the p-value for the second principal component was very close to being significant ( $p=0.057$ ). For individuals aged 1.0 to 3.9 years, no significant differences in AS morphology were found (see Figure 4.11). Finally, male and female specimens with 4.0 to 6.9 years presented slight statistically non-significant shape differences in the AS (see Figure 4.13). These results point to the presence of sexual dimorphism in AS morphology at early ages. Contrarily, in individuals over one year, the biological signal reflecting sexual dimorphism seems to fade, since males and females present similar AS. Sexual dimorphism still exists, but the differences between males and females may be so subtle that this technique does not capture its biological signal (Olivares & Aguilera, 2016; Ubelaker & DeGaglia, 2017; Wilson & Humphrey, 2017). The display of sexual dimorphism at early ages may be explained by what some authors call 'minipuberty' (Lanciotti et al., 2018; Monge Calleja et al., 2020) – or neonatal hormonal surge (Knickmeyer & Baron-Cohen, 2006; Stull & Godde, 2013; Clarkson & Herbinson, 2016). Some hours after birth, males experience a surge of testosterone (Clarkson & Herbinson, 2016; Lanciotti et al., 2018). Hormonal levels reach their highest peak between the first and the third month and then they gradually decline until the sixth and the ninth month of life (Lanciotti et al., 2018). In turn, females experience a post-natal surge of oestradiol (Knickmeyer & Baron-Cohen, 2006), which starts a week after birth. Hormonal levels will remain elevated for around 6 months with significant fluctuations (Lanciotti et al., 2018; Luna et al., 2021). Later, between

the end of the second year of life and until the beginning of puberty – around 12 to 14 years of age in the current Portuguese population (Rocha et al., 1998) – there is a period of low hormonal concentration levels, also designated as 'prepubertal hiatus' (Cummings & Kavlock, 2004).

So, the results obtained in this study may be related to the neonatal hormonal surge, which might explain the presence of sex-related variations in the AS morphology in infants under one year. At the same time, the 'prepubertal hiatus' may be reflected in the lack of differences in individuals over one year. It is also tentatively suggested that the biomechanical forces associated with bipedal locomotion – usually reached at 12 to 15 months of age (Cowgill & Johnston, 2018) – may also influence AS morphology. Specifically, infants begin developing bipedal locomotion approximately when the sex differences in AS shape disappear. Thus, comparable mechanical stresses and strains associated with developing walking may prompt bone mechanical adaptation that results in the convergence of AS shape between males and females. This is consistent with authors stating that the sacroiliac joint morphological changes at the onset of locomotion are possibly due to mechanical forces associated with supine positioning and transfer of body weight (Solonen, 1957; Vleeming et al., 2012; Yusof et al., 2013). Garvin and colleagues (2021) obtained similar results when examining the overall ilium outline and the sciatic notch. They established a probable relationship between their results and the neonatal hormonal surge. Moreover, the authors suggested that the lack of biomechanical forces related to bipedal locomotion may also explain the shape differences found between sexes before one year of age.

Monge Calleja and colleagues (2020) also referred to the neonatal hormonal surge. Following the methodology proposed by Luna and collaborators (2017), these authors found sex-related variations in the AS for individuals under 19 years of age. However, sex estimation accuracy was higher in individuals under 2 years of age. Interestingly, Luna and collaborators (2021), employing the same method, also observed morphological differences in the AS between males and females under 6 years, but accuracy was higher in fetuses. As for Marino and co-authors (2021), they detected sex-related variations in the AS morphology in non-adults under 18 years. However, the same technique was less successful in infants under one year, possibly due to the high variability of this anatomical region during the first year of life.

For decades other studies have also investigated whether the AS is a reliable sex indicator in non-adults, but the results are contradictory. Weaver's (1980) pioneer study reported the presence of sexual dimorphism in the AS in infants under one year. Promising results led Hunt (1990) to test Weaver's technique, but the outcomes suggested that differences in AS morphology were age-related and not sex related. Mittler & Sheridan (1992) conducted yet

another study involving a sample of individuals from birth to 18 years of age. Even though their results were similar to those observed by Weaver, they pointed out that the method's accuracy was higher in children over 9 years old. Likewise, Sutter's study (2003) disregarded the AS as a sexually dimorphic feature in non-adults under 16 years old.

García-Mancuso and González (2013) evaluated five iliac features in infants under 5 months. Their outcomes indicated that the AS presented sex-related differences. Years later, a new study by García-Mancuso and co-authors (2018) investigated eight features of the same bone in non-adults under 17 years old. Results discarded the AS as a reliable feature for sex estimation in subadults. Previously, Wilson and collaborators (2008) were able to distinguish males and females under 9 years through AS morphology. In a subsequent study, Wilson and colleagues (2011) classified the AS morphology as a poor sex indicator in non-adults younger than 8 years. Despite the varying outcomes found in previous studies, certain key aspects need to be considered. First, the sample size of these studies is quite distinct, as is the age representation and sex proportion. Second, sampled individuals were from geographically, temporally, and sometimes culturally distant populations (Ubelaker & DeGaglia, 2017; Wilson & Humphrey, 2017). Third, the techniques employed by these studies were diverse – some used a combination of conventional morphometrics and visual assessment of AS features (Luna et al., 2017; Monge Calleja et al., 2020; Luna et al., 2021; Marino et al., 2021). Several have evaluated the AS elevation (e.g. Weaver, 1980; Hunt, 1990; Mittler & Sheridan, 1992; Sutter, 2003; Wilson et al., 2008; Wilson et al., 2011; Blake, 2019). A small portion of studies analysed the AS partial outline through 2D GM (e.g. García-Mancuso & González, 2013; Estévez et al., 2017; García-Mancuso et al., 2018). Therefore, it is no surprise that the results of those studies quantifying the expression of sexual dimorphism in the abovementioned samples are variable in degree and pattern (Ubelaker & DeGaglia, 2017; Wilson & Humphrey, 2017).

Concerning the methodology, this study represents the first use of 3D GM to assess the AS's complete outline and its potential for estimating sex in non-adults. This non-invasive and non-destructive technique uses virtual replicas – 3D models – instead of dry bone specimens (Wilson & Humphrey, 2017), contributing to their preservation (Kuzminsky & Gardiner, 2012; Godinho & Gonçalves, 2020; Godinho & Duarte, 2022; Godinho et al., Accepted). Although scanning technology and computer power are still expensive, over the last few years the costs of these resources decreased significantly. A surface scanner presents some benefits when compared to other 3D acquisition equipment, such as computed tomography (CT) or micro-CT (mCT). It is a less expensive piece of equipment and has the advantage of being portable. Additionally, it rapidly generates post-processing free (or minimally demanding) 3D data, creating a permanent

record of the scanned specimen for multiple uses (Wilson & Humphrey, 2017; Godinho & Gonçalves, 2020; Godinho & Duarte, 2022; Godinho et al. Accepted).

