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Hard tissue thin-sectioning techniques in vertebrate paleohistology: Review, synthesis and improvements

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Abstract

1. Paleohistology provides a significant amount of information on the physiology and evolution of ancient organisms through the study of their fossilized tissues' microstructure. Such discipline has been increasingly applied in vertebrate research over the years, however comprehensive and up-to-date methodological resources are lacking. In the specific, technical expedients associated with mammal research are little mentioned and some standards and protocols are still needed for the homogenization of paleohistological research.
2. This work includes a review and summary of current paleohistological techniques, and new proposals for the improvement of the most widely used protocols for the preparation and sectioning of hard tissues. We further expand the focus beyond the traditional techniques including expedients for dentognathic remains, fossil and extant.
3. The guide provided herein covers the techniques necessary to produce a paleohistological thin section, providing step-by-step instructions along with insights for technicians and both beginners and experienced scientists.
4. Although paleohistology is widely applied, its technical aspect is often overlooked. With this work, we considerably improve the learning process, help standardize paleohistological techniques and make them accessible to researchers, increasing scientists' awareness of thin-sectioning techniques and encouraging the exploration of new methodologies in this field.

KEYWORDS
incremental structures, methodological review, paleohistology, paleontological techniques, skeletal histology

1 | INTRODUCTION

Paleohistology is the study of fossilized tissues whose features provide insights into the growth, development and physiology of ancient organisms (Chinsamy, 2023; Chinsamy et al., 2014; Cubo et al., 2012; De Ricqlès et al., 2004; Downs & Donoghue, 2009; Houssaye et al., 2008; Nacarino-Meneses et al., 2017; Orlandi-Oliveras et al., 2019; Sander et al., 2011; Sander & Andrássy, 2006; Scheyer & Sander, 2004; Walker et al., 2020). Skeletal tissues are the most common fossil tissues in vertebrate paleohistology since their

mineralization (Francillon-Vieillot et al., 1990) improves the chances of successful fossilization. They include bone, calcified cartilage and dental tissues. Vertebrate paleohistology has become increasingly popular, especially in dinosaur-related research (Bailleul et al., 2019; Cerda et al., 2021; Sander et al., 2011; Woodward et al., 2015). However, paleohistology applied to other vertebrates such as fish (Meunier et al., 2010; Mondéjar-Fernández et al., 2021; Mondéjar-Fernández & Meunier, 2021), squamates (De Ricqlès et al., 2004; Houssaye et al., 2008), amphibians (McHugh, 2014, 2015) and mammals (Funston et al., 2022; Jannello & Chinsamy, 2023; Kolb et al., 2015; Orlandi-Oliveras et al., 2019) has also seen increasing traction. The reason behind this success is the amount of information that it is possible to obtain from fossilized hard tissues. Bone and teeth microstructures hold extensive phylogenetic and biomechanical information and have been used for systematics and taxonomical purposes (Alba et al., 2010; Canoville & Laurin, 2010; Chinsamy et al., 2014; Scheyer & Sander, 2004). They are called 'recording structures' as they react to physiological changes in the organism by changing their structure as they grow (Klevezal, 1995). Due to this property, an increasingly common application of paleohistology is to provide fine chronological records used to interpret biogeochemical intra-tissue variations in terms of paleobiology, including aspects such as diet, physiology, growth dynamics and ecological adaptations (Chinsamy, 2023; Funston et al., 2022; Hogg, 2018). For these reasons, paleohistology of mammalian hard tissues has been successfully used in many different studies, such as the nature and growth patterns of ruminant cranial appendages (Azanza et al., 2003, 2011, 2022; Azanza & Ginsburg, 1997; Bubenik, 1983, 1990; Rössner et al., 2021), biomechanical and physiological adaptations to particular lifestyles (Amson et al., 2014; Hayashi et al., 2013; Walker et al., 2020), and life history events and traits (Funston et al., 2022; Jannello & Chinsamy, 2023; Köhler & Moyà-Solà, 2009).

To extract such information, it is necessary to employ a method that helps reveal the tissue's internal microstructure with the appropriate resolution (Figure 1). Although new technologies—such as high-resolution synchrotron-based X-ray microtomography (Knoll et al., 2018; Nava et al., 2022; Sanchez et al., 2012)—have recently emerged in the field, the primary and most suitable methodology in paleohistology remains the production of petrographic thin sections. Thin-sectioning methods applied to fossil organisms found their roots in the early 19th century with works on fossilized trees by Nicol (1831) and Witham (1831). The technique emerged as an application of the petrological thin section method carried out about 10 to 15 years earlier by George Sanderson, a lapidary, and the scientists David Brewster and William Nicol (Falcon-Lang & Digrius, 2014). The technique consisted of transversally slicing a fossil, polishing one of the surfaces to glue it to a glass plate and then grinding the opposite side to a thickness that allowed for the microstructure to be seen (see the below section for further information about the thin-sectioning method). Since then, this method has increasingly gained momentum, nonetheless, as dinosaurs-focused research increased hugely, it evolved consequently. Dinosaur remains are usually larger and more mineralized by a longer taphonomical history than many mammal ones and, in the former, dental histology is not as popular as bone histology (Bailleul et al., 2019; García & Zurriaguz, 2016; Hwang, 2005). This causes a lack of methodological resources for techniques largely applied in paleohistology of mammals, such as dental thin-sectioning or smaller and poorly fossilized—even extant—bone processing, in spite of its major application. In this regard, as most works only mention the major steps of the sectioning protocol in the methods section (Jordana & Köhler, 2011; Kolb et al., 2015; Skinner & Byra, 2019), comprehensive and up-to-date methodological resources are still needed. The result is a difficult and time-consuming learning process for early career scientists who need to implement paleohistological analysis in their research. Since the techniques for producing thin sections are closely tied to the final results, a lack of awareness directly limits paleohistological research. In fact, the process gives better outcomes if cooperatively carried out by both researcher(s) and technician(s), especially when the former possess a strong knowledge of the methodology and can adapt or modify it as needed.