By using 3D models, GM can capture and quantify variations in skeletal features that are not accessible through traditional morphometrics or morphological techniques (Wilson & Humphrey, 2017). This approach employed both traditional LMs and sSLMs to collect quantitative data – less prone to subjectivity and observer bias – on the AS morphology (Mitteroecker & Gunz, 2009; Conner et al., 2024). The incorporation of sSLM into the analysis allowed to overcome the limitations of using only conventional LM by providing a better quantitative representation of the AS (Bookstein, 1991; Gunz et al., 2005; Mitteroecker & Gunz, 2009). This is an advantage over previous investigations that have used conventional morphometrics to study AS morphology (e.g. Luna et al., 2017, 2021; Monge Calleja et al., 2020; Marino et al., 2021). Although these approaches are very useful, they can only examine the morphological information of the measured segments, overlooking what lies in between the anatomical LMs used as references for linear data collection. Ultimately, the present investigation demonstrates, alongside previous studies (e.g. Bilfeld et al., 2013; Mokrane et al., 2013; Wilson et al., 2017; Garvin et al., 2021), how 3D GM can be a powerful tool when researching sex estimation in infants.

## **5.2. Archaeological Sample Results: Significance and Interpretation**

Given the outcomes obtained for the reference sample, the second part of this study – the application of the technique here developed to a smaller Mesolithic archaeological sample comprising eight individuals of unknown sex – could then ensue. Therefore, a second morphological analysis was conducted including the 54 specimens from the reference and the archaeological samples. The age-at-death of the archaeological infants was confirmed using the AS centroid size of the LLSC sample as a reference (see Figure 4.14). Three infants were estimated to have between 1.0 and 3.9 years, while the remaining five individuals were confirmed as infants under one year (see Figure 4.15), consistently with the maximum iliac length-based estimates (Molleson & Cox, 1993).

The results of the morphological analysis were inconclusive across all age groups, so the sex of the archaeological individuals could not be accurately estimated. Of the five archaeological specimens under one year of age-at-death, only one fell within the reference group's range and could, therefore, be classified as a female (see Figure 4.16). Since the second principal component clearly separates the male and female groups (females are closest to the maximum

scores and males tend to be closer to the minimum scores), some extrapolations about the sex of the remaining four archaeological specimens could be made. Specimens with lower PC2 scores are more likely to be male. On the other hand, specimens closest to the PC2 maximum scores are probably females. Therefore, it is tentatively assumed that of the five archaeological infants under one year, two are probably females (2/5; 40.00%) and the remaining three are possibly males (3/5; 60.00%).

The results of the archaeological specimens in the age group from 1.0 to 3.9 years are more ambiguous due to the significant overlap of the reference's male and female groups and little group-specific morphospace (see Figure 4.17). Even though two archaeological specimens are closer to exclusively male morphospace, it is not possible to conclusively classify them as males. The third archaeological individual in this age group fell in the exclusively male morphospace and so was classified as a possible male. Because of these outcomes, the sex estimation method presented here cannot be considered, at this stage, a substitute for biomolecular techniques such as ancient DNA analysis and paleoproteomics.

Although the presented results are inconclusive, there seems to be a relatively balanced representation of both sexes among individuals under one year of age, even though there are slightly more males (three) than females (two). For the age group 1.0-3.9 years, the results are less certain than for the previous age cohort. While the sex estimation suggests the presence of three males, the considerable overlap between male and female reference groups raises significant doubts about this classification. If these outcomes were definitive, this would suggest a similar representation of male and female infants under one year, but a total absence of females in the 1.0-3.9 years age group. These results could then be further explored to investigate other cultural or biological variations related to sex within the Muge Mesolithic communities. For example, why infant mortality is higher for males than for females? Is it related to cultural practices like infanticide or selective childcare? Or can it be explained by a higher susceptibility of males to certain diseases?

Other questions, such as sex-related differences in weaning and post-weaning practices or dietary patterns, could also be investigated. The isotopic studies indicate that the Muge diet consisted of both terrestrial and marine proteins, with the latter being predominant (Lubell et al. 1994; Umbelino, 2006; Umbelino et al., 2007, 2015; Peyroteo-Stjerna, 2016). Differences in dietary patterns between male and female adults in Muge have also been noted (Umbelino, 2006). Can these differences extend to non-adult males and females as well? Estimating sex in non-adult individuals would facilitate the investigation of this question, as there is already some data regarding isotopic analysis of Muge infants (Umbelino et al., 2015).

Other lines of investigation could also be explored, such as distinctions in indicators of growth and developmental disruptions (e.g. enamel hypoplasia or *cribra orbitalia*) or mortuary practices (e.g. body positioning, orientation, or sex-specific grave goods). This aspect would be particularly interesting to investigate at Moita do Sebastião shell midden, as a specific burial area for infants has been documented (Roche, 1972; Carvalho, 2009; Jackes & Meiklejohn, 2012). Knowing the sex of these infants would enable the evaluation of potential differences in burial rituals between males and females.

Although these results appear promising, suggesting that the method developed in this study can potentially estimate the sex of infants under one year of age from archaeological contexts, an important key aspect must be considered. The archaeological sample and the reference sample are chronologically distant – ~8000 years apart. The target sample represents a pre-historic population, while the reference sample represents a modern one. Besides differences in, for example, living conditions, nutrition, life expectancy and morbidity, which possibly impacted skeletal ontogeny and morphology (Ubelaker & DeGaglia, 2017; Wilson & Humphrey, 2017; Corron et al., 2021), those two groups have significantly different population histories (e.g. Haak et al., 2015; Olalde et al., 2019; Vilallba-Mouco et al., 2019; Carvalho et al., 2023). Possibly, the extent and degree of sexual dimorphism is not the same in both samples. The outcomes observed for individuals under one year may reflect those differences since most archaeological specimens do not overlap with the reference female and male groups. Considering there is no information regarding the pattern of sexual dimorphism in the archaeological sample, comparisons with the reference sample results may be challenging (Wilson & Humphrey, 2017). Even though the current study outcomes are not quite the same for both samples, there is still evidence that the AS possibly has sex-related morphological variations at early ages.

Given this constraint, it would be both premature and potentially misleading to make definitive assumptions about the sex of the archaeological specimens analysed. Cintas-Peña and Herrero-Corral (2020) have made pertinent observations about this issue. When analysing the data of 2410 individuals from 62 archaeological sites in the Iberian Peninsula, chronologically framed between the 8th and 3rd millennia BCE, they noted a clear prevalence of males over females. In Neolithic sites, for example, the data showed a sex ratio of 150.6, meaning there were 151 males for every 100 females. Males outnumbered females in every period they looked at. So, what caused these results? Without excluding possible differences in funerary treatment, the authors pointed out methodological problems that led to females being misclassified more often than males. Because prehistoric skeletal remains are often incomplete, poorly preserved or

commingled, the accuracy of sex estimation methods is greatly reduced. The likelihood of misclassifying a male as a female, or vice versa, increases significantly. Therefore, as suggested by Cintas-Peña and Herrero-Corral (2020), it is important to consider possible methodological biases or problems before making interpretations about the data. Otherwise, the conclusions drawn will be questionable.