The work of Padian and Lamm (2013) has greatly contributed to filling the methodological gap in the paleohistology of vertebrates, so it played a fundamental role in the development of this manuscript. This book is a must-have for every paleohistology researcher and as such, the structure we adopt is largely based on it. The goal of this review is to provide a general guide for preparing thin sections, comprehensive of various protocols we adopted in our laboratory, with the aim of not just describing the recommended steps to follow but also updating current methodologies and explaining the reason behind the application of the most popular (and also critical) methods.

2 | MATERIALS AND METHODS

Every laboratory has its own methodological flowchart with tips and tricks given by years of experience, specifically with certain equipment. In fact, the available tools are one of the numerous variables to consider when producing a thin section. Indeed, including all the possible variables is not practical, however, it is still possible to include the most important ones and draw the general steps to follow. As reported by Lamm (2013), in thin-sectioning it is important to look at the process a few steps ahead, since considering the steps independent from each other could lead to poor outcomes. Table 1 shows a list of the necessary equipment and, to better follow the proper process, Figure 2 depicts the flowchart we use for the elaboration of thin sections. The flow diagram is only a guide and the expertise of an experienced technician is extremely valuable (if not necessary).

We differentiate the whole process into three macro-stages: Preliminary preparation, intermediate block elaboration and procedures for mounted block. To avoid misleading terminology and to clarify the text, especially for the least experienced, the terms used in this work for referring to the specimen and/or its derivatives are depicted in Figure 3. In particular, the word specimen refers to the

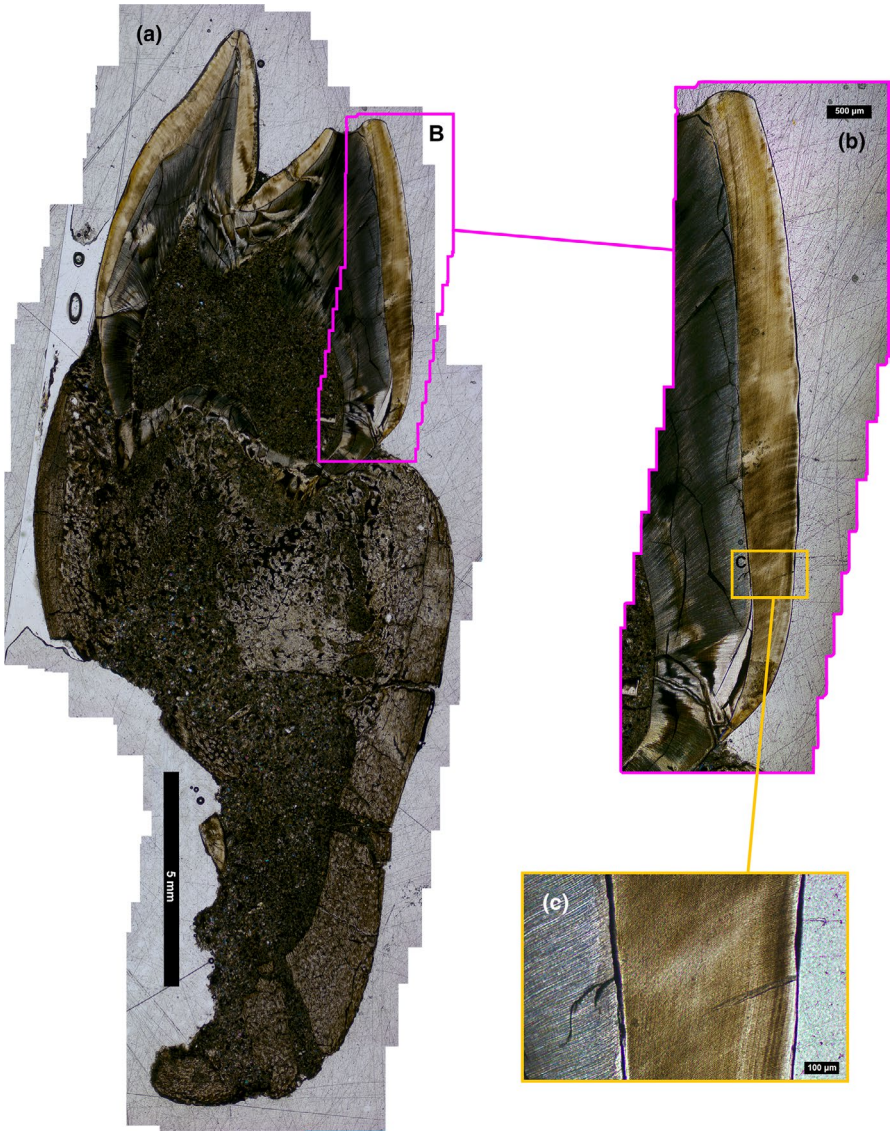

FIGURE 1 Thin section of a left lower second molar of the Miocene deer *Procervulus ginsburgi* (MPZ 2024/309), transversal section of metaconid (left) and protoconid (right) at 50x magnified thin section (a). Detail of the labial side of the protoconid (b). Portion of dentine, on the left, and enamel, on the right, of the labial side of the protoconid (c). Note how the incremental structures are seen at different resolutions.

whole object of study, while the sample is referred to as the part extracted from the specimen (see Section 2.1.2). Bear in mind that thin-sectioning procedures involve the manipulation of potentially toxic chemicals as well as fast-moving objects such as blades or grinders, which can cause serious injuries. Providing safety guidelines when working in a thin section laboratory falls outside the scope of this work, however, it is important to stress that basic safety measures must be adopted. Reading the owner's manual for the machinery as well as the safety data sheet of the chemicals employed is mandatory, moreover, we recommend the safety suggestions made by Lamm (2013) which are valid for all laboratories.