That being said, the study of non-adult skeletal remains can greatly enrich archaeological knowledge about the dynamics and cultural behaviour of past human societies. Research into new methods of sexing them is essential for answering questions such as "Are there differences in funerary treatment between males and females" or "Is infant mortality the same for both sexes"? Although the contributions of the present study in this regard are limited, previous studies have shown how the sex estimation of non-adults enhances the information about past communities' ways of life. For example, Rebay-Salisbury and co-workers (2022) have recently investigated non-adult burial practices in Franzhausen I, an Austrian Early Bronze Age necropolis. Using paleoproteomics, the authors were able to estimate the sex in 70 non-adults aged 0-12 years and to confirm that the burial treatment was received according to biological sex. Based on funerary rituals, sex was then assessed for all non-adults (N=210), aged 0 to 20. This was followed by an analysis of the sample demographics, which showed that the mortality rate was higher for males under the age of 5. Mortality was similar for males and females between 5 and 12 years of age. From puberty to adulthood, female mortality increased. The authors pointed out that newborns and infants less than 2 years old were underrepresented, so the small sample size could explain why mortality was higher for males under 5 years old. Alternatively, the sex ratio at birth could favour males, as is currently the case. No discrepancies were found in skeletal indicators of stress (e.g. enamel hypoplasia), trauma and disease, although the high incidence of *cribra orbitalia* in males may indicate some dietary differences. In addition, Bas and collaborators (2023), found slight differences in the microwear of female and male teeth, supporting the hypothesis of differences in early childhood nutrition. Another alternative could be that the weaning process of the female infants from Franzhausen I started at an earlier age.

The present study represents the first attempt in Portugal to directly apply a method for sex estimation in infants to an archaeological sample. Although the selected sample comes from a Mesolithic shellmidden, this method can be applied to skeletal remains from different burial contexts and chronologies. Furthermore, this method can be used for both articulated and commingled skeletal remains – e.g. when skeletal elements from multiple individuals are mixed (Osterholtz, 2018). Given the undeniable importance that human skeletal remains have assumed

in recent decades for reconstructing past human dynamics and cultural behaviour (Cintas-Peña & Herrero-Corral, 2020), this method should be further developed and tested (see section 5.3.). It is important to note that other investigations have already used similar approaches when analysing archaeological skeletal remains. For instance, Conner and co-authors (2024) used 2D GM to assess the reliability of two pelvic features in adult sex estimation. Their approach's accuracy reached 90% in a modern reference sample, and afterwards, the same method was used to estimate the biological sex of 73 archaeological specimens, 71 from the Early-Late Medieval period (5th-15th century) and two from the Roman period (3rd-4th century).

### **5.3. Limitations and Future Prospects**

When interpreting the results of the present study, it is important to consider a major constraint: the sample size. This study gathered a total of 46 specimens from the Luis Lopes Skeletal Collection, all under 7 years old (see Figure 3.2). Initially, a total of 57 individuals was selected but the poor preservation of the AS resulted in the exclusion of 11 specimens from the study. The sample sex composition shows a slight predominance of male individuals (26 out of 46; 56.52%) over female individuals (20 out of 46; 43.38%). Upon examining the distribution of the individuals according to their age-at-death per age cohorts, it is clear the sample is unbalanced. The groups range from a minimum of three individuals observed in the 6.0-6.9 age range (3 out of 46; 6.52%) to a maximum of 14 individuals in the 1.0-1.9 age range (14 out of 46; 30.43%). Given that the number of individuals per age categories of 1 year was relatively small, the sample was rearranged into age groups (see section 3.2.3.). Therefore, the chosen age groups were as follows: < 1.0 years, 1.0-3.9 years and 4.0-6.9 years (see Figure 3.8). Nevertheless, it was not possible to overcome the sample imbalance and that most of the individuals were in the 1.0-3.9 years group (27 out of 46; 58.70%). Only seven individuals were in the < 1.0-year group (15.22%) and 12 in the 4.0-6.9-year group (26.07%). For this reason, interpretations regarding this study's results should be cautious as overall accuracy might be affected by the small sample size and age distribution.

Many studies attempting to estimate the sex of non-adults face the same limitation. The underrepresentation of well-preserved sub-adults, particularly infants, of known sex and age is transversal to many documented skeletal collections (H. F. Cardoso, 2008; Willson & Humphrey, 2017; Sharman & Albanese, 2018; Stull et al., 2020; Ortega et al., 2021). For example, in the LLSC, the largest in Portugal and the one used in the present investigation, the percentage of individuals under 20 years old is only 13.16% (92 specimens out of 699, for

whom the data was treated back in 2006; H. F. Cardoso, 2006a). Likewise, the Coimbra Identified Skeletal Collection, presently curated at the Department of Life Sciences of Coimbra University, lacks subadults under 7 years of age (Sharman & Albanese, 2018). The 21st Century Identified Skeletal Collection, also housed at Coimbra University, is composed exclusively of adults over 28 years of age (Ferreira et al., 2014; Ferreira et al., 2021). Another example is the Identified Skeleton Collection of Évora, curated at the Laboratory of Biological Anthropology of Évora University, which only accounts for 3.37% of subadults (7 out of 208) (Lopes & Fernandes, 2022). This underrepresentation is bound to be reflected in studies aiming to develop or test non-adult sex estimation techniques (Albanese, 2018; F. A. Cardoso, 2018; Sharman & Albanese, 2018; Lopes & Fernandes, 2022). For example, Luna and colleagues (2017) resorted to the Coimbra Identified Skeletal Collection for the development of their sex estimation method. Therefore, they could only gather a sample of 34 individuals over 7 years old (21 females and 13 males). Monge Calleja and co-authors (2020) tested the abovementioned method in a sample of 61 individuals under 19 years old from the Luis Lopes Skeletal Collection. However, only 23 individuals (37.71%) correspond to the age range analysed in the present study (0-6 years). Wilson and colleagues (2008) evaluated a sample comprising 25 infants under 9 years of age (17 males and eight females) from the London Spitalfields Skeletal Collection. Thus, despite the above-mentioned constraints, the sample used in this study is, to the best of my knowledge, one of the largest gathered thus far in any comparable study.

Besides sample size, another significant limitation is related to the cross-application of the presented methodology. Since GM techniques do not require the scoring of skeletal features based on previously existing standards (e.g. Ferembach et al, 1980; Buikstra & Ubelaker, 1994; Bruzek, 2002) or a highly experienced observer (Wilson & Humphrey, 2017), interobserver error testing was not necessary. On the other hand, the identification of LMs may be affected by the observer's subjectivity, so an intra-observer error test was performed. This was particularly important since the 3D models lacked information regarding texture and colour. The outline of the AS was not always clear and had to be confirmed through digital photography. The test results showed that the error introduced by the observer was negligible. Therefore, the landmarking protocol proposed here is reliable. Nevertheless, a cross-validation test in other documented skeletal samples is still necessary to assess the success rate of this technique.

Body size, robustness, and the degree of sexual dimorphism are known to vary among different populations. Since these features impact skeletal morphology, multiple studies had limited success when testing non-adult sex estimation across populations (Ubelaker & DeGaglia, 2017;

Wilson & Humphrey, 2017; Corron et al., 2021). For instance, the method for the morphological features of the ilium was originally proposed by Schutkowski (1993). This author used a small sample from the London Spitalfields Skeletal Collection. This technique was posteriorly tested in three distinct reference collections, Chile (Sutter, 2003), Lisbon (Vlak et al., 2008) and, more recently, Granada (Olivares and Aguilera, 2016). Accuracy levels varied across studies – 49% in the Lisbon sample and 94% in the one from London – but all pointed to the sciatic notch as the most significant sex-related feature. The discrepancy between these studies' results may be due to inter-population differences in sexual dimorphism (Wilson & Humphrey, 2017).