2.1 | Preliminary preparation

This is the most complex macro-stage of the protocol and is divided into four stages: Planning and documentation, non-fossil tissues treatment (Figure 4a), stabilization and extraction (Figure 4b) and embedding (Figure 4c–e). It requires thinking about the whole process, documenting adequately the specimens and preparing the material for any process to come. Since conducting a study based on extant specimens is crucial for direct comparison, we included extant specimen preparation in our research (Figure 4a). When preparing a specimen, especially if fossil or sub-fossil, it may be necessary to stabilize it before extraction (for example by reinforcing it with an adhesive), whereas when the material comes from an extant organism, the process involves various preparations that take place after the extraction and, often, before the embedding.

2.1.1 | Planning and documentation

One of the main constraints for paleohistological studies involving thin-sectioning is the destructive nature of these techniques, which limits the availability of specimens. Moreover, the production of different material resulting from the same specimen is quite common. Effective planning and documentation are then essential to ensure that the specimen will be appropriately processed for downstream

TABLE 1 Material used in the thin-sectioning process, colours represent the three macro-stages: Preliminary preparation (Green), intermediate block elaboration (Yellow) and procedures for mounted block (Red).

Preliminary preparation	Intermediate block elaboration	Procedures for mounted block
Calliper	Manual high-speed saw	Automatic feed trim saw
Scale bar	Diamond-edge blade	Trim saw diamond-edge blade
Digital camera	Automatic feed trim saw	Epoxy resin (Epo-Tek 301)
Moulding and casting material	Trim saw diamond-edge blade	Beckers for mixing
Alcohol (70%, 96%, 100%)	Cutting liquid	Wooden sticks
Containers for dehydratation	Rotary tool (dremel)	Thin section machine (PetroThin)
Cyanoacrylate glue (Paleobond)	Epoxy resin (Epo-Tek 301)	Grinding discs (p1000/p1500/p2500)
Epoxy resin (Epo-tek 301/Epo 150)	Beckers for mixing	Glass coverslip
Beckers for mixing	Wooden sticks	Loctite 350
Wooden sticks	Grinding/polishing machine	UV lamp
Containers for embedding	Grinding discs (p600/p1000/p1500)	
Vacuum oven	Glass slide	
Manual high-speed saw		
Diamond-edge blade		
Rotary tool (dremel)		
Ruler and pencil		
Loctite		

analyses as well as to obtain the frequently compulsory permits. This also involves the selection of the best specimens for the upcoming analysis while considering the potential consequences for the remnant of the specimens once the process is finished, which is critical since it implicates irreversible procedures like embedding. A critical objective is to anticipate issues that may occur, adopting a strategy based on the specimen under investigation. An in-depth understanding helps to develop an effective plan accounting for the chemical and physical properties of the specimen under investigation (such as dimensions, firmness, and presence of soluble material). Knowledge of the laboratory's available resources is also crucial, as these are limiting factors; typically including the size of the glass slides, type of cutting liquid, size of the embedding containers, block holders, slide holders and so forth. It might sound trivial nonetheless, when dealing with a large number of variables, even the smallest detail can affect the whole process at more than one level.

Once the plan has been conceived, we must document the initial status of each specimen—that is recording and storing as much information as possible about the specimen before it is processed. Since the methods of acquiring information change over time, there are today many ways of recording data. The most classical and still widely used way to document are digital photographs (Botha & Chinsamy, 2005; Houssaye et al., 2008; Witzel et al., 2018), measurements (Calderón et al., 2021; Hayashi et al., 2023) and moulds (Cerda et al., 2020; Houssaye et al., 2008), but some other methodologies can be suitable depending on the nature of the specimen and the data to be stored, such as 3D imaging (including photogrammetry and superficial scanning of the individuals) or X-ray tomography (Witzel et al., 2018). With regards to the latter, it is worth mentioning that a better understanding of the internal structure (fracturing, filling and bone microstructure) often assists the successive stabilization stage and might also be useful in finding the best part to be sectioned (or homologous part in case our specimen is a fragmented skeletal part).