Other factors to consider in cross-validation tests include inter-population secular changes in body mass and stature. Past and present populations experience different living conditions, diet, and healthcare, all impacting skeletal development and growth. Successful methods in contemporary populations may not produce the same results in pre-historical or historical populations, and vice-versa (Wilson et al., 2011; Wilson & Humphrey, 2017; see section 5.2.). For example, Schutkowski (1993) used a mid-1700s to mid-1800s sample in his study. The tests on his technique were conducted in populations not only from different geographic regions and cultural contexts but also from different time periods. This can explain why the outcomes were not consistent. Sutter (2003) used a pre-Colombian sample from the Atacama Desert region in northern Chile. Vlak and collaborators (2008) analysed a post-industrial sample from Lisbon (late 19th century – late 20th century). Finally, Olivares and Aguilera (2016) examined contemporary non-adults from the Cemetery of San Jose in Granada (20th century).

Regarding this investigation's limitations, it is still important to note that only one region of the ilium was analysed, the AS. The selection of this particular area relates to the positive outcomes of the method proposed by Luna and co-authors (2017) and the subsequent tests by Monge Calleja and collaborators (2020) and Luna and colleagues (2021). However, several studies have reported that other features of the ilium can also be dependable sex indicators in non-adults, like the sciatic notch (e.g. Boucher, 1957; Fazekas & Kosá, 1978; Schutkowski, 1987; Schutkowski, 1993; Adalian et al., 2001; Sutter, 2003; Wilson et al., 2008; García-Mancuso, 2009; Wilson et al., 2011; García-Mancuso & González, 2013; Olivares & Aguilera, 2016; Estévez et al., 2017; García-Mancuso et al., 2018). Some researchers have also discussed that sex discrimination is more successful when two or more features are analysed together. For example, Wilson and collaborators (2011) evaluated three iliac skeletal traits, the AS, the sciatic notch and the iliac crest. Despite the author's suggestion about not using the AS as a sex indicator, they concluded that the best outcome was based on all the evaluated features. As for Estévez and co-authors (2017), their study assessed the reliability of the sciatic notch and the

partial outline of the AS. Both features displayed sexual dimorphism, but the researchers found that sex discrimination accuracy increased when both traits were analysed together. The possibility of evaluating the reliability of more than one skeletal feature should be considered for future studies.

To conclude, this study represents the first effort to use 3D GM to assess the AS's complete outline and its potential for estimating sex in non-adults under 7 years old. After the initial analysis performed on a modern reference sample, a cross-application test was conducted on a small pre-historic sample. Results are promising and point to the presence of sexual dimorphism in the AS morphology at early ages. In individuals over one year, the biological signal reflecting sexual dimorphism seems to fade. The next steps should include increasing sample size and conducting cross-validation and cross-application tests. Additionally, more skeletal features ought to be included in the study. This would allow to compare the outcomes for different iliac features and to evaluate if sex discrimination accuracy increases when more than one skeletal trait is used. Finally, AI techniques (Deep learning and Machine learning) could be also considered for future studies. Some investigations have already assessed the performance of these techniques for sex estimation in non-adults (e.g. Ortega et al., 2021) and adults (e.g. Bewes et al., 2019; Cao et al., 2022; Bidmos et al., 2023; Knecht et al., 2023; Díaz-Navarro et al., 2024; Kuha et al., 2024) and the results are very promising.

## 6. Final Considerations

Estimating biological sex is an essential tool for studying the dynamics and cultural behaviour of past populations. However, it is not usually performed on non-adult individuals before the onset of puberty, which limits the knowledge that can be gained from immature archaeological skeletal remains. Bearing this in mind, this thesis aimed to address the following questions: Is it possible to detect sexual dimorphism in infant skeletal remains using 3D GM? Can the same technique be applied to non-adults from archaeological assemblages? How can this method contribute to future studies regarding sex estimation in non-adults?

The first part of the original work of this thesis presented the development, testing and validation of a 3D GM technique to estimate the biological sex of infants aged 0-6 years using the AS. A contemporary sample with known sex and age from the LLSC, the largest identified Portuguese osteological collection, was used for this purpose. There were no differences in the size of the AS between males and females when using the full sample. When grouping the specimens by age groups, then sex differences in the size of the AS emerged. Sex-related shape differences in this anatomical region were observed in infants under one year of age, but not in older individuals. As the results showed sex-related differences in AS morphology, the next step in this study was to attempt to estimate the sex of the eight infants within the same age range from the Muge Mesolithic shellmiddens.

Of the five archaeological specimens under one year of age-at-death, only one was within the range of the reference female group and was, therefore, classified as a female. Of the other four individuals, one was closer to the female morphospace and was classified as a probable female. The remaining three individuals were closer to the male morphospace and were, thus, classified as possible males. Therefore, it is tentatively assumed that of the five archaeological infants under one year, two are probably females (2/5; 40.00%) and the remaining three are possibly males (3/5; 60.00%). As for the individuals in the age group 1.0-3.9 years, results are more ambiguous due to the significant overlap of the reference's male and female groups and little group-specific morphospace. Nevertheless, one of the specimens fell in the male reference group morphospace and was classified as a probable male. As for the remaining two archaeological individuals, although they are closer to the exclusively male morphospace, it was not possible to conclusively classify them as males. Given the morphological analysis results were inconclusive, particularly for the 1.0-3.9 years age group, it would be premature, at this stage, to make definitive assumptions about the sex of the archaeological specimens analysed.

These findings suggest the presence of sexual dimorphism in the AS at an early age, and that its biological signal can indeed be used to estimate sex. However, in individuals over one year of age, the morphology of the AS is similar in males and females, suggesting that the biological signal reflecting sexual dimorphism fades. The neonatal hormonal surge, or 'minipuberty' as some authors call it, may be responsible for these findings. At the same time, it is tentatively suggested that the biomechanical forces associated with bipedal locomotion may also influence AS morphology, reducing sex-related differences in individuals over one year.

Although these outcomes are promising, caution should be exercised when interpreting them. Even though the reference sample used in the present study is, to my knowledge, one of the largest in any comparable research to date, the small sample size per age cohort limits the results and conclusions. In addition, the results and (some) success of the methodology presented here need to be assessed by performing cross-validation and cross-application tests in different samples. Nevertheless, the application test carried out on the archaeological sample is promising. Despite the results are not quite the same as those observed for the reference sample, possibly due to inter-population variation in the extent and degree of sexual dimorphism, there is still evidence that this method can be used to distinguish males and females in different populations. Finally, it should be noted that only one anatomical region, the AS, was analysed in this study. Previous studies have shown that other iliac features can also be reliable sex indicators (e.g. the sciatic notch). Including these might increase the accuracy of sex discrimination in all age cohorts. Another key aspect to consider for future studies is the application of AI techniques (Deep and/or Machine learning). Some investigations have had promising results when applying this technique to sex estimation, either in adults or non-adults, and such tools may be used to provide probability-based classification of the archaeological specimens.

In conclusion, this study has demonstrated the potential of using 3D GM for sex estimation in archaeological non-adult skeletal remains. It has also shown that the AS is a reliable sex indicator for sex estimation in infants under one year of age. In individuals over one year of age, the biological signal reflecting sexual dimorphism appears to fade. These findings should be investigated in more detail and further studies should include the analysis of other iliac features, a larger sample integrating other populations to examine if there are inter-population differences, cross-validation and cross-application testing, and eventually the use of AI techniques.

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## **Appendix A – Supplementary Material**

Table A – List of non-adult sex estimation studies that evaluated features of the ilium, or the complete bone (outline or linear measurements). NA stands for non-applicable. ✖ indicates the studies that did not approach that (those) feature(s). ✔ indicates studies that evaluated that (those) trait(s).

<i>Study</i>	<i>Method</i>	<i>Age interval</i>	<i>Nr. Specimens</i>	<i>Ilium</i>	<i>Iliac crest</i>	<i>Auricular surface</i>	<i>Sciatic Notch</i>	<i>Acetabulum</i>	<i>Arch criterion</i>
<i>Boucher (1957)</i>	Morphometrics	Foetuses	129	✖	✖	✖	✔	✖	✖
<i>Fazekas &amp; Kósa (1978)</i>	Morphometrics	Foetuses	104	✔	✖	✖	✔	✖	✖
<i>Weaver (1980)</i>	Morphometrics and Morphology	Foetuses 0-6 months	153	✔	✖	✔	✔	✖	✖
<i>Schutkowski (1987)</i>	Morphometrics	Foetuses	104	✔	✖	✖	✔	✖	✖
<i>Hunt (1990)</i>	Morphology	NA	NA	✖	✖	✖	✔	✖	✖
<i>Mitler &amp; Sheridan (1992)</i>	Morphology	0-18 years	58	✖	✖	✔	✖	✖	✖
<i>Majò et al. (1993)</i>	Morphometrics	7-16 years	41	✔	✖	✖	✔	✖	✖
<i>Schutkowski (1993)</i>	Morphology	0-11 years	61	✖	✔	✖	✔	✖	✔
<i>Holcomb &amp; Konigsberg (1995)</i>	2D GM	Foetuses	133	✖	✖	✖	✔	✖	✖
<i>Adalian et al. (2001)</i>	Morphometrics	Foetuses	83	✖	✖	✖	✔	✖	✖
<i>Sutter (2003)</i>	Morphology	0-15 years	85	✖	✔	✔	✔	✖	✔
<i>Cardoso &amp; Saunders (2008)</i>	Morphology	0-15 years	97	✖	✖	✖	✖	✖	✔
<i>Vlak et al. (2008)</i>	2D GM	0-16 years	56	✔	✖	✖	✔	✖	✖
<i>Wilson et al. (2008)</i>	2D GM	0-8 years	25	✖	✔	✔	✔	✖	✖

<i>Study</i>	<i>Method</i>	<i>Age interval</i>	<i>Nr. Specimens</i>	<i>Ilium</i>	<i>Iliac crest</i>	<i>Auricular surface</i>	<i>Sciatic Notch</i>	<i>Acetabulum</i>	<i>Arch criterion</i>
<i>García-Mancuso (2009)</i>	Morphometrics	Foetuses	62	✓	✗	✗	✓	✗	✗
<i>Wilson et al. (2011)</i>	2D GM	0-15 years	126	✗	✓	✓	✓	✗	✗
<i>García-Mancuso &amp; González (2013)</i>	2D GM	0-4 months	21	✓	✓	✓	✓	✓	✗
<i>Mokrane et al. (2013)</i>	3D GM	Foetuses	93	✓	✗	✗	✗	✗	✗
<i>Olivares &amp; Aguilera (2016)</i>	Morphology	Foetuses 0-6 years	185	✗	✓	✗	✓	✗	✓
<i>Estévez et al. (2017)</i>	2D GM	0-1 year	71	✗	✗	✓	✓	✗	✗
<i>Luna et al. (2017)</i>	Morphometrics and Morphology	7-18 years	34	✗	✗	✓	✗	✗	✗
<i>Wilson et al. (2017)</i>	3D GM	0-15 years	34	✗	✓	✗	✗	✗	✗
<i>García-Mancuso et al. (2018)</i>	2D GM	Foetuses 0-16 years	216	✓	✓	✓	✓	✗	✗
<i>Blake (2019)</i>	Morphology	Foetuses 0-16 years	197	✗	✗	✓	✗	✗	✗
<i>Monge Calleja et al. (2020)</i>	Morphometrics and Morphology	0-18 years	61	✗	✗	✓	✗	✗	✗
<i>Garvin et al. (2021)</i>	3D GM	0-14 years	397	✓	✗	✗	✓	✗	✗
<i>Luna et al. (2021)</i>	Morphometrics and Morphology	Foetuses 0-5 years	197	✗	✗	✗	✓	✗	✗
<i>Marino et al. (2021)</i>	Morphometrics and Morphology	0-17 years	127	✗	✗	✗	✓	✗	✗
<i>Ortega et al. (2021)</i>	AI	Foetuses 0-6 years	135	✗	✓	✗	✓	✗	✓

Table B – Procrustes distances of the three landmark sets collected from seven specimens of the LLSC sample, used for the intraobserver error test.

	389	389_2	389_3	521	521_2	521_3	600_4	600_5	600_6	1160_2	1160_3	1160_4	1399_3	1399_4	1399_5	1557_4	1557_5	1557_6	1660	1660_2	1660_3	
<b>389</b>	0																					
<b>389_2</b>	0,0203112	0																				
<b>389_3</b>	0,0200132	0,0142909	0																			
<b>521</b>	0,0879426	0,091049	0,08649	0																		
<b>521_2</b>	0,0917614	0,0976518	0,0916632	0,0386005	0																	
<b>521_3</b>	0,0959452	0,1013073	0,0953921	0,0324328	0,0223819	0																
<b>600_4</b>	0,1424486	0,1444968	0,1380812	0,1505015	0,1341521	0,1411251	0															
<b>600_5</b>	0,1316684	0,1342042	0,1277123	0,1407827	0,1255433	0,133042	0,0181413	0														
<b>600_6</b>	0,1501288	0,1527182	0,1457454	0,1574773	0,139282	0,1461075	0,0180506	0,0282492	0													
<b>1160_2</b>	0,0940137	0,0930814	0,0942648	0,1438128	0,1451866	0,1508955	0,1515978	0,1445035	0,1632133	0												
<b>1160_3</b>	0,1012518	0,0988012	0,1014678	0,1493657	0,1506534	0,1566844	0,1562011	0,1498054	0,1686466	0,0212897	0											
<b>1160_4</b>	0,1136232	0,1117515	0,1130011	0,1596346	0,158213	0,1633484	0,1432814	0,1388907	0,155408	0,036852	0,0337583	0										
<b>1399_3</b>	0,137557	0,1416656	0,1334739	0,1343416	0,1219962	0,1296693	0,0911027	0,0822675	0,0936307	0,1574534	0,1647803	0,1600926	0									
<b>1399_4</b>	0,138667	0,140496	0,1325007	0,1334086	0,1245339	0,1318639	0,1009764	0,0918663	0,1043281	0,1566683	0,1636351	0,1608326	0,024857	0								
<b>1399_5</b>	0,1416956	0,1435734	0,1358897	0,1355402	0,1249866	0,1320337	0,0948236	0,0867041	0,0978364	0,158051	0,1642269	0,1595664	0,0240115	0,0171827	0							
<b>1557_4</b>	0,093695	0,0926977	0,087785	0,1022254	0,1107442	0,1142296	0,1257019	0,1151032	0,1349405	0,1091652	0,1177939	0,1197368	0,1048296	0,1008789	0,1043672	0						
<b>1557_5</b>	0,0928903	0,0929388	0,0877178	0,1030872	0,107488	0,1113796	0,1132172	0,1027207	0,1220564	0,1091592	0,1176968	0,1169002	0,0993526	0,0977563	0,0992418	0,0196421	0					
<b>1557_6</b>	0,0990402	0,0995894	0,0932857	0,1054142	0,1111151	0,1143538	0,1185334	0,1078762	0,1265017	0,1163509	0,1262493	0,1259265	0,0981441	0,0955452	0,0981372	0,0203954	0,0176297	0				
<b>1660</b>	0,0795494	0,0766135	0,0762734	0,0995425	0,1059698	0,110514	0,1298698	0,1199646	0,1415803	0,0864964	0,0877314	0,0971805	0,1164417	0,1122782	0,114961	0,0883887	0,0901537	0,0966261	0			
<b>1660_2</b>	0,0864065	0,085239	0,0814908	0,1012451	0,1018498	0,1056252	0,1080289	0,0991009	0,1188374	0,0916234	0,0963749	0,0963423	0,0992627	0,0966755	0,0984721	0,085281	0,0843617	0,0903274	0,0361788	0		
<b>1660_3</b>	0,0806082	0,079675	0,0757551	0,0961601	0,0962279	0,1010118	0,1090163	0,0995284	0,1195881	0,0938963	0,0985093	0,1010723	0,0973278	0,0947629	0,0971761	0,0843747	0,0834934	0,0893238	0,0330124	0,014379	0	