2.1.2 | Stabilization and extraction

It is vital to remember that the safety of the specimen is paramount. In this regard, this step is not only beneficial for facilitating the handling of the fossil but also for preserving its remaining part(s). Stabilization is required in the case of fragile specimens or partially mineralized subfossils, yet, even extant specimens might need a certain degree of preparation prior to extraction. In the case of specimens with void spaces (such as tusks, horns or the marrow cavity of a bone), they might need to be stabilized by filling the empty spaces with glue or resin (Lamm, 2013) that otherwise can collapse during extraction. For medium to large and especially well-preserved specimens, after cleaning the surface with alcohol, epoxy putty can be used to reinforce the area before sectioning (Pereyra, 2021; Cerda et al., 2020). It has the advantage of being removable by applying heat and has also been used as a cheaper alternative for embedding

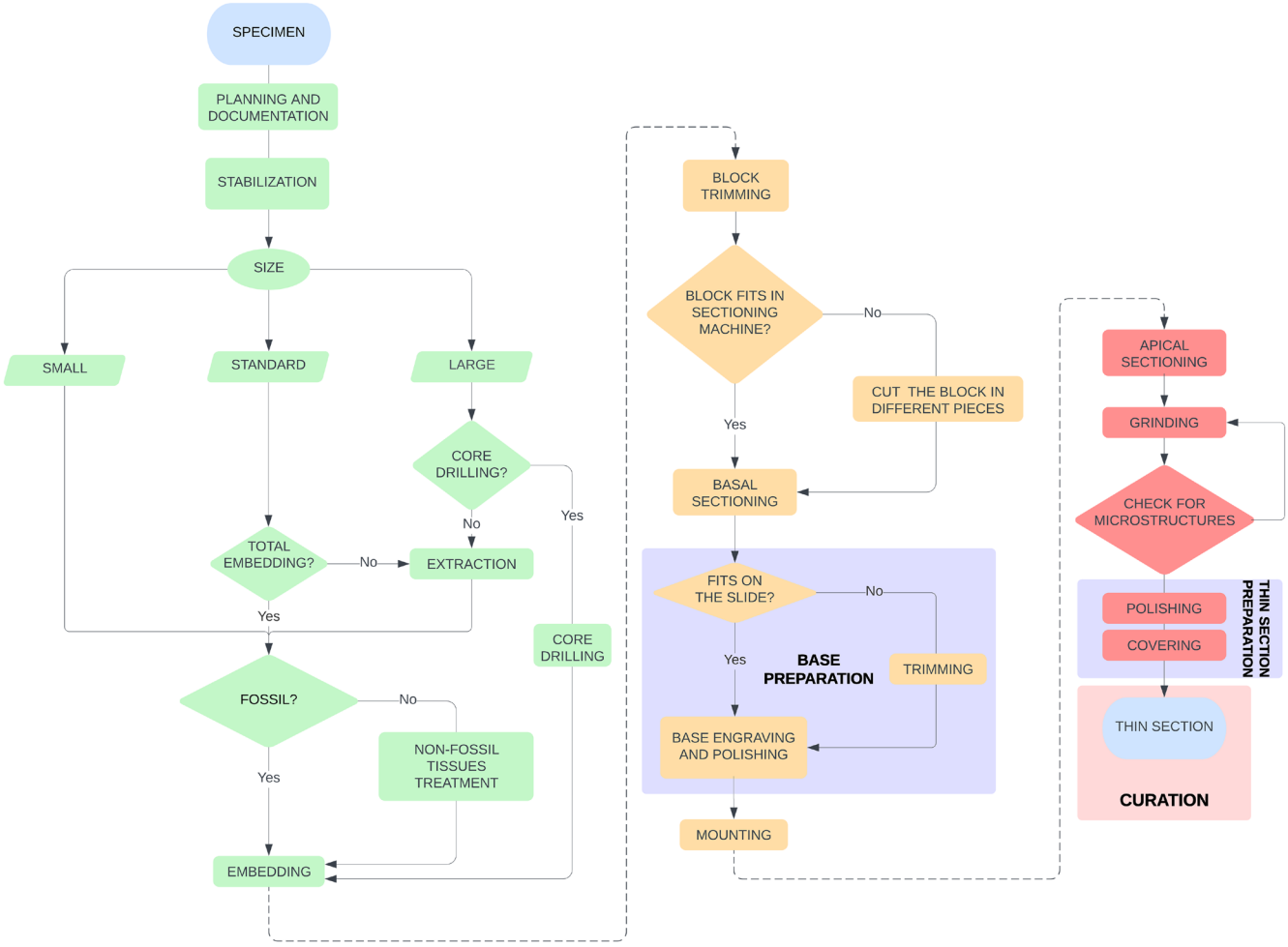

FIGURE 2 Flowchart of the thin-sectioning process. The colours represent the three macro-stages: Preliminary preparation (Green), intermediate block elaboration (Yellow), and procedures for mounted block (Red). The purple squares refer to the name used to group a series of different steps as appears in the text.

(Pereyra, 2021; see Section 2.1.4). For especially damaged fossils, an adhesive like cyanoacrylate (Lamm, 2013; Maat et al., 2001; Schwartz et al., 2002, 2005) can be used to keep them in place, and epoxy resin (Epo-Tek 301) to reinforce the areas where the saw blade will section during extraction. However, caution is needed, as these substances might interfere with the proper visualization of the histological features or with the following procedural stages. In general, minimizing the use of substances other than the embedding medium (see Section 2.1.4) is preferred. Nevertheless, when the use of other substances is necessary (as mentioned earlier), avoid applying them near the area where the histological section is planned. If a fossil specimen has been previously stabilized (e.g. with Paraloid), an option is to remove the chemical substance from the area of interest using its specific solvent, if available. However, this may not be as effective for porous specimens, in which case selecting another specimen is advisable.

When required, subsequent to the initial stabilization, the isolation of the anatomical part to be the object of study is achieved through extraction. Extraction is an optional step and it is significantly associated with the size of the specimen as it influences the possibility of its complete embedding (see Section 2.1.4) and directly poses limitations to the process. Accordingly, we classify the specimen based on the largest dimension into 3 indicative categories, similar to Lamm (2013): Small <2 cm; standard from 2 to 6 cm; and large >6 cm.

When dealing with a small specimen, such as an isolated dentognathic remain, a complete embedding (Kierdorf et al., 2019) to prevent the specimen from shattering is recommended. A standard-sized specimen can either be fully embedded in resin or a part of it can be extracted and then embedded. On the contrary, for large-sized specimens, the extraction of a sample prior to embedding is always required. It is vital to stress that the size categories are just a general guide, as the shape of the specimen also determines the process ahead. In the case of long specimens (usually complete long bones or diaphysis), the extraction is performed by sawing the specimen and extracting a cylindrical section (Figure 3). The base and top of this cylindrical section are frequently damaged by the saw, hence it is recommendable to extract a long enough cylindrical section to preserve the central part, from which the thin section is produced. Usually, this step is made using a manual high-speed

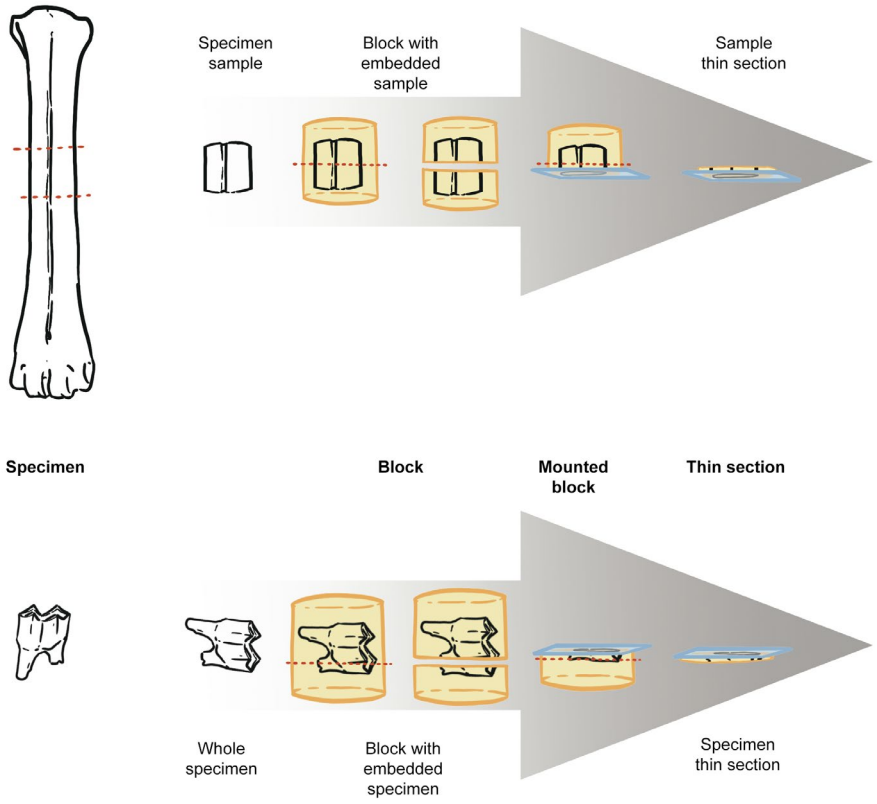

FIGURE 3 Terminology used for the different phases undertaken by the specimen and/or the specimen sample. The dotted lines correspond to the section planes. The III–IV metapodial (above) and the inferior molar (below), respectively represent a large-sized and a small-sized specimen. Between the two specimens, in bold, the terminology used is based on the irreversible changes occurring during the process: "Block" refers to an embedded specimen/sample; "Mounted block" refers to a sectioned block glued to a slide; "Thin section" refers to a mounted block that underwent an apical section or severe grinding.

saw (Figure 4b) with a diamond-edge blade and it is useful to reinforce the sectioned edges with epoxy resin to prevent shattering. In the case of extant teeth embedded in the bone, we recommend the extraction of each individual tooth to easily handle each of the specimens. Consider that sawing implies a loss of material due to the thickness of the blade (which can range from approximately 0.15 mm to over 1 mm) and its action on the specimen, called kerf loss. Lamm (2013) suggests the breakout method, proposing that, if the fossil to be sectioned has an existing glue line or crack, it can be broken out at that point. Although this method can be useful for limiting kerf loss and facilitating further specimen restoration, it needs to be contextualized to the study of particularly well-preserved large bones (Sander, 2000) since small or fragile specimens might shatter during the breakout process. Finally, the largest specimens, at least 30 cm long according to Stein and Sander (2009), can be object of a special extraction: the histological coring method consists of drilling a small core out of the specimen (Sander, 2000; Stein & Sander, 2009) and has been mostly applied to dinosaur remains however, virtually, any vertebrate hard tissue can be sampled this way (Kolb et al., 2015; Stein & Sander, 2009). This technique allows for the preservation of most of the specimen and it is therefore useful when dealing with limited material. Nonetheless, compared with the complete sectioning of the specimen, the obtained data is lower as the extracted core is only a narrow segment of the specimen. In case of long bones, the information might not be recorded uniformly along the cortex (Calderón, 2023; Calderón et al., 2021) and when core drilling subfossil or poorly preserved specimen the trabecular bone can be damaged in the process (Stein & Sander, 2009).