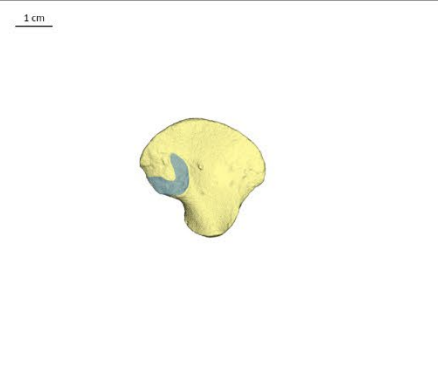
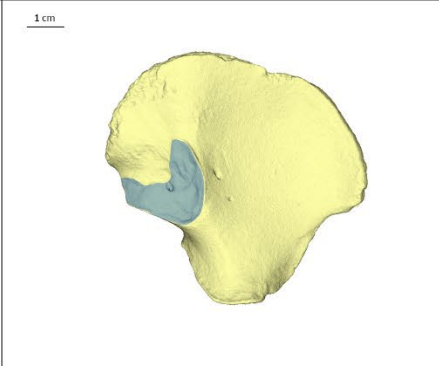
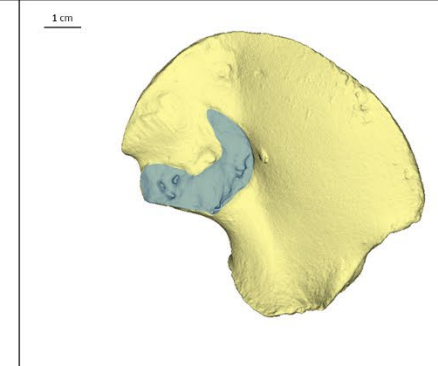
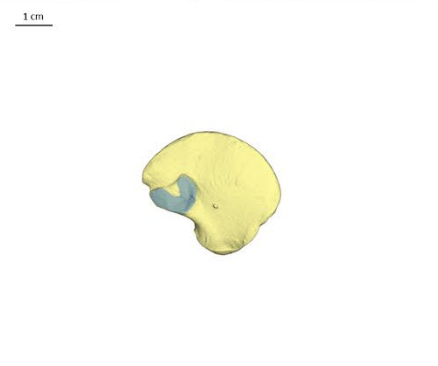
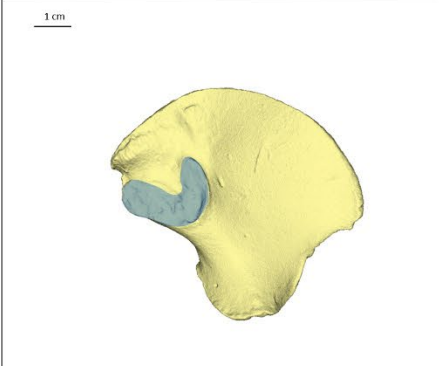
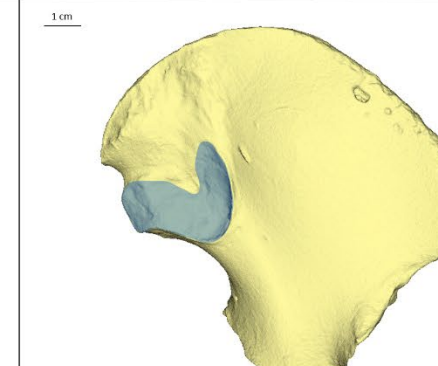
	< 1 year	1.0-3.9 years	4.0-6.9 years
Males			
Females			

Figure A – 3D models of the reference sample ilia, by sex and by age group, with the AS highlighted in blue (scale of 1 cm). There is an increase in the size of the AS according to age. When the AS are compared between the sexes within the same age group, no differences are observed for individuals under 1 year. In the 1.0-3.9 years age range, the AS is slightly larger for females and in the 4.0-6.9 years age group this tendency is reversed.

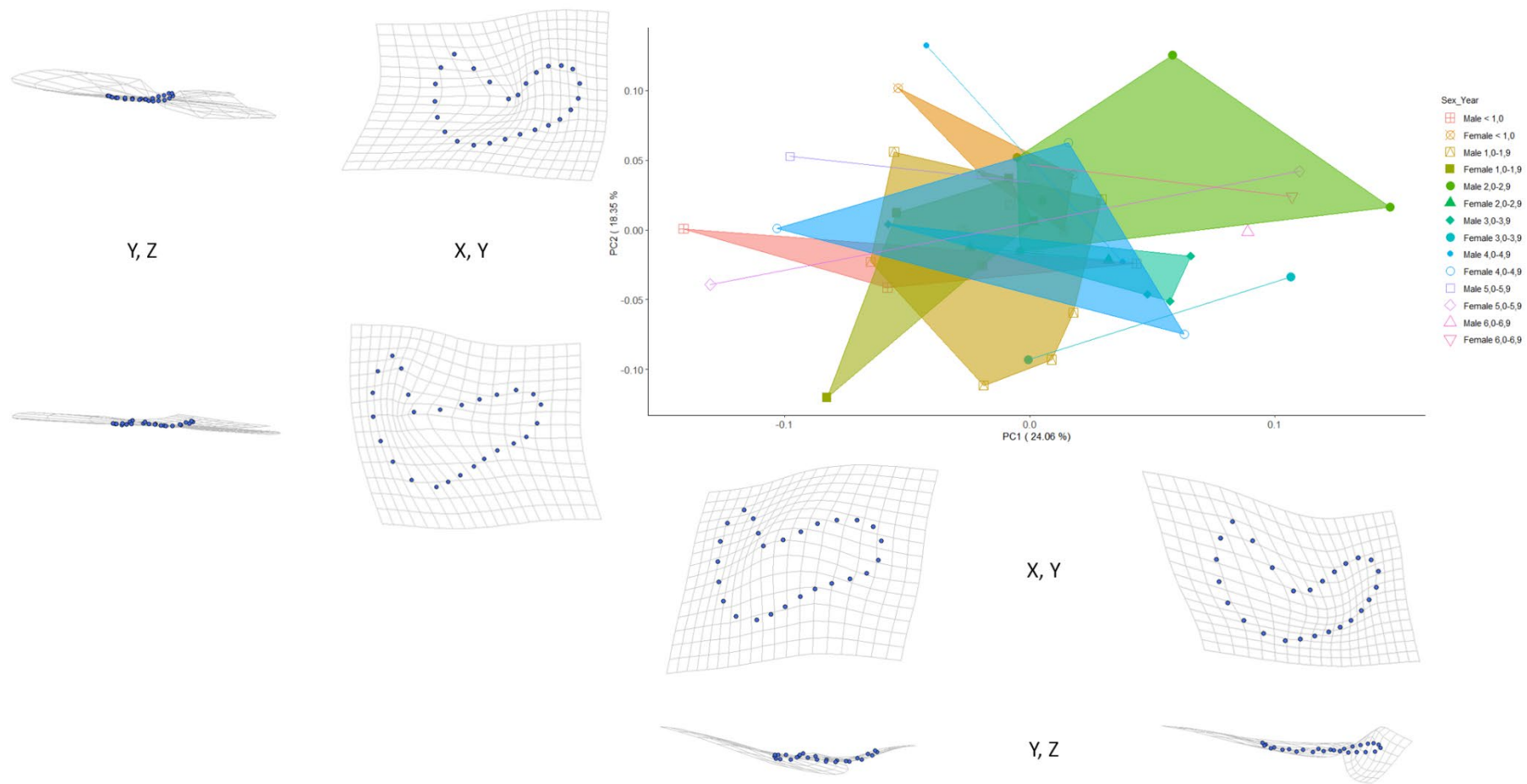


Figure B – PCA of the AS shape of the LLSC sample, with individuals grouped by sex and by age categories of 1 year. The data shows a substantial overlap between the groups. The deformation grids on the bottom represent the AS shape of the specimens closest to the minimum (on the left) and maximum (on the right) PC1 scores on the X and Y axes and the Y and Z axes. The grids on the left represent the AS shape of the specimens closest to the minimum (at the bottom) and maximum (at the top) PC2 scores on the X and Y axes and the Y and Z axes. Note that the visual differences in the deformation grids are magnified two times. Also note that some groups do not present convex hulls. This is because the number of specimens is lower than three, thus not providing morphospace area to classify archaeological specimens.

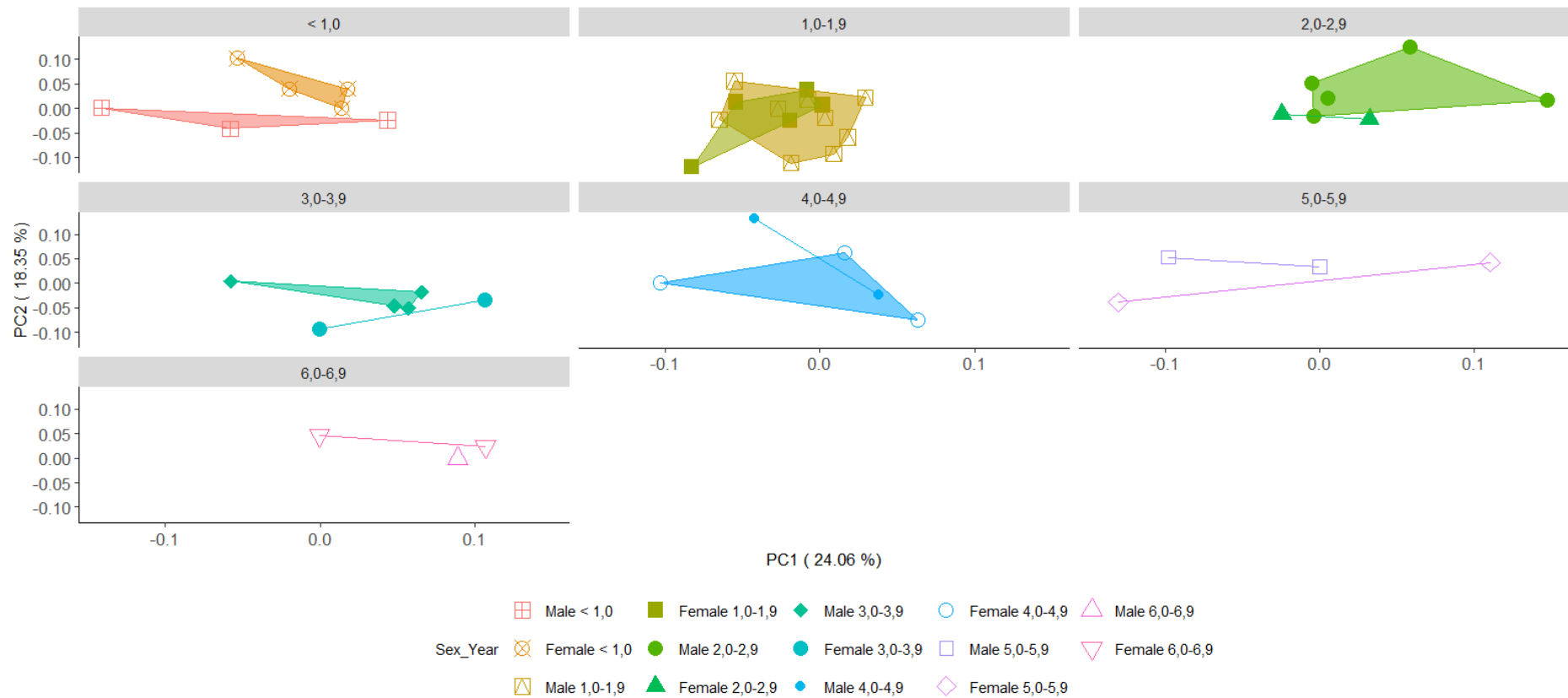


Figure C – PCA of the AS shape of the LLSC sample, with individuals grouped by sex and by age categories of 1 year separately. Morphology differences between sexes are observed in some age categories, but the groups are small and interpretations regarding these results should be cautious. This plot is comparable to Figure B (but faceted/separated by age categories) and so the deformation grids are the same and can be consulted in Figure B. Also note that some groups do not present convex hulls. This is because the number of specimens is lower than three, thus not providing morphospace area to classify archaeological specimens