2.1.3 | Non-fossil tissues treatment

Processes for non-fossilized tissues show considerable variability. Degrease and dehydration (Figure 4a) are the most diffused techniques and consist of the removal of fat and successive dehydration in ethanol to remove water (e.g. Bromage et al., 2016; Legendre & Botha-Brink, 2018; Montoya-Sanhueza et al., 2020; Nacarino-Meneses et al., 2017) since they are contained in bones and could later cause issues during the embedding. Today, many authors use acetone (Cerrito et al., 2021; De Margerie et al., 2004; Montoya-Sanhueza et al., 2020) or soap (Legendre & Botha-Brink, 2018) to remove fat or they add enzymes to the detergent (Zedda et al., 2015, 2019), while some decades ago the specimens were simply cleaned with running water (Morris, 1970).

Decalcification is a technique (e.g. Fogl & Mosby, 1978; Klevezal & Mina, 1990; Klevezal, 2002; An & Martin, 2003; Sinsch, 2015) whose primary objective is to soften hard tissues when paraffin is used for embedding (see Section 2.1.3) since the latter is significantly softer compared to bone (An & Martin, 2003). However, embedding with substances, such as resins, permits us to avoid decalcification and, consequently, a potential loss of information due to over-decalcification (An & Martin, 2003).

The specimen may require prior fixation to prevent tissue decomposition, in this case, formalin is the most used compound (An & Martin, 2003; Luque, Learmonth, et al., 2009; Luque, Pierce, et al., 2009; Meister, 1956; Schweitzer et al., 2007).

Finally, there are other preparations that, in contrast to what is applied with fossil material, are applied almost at the end of the

FIGURE 4 'Preliminary preparation' macro-stage. Teeth from extant specimens are dehydrated in successive alcohol solutions of 70%, 96%, and 100% (a). Extraction of a sample from a fossil specimen with a high-speed saw (b); although it is out of the scope of this work, it is worth mentioning that, when operating certain machines, protective gloves must not be used due to the risk of them getting hooked. Yellowing due to heat during resin curation (c). Resin cured at room temperature (c1). Resin cured in a refrigerator (c2). Specimens requiring a precise section are mounted on cured resin bases with the section plane marked on them (d). Specimens are placed in moulds in which the resin is poured until it covers them (e).

thin-sectioning process (such as blood-derived pigment removal, Nakai & Boyde, 2024; or cross-section staining, see Meister, 1956; Klevezal & Mina, 1990; Sinsch, 2015) while others, like fluorescent labeling, are applied at the beginning when the animal is still alive (Calderón et al., 2021; Castanet et al., 2004; De Margerie et al., 2002, 2004; Emken et al., 2021; Kierdorf et al., 2013; Montoya-Sanhueza et al., 2021; Starck & Chinsamy, 2002). Since the selected procedures depend on the desired outcomes, various methods can be employed and, most importantly, in different order. Therefore, it is highly recommended to conduct comprehensive research on specimen preparation associated with the specific aims of the study.

2.1.4 | Embedding

Embedding is the final part of the macro-stage and it results in our specimen (or the sample previously extracted) being incorporated into what will sustain it during the sectioning. In the case of extant specimens some authors do not embed (Brits et al., 2013; Maat et al., 2001; Mayya et al., 2016), however we consider embedding necessary to maintain bone/tooth structural integrity during later stages. A broad range of embedding substances are used, most of which, once cured, cannot be removed without damaging the specimen. Although some authors use paraffin wax (exclusively for extant specimens/samples, see Barker et al., 2003; Luque, Pierce, et al., 2009; Sinsch, 2015; Slooten, 1991), polymethylmethacrylate (PMMA) or methylmethacrylate (MMA; Bromage et al., 2009; Cerrito et al., 2021; Goldman et al., 2006; Ries, 2003), the most common substances are polyester (Cubo et al., 2012; Heck et al., 2020; Schwartz et al., 2002, 2005) and epoxy resins (Calderón et al., 2019; Jordana & Köhler, 2011; Nacarino-Meneses et al., 2017; Schweitzer et al., 2007). As mentioned earlier, a cheaper alternative embedding substance for well-preserved specimens is epoxy putty (Pereyra, 2021). However, it is important to note that, although it is partially reversible, the epoxy putty has a lower capacity to penetrate cavities and tends to produce a paste during polishing or grinding (Cerda et al., 2020), which must be washed away after each polishing or grinding session (see Sections 2.2.2, 2.3.1, 2.3.2). For these reasons, when affordable, we recommend using resins instead. In the specific, we recommend embedding with epoxy-type resins (e.g. Araldite, Epo-tek) and although the costs are higher with respect to other substances, some cheaper alternatives give excellent results (e.g. Epo-150).

Before the resin is prepared, it is important to ensure that the specimen/sample fits into the container used for embedding, especially considering that it is recommendable to include a cast resin

base of about 0.5 cm. This base is previously prepared using the same epoxy and the same container used for the following embedding, and allows for complete inclusion of the specimen/sample. When choosing the container, it is important to consider the laboratory available infrastructures and the anatomical cut to perform, since different equipment have different requirements, such as block size and/or orientation. Before proceeding with the embedding, it is important to treat the container to prevent the resin from sticking to it. The substances used for this purpose are called mould release agents and they are mainly sold as sprays or spreadable wax. However, Vaseline has always proved an excellent and cheaper alternative. Note that the coating layer should be as thin as possible since a high amount of Vaseline could cause rugged sides. It is important to mention that a practical alternative is using 'self-removable' moulds, such as silicone ice trays. These moulds naturally prevent resin from sticking without any treatment. However, applying a release agent can further extend their lifespan.