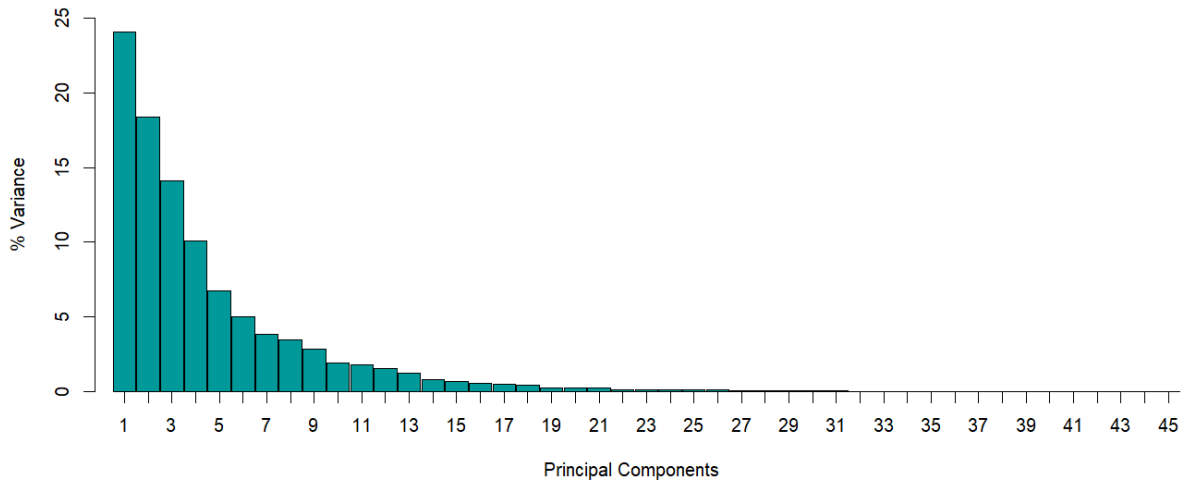


Figure D – Percentage of variance explained by the Principal Components obtained from the PCA performed to the overall reference sample.

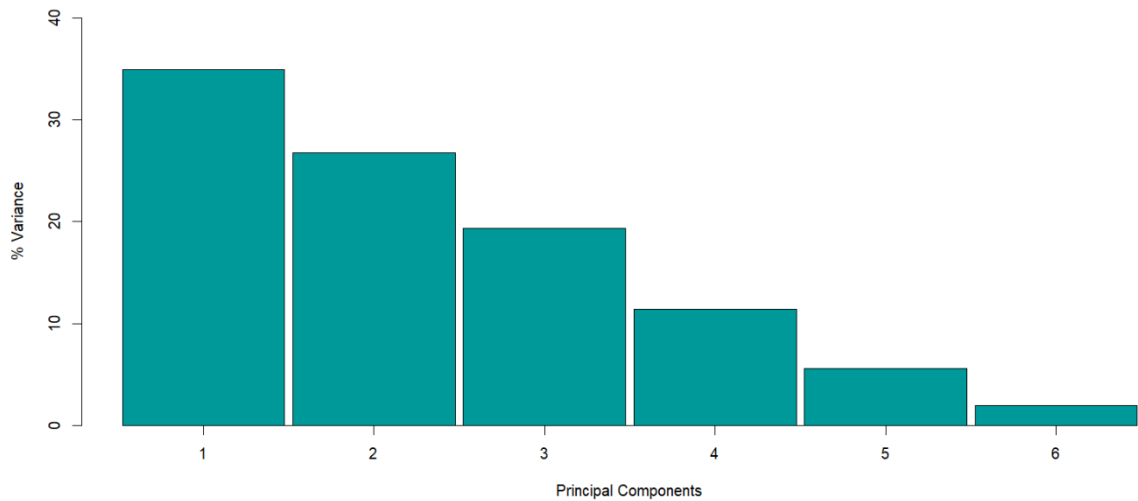


Figure E – Percentage of variance explained by the Principal Components obtained from the PCA performed on individuals under 1 year from the reference sample.

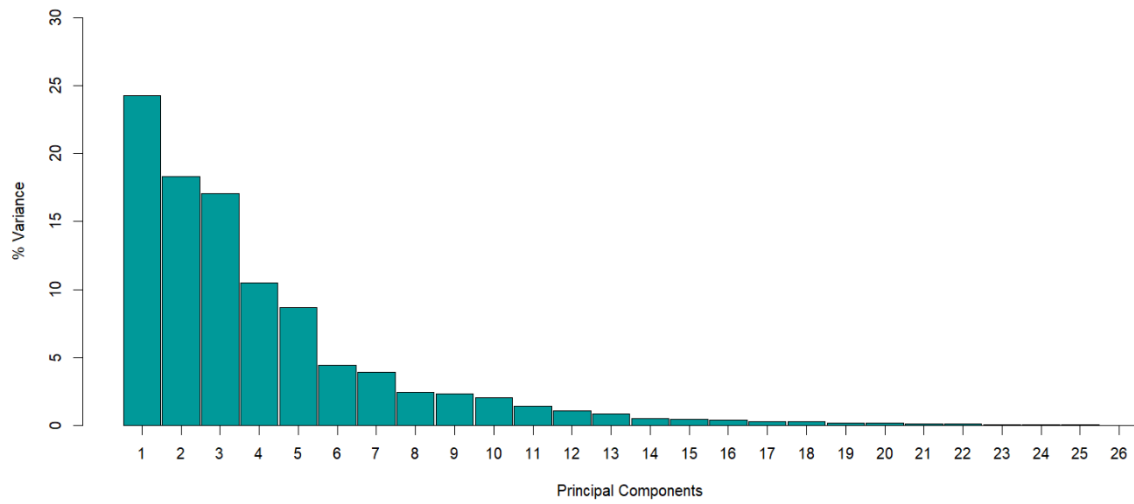


Figure F – Percentage of variance explained by the Principal Components obtained from the PCA performed on individuals in the 1.0-3.9 years age range from the reference sample.

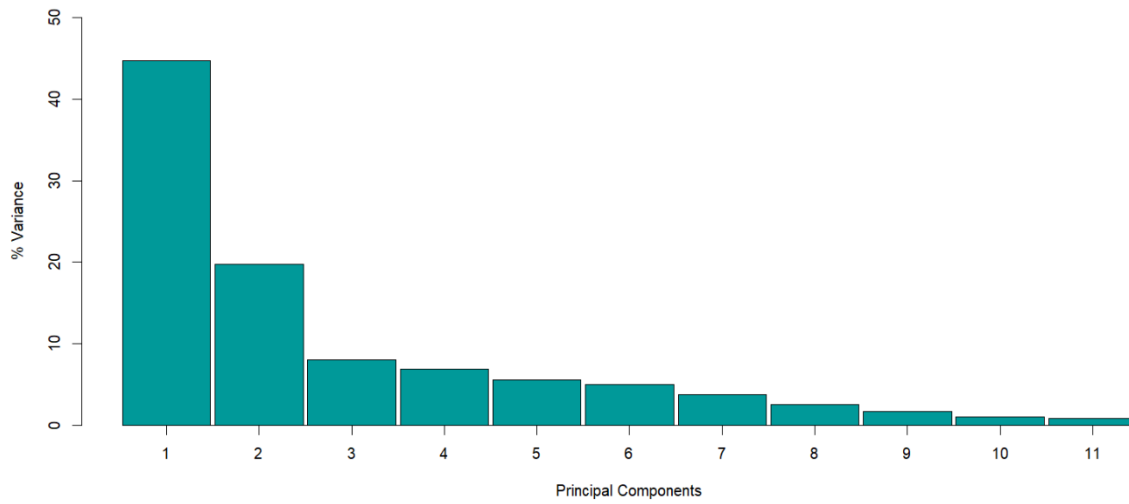


Figure G – Percentage of variance explained by the Principal Components obtained from the PCA performed on individuals in the 4.0-6.9 years age range from the reference sample.