When embedding, it is important to consider that each resin has different characteristics such as viscosity, colour and refraction. In porous bones or materials characterized by numerous cavities, the resin viscosity determines the extent to which the empty spaces can be adequately filled, consequently influencing the incidence of trapped air bubbles. A resin with a low viscosity easily allows the tiniest cavity to be filled and also facilitates the expulsion of jammed air when subjected to a vacuum. Epoxy resins are normally transparent to semi-transparent, but it should be emphasized that they can turn yellow due to the heat generated by exothermic reactions, which can make the specimen/sample hard to observe. The yellowing may occur when pouring large quantities of resin or banally at high room temperatures. In such cases, keeping the resin in the refrigerator allows the block to cure correctly and stay transparent (Figure 4c). However, over the long term, yellowing is not avoidable. The last parameter to be considered is refraction, and it can affect our ability to effectively locate the specimen/sample inside the block, especially for those sections that require high precision to be carried out. It is generally recommended to draw the section plane on the resin surface before sectioning, especially when the material is opaque (for this purpose, 3D imaging and X-ray tomography have proved valuable tools to determine the section plane). However, when operating with small specimens and extremely precise sections in which the apparent displacement has a bigger impact on the final result, we propose and recommend an approach to significantly limit this effect. It consists of drawing a line, representing the section plan, directly on the cast resin base (Figure 4d). This line, drawn with a pencil (depending on the resin being used), allows for an exceptionally precise cut, despite the refractive index that tends to create an apparent displacement of the specimen/sample within the block. The specimen or sample to be sectioned will be directly fixed on the base, with the section plane right below. In order to hold it, we recommend the use of any *instant adhesive* (e.g. Loctite® Super Glue), but instead avoid it when applied to highly porous tissues as it might penetrate. In the case of irregularly shaped specimens/sample, resin edges can be fixed on the cast resin base to help with the spatial orientation. These expedients are especially useful when dealing with precise sections, such as dental thin sections.

A last, crucial step after embedding is to remove eventual air bubbles using a vacuum oven (Lamm, 2013; Ray et al., 2005; Sander & Andrássy, 2006). Here, we highly recommend performing this process a couple of times and brushing away the bubbles that will rise to the surface with a small stick after each 10-min vacuum session.

2.2 | Intermediate block elaboration

This stage aims at obtaining a block mounted on a slide that will be later sectioned to obtain a thin section (see Section 2.3). To do so, the block is sectioned along the region of interest and the sectioned surface of one of the two halves, after being polished, is fixed onto a slide serving as the base of our thin section. This stage can be divided into block trimming and basal sectioning (Figure 5a,b), and base preparation and mounting (Figure 5c–e).

2.2.1 | Block trimming and basal sectioning

Block trimming is recommended but not always necessary. It involves removing the excess resin from the block (Figure 5a), leaving a margin of at least 0.5 cm around the specimen/sample; a buffer zone that will ensure protection and mechanical sustain. This step holds great importance because it allows to work with smaller blocks, simplify handling and achieve a finer visibility of the specimen/sample. Note that if the latter still does not fit the saw after trimming the excess resin, it is necessary to cut the block into smaller pieces with a manual high-speed saw and then process every piece by itself (Lamm, 2013).

For sectioning (Figure 5b), an automatic feed trim saw (e.g. Accutom) with diamond edge blades, provides cleaner cuts, limits human error and allows control over different parameters. Besides the blade rotational speed (measured in rotations per minute), it is important to check the feed speed (i.e. the speed at which the blade moves toward the block). When cutting hard materials, a slow feed prevents the disc from advancing 'past the cutting depth'. However, many automatic sawing devices (for example, Buehler Isomet 5000) allow specifying the cutting length and automatically stops when it has been reached. For embedded specimens/samples with a significant amount of resin, and hence a somewhat elastic behaviour, a low feed speed is especially required. Failure to control the feed can result in the blade deviating, getting stuck, or breaking. Some sawing devices (such as the Buehler Isomet 5000), offer an automated option ('Smartcut') that regulates the feeding speed depending on the elasticity of the material. If an automated trim saw is not available, there are other options that can be considered: The Struers Minitom (e.g. Barker et al., 2003) is a good alternative since features a gravity feed system that can also be regulated.

In order to execute a complete cut, the section length cannot be bigger than the distance between the blade's edge and its arbor flange

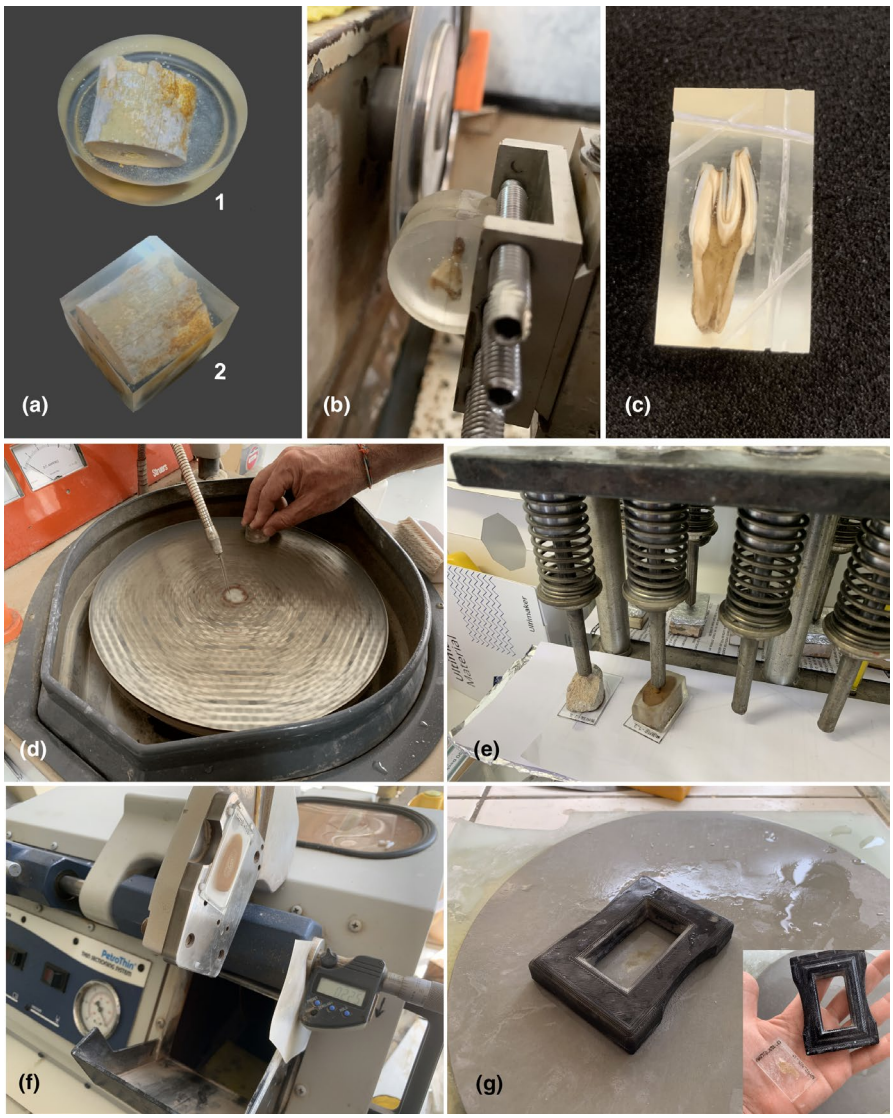

FIGURE 5 'Intermediate block elaboration' and 'Procedures for mounted block' macro-stages. Trimming process (a). A resin block is produced during embedding (a1) and the excess resin is trimmed off (a2). Sectioning of a block with an automated feed trim saw (b). The block's basal surface shows grooves made during the engraving stage (c). Polishing on an automated grinding/polishing machine (d). A press is used to keep in place the mounted blocks (e). The thin section is ground until the optimal thickness is achieved (f). Thin sections are manually polished using a holder and paper-grinding discs (g).

(i.e. the support at the centre of a blade, to which the motor shaft attaches) so both blade and block sizes must be considered. When it is not possible to perform a complete cut, the block can be reversed and the section completed starting from the opposite side. This method, however, may cause a slightly uneven cut since the blade does not go perfectly straight. In such instances, we recommend grinding/polishing the surface to make it even.

The use of fluids (water, oil and others) is essential for a correct cut and performance of the equipment since a dry cut usually generates heat, which can damage the machinery as well as the specimen/sample, for example by altering the minerals of fossils. Selecting the right liquid is important for two reasons: first, the specimen could contain soluble material, and second, some substances may be corrosive. For instance, distilled water is the most common and cheap liquid used for cutting, however, it might partially dissolve the specimen/sample as well as corrode the equipment. Generally, several oil-based liquids are added to water due to their coolant, lubricant, and antioxidant proprieties, however, they are mainly directed toward machine preservation. Among these liquids, ethylene glycol is recommended since it also assures that no water-soluble material of the specimen/sample is lost during the process.

After sectioning the block, it is essential to check whether additional resin needs to be poured on the exposed tissues. This is particularly important for delicate specimens/samples, as they might fracture during the sectioning process. Additionally, it may reveal empty cavities, such as the pulp cavity in teeth. After applying the additional layer, allow it to cure before moving to the next step.

2.2.2 | Base preparation and mounting

Once the block is sectioned, the next step is to set up the base for mounting, via base preparation; a series of three processes: Trimming, engraving and polishing. Firstly, check if the block fits the slide. If not, trim the excess resin or use a larger slide. After that, engrave the base surface with a rotary tool (Dremel). The engraving (Figure 5c) consists of carving linear grooves on the resin, around the specimen/sample, in order to limit the traction effect of the block glued to the slide. This step

is vital because dilatation and shrinkage (due to variations in temperature and humidity) might potentially fracture the glass slide. According to Smith (2020) and Jannello and Chinsamy (2023), a recommendation for preventing slide cracking is to let the cut-off thin sections dry completely overnight before grounding them. After engraving, it is important to polish the base with a grinding/polishing machine using resin-bonded diamond grinding discs with grit sizes from p1000 to p1500 (FEPA P scale). The purpose is to obtain a smooth surface (Figure 5d), however, the speed of the disc should not be too high, since the specimen/sample may be damaged. Alternatively, the surface can also be manually polished with a succession of paper grinding discs of the same sizes. Although this method is slower and time-consuming, it leads to a more delicate polishing. For any of these two protocols, polishing should be carried out through an infinity symbol (∞) movement, since it allows for more uniform wear.

Once the surface is polished, cleaning is crucial in order to remove or minimize the presence of external elements from equipment, blocks and slides. The standard procedure involves cleaning with ultrasonic and finishing with acetone in the case of the slides.

Mounting the block onto the slide is carried out after polishing. Many types of adhesives can be used in this step, such as Loctite 358 (García-Martínez et al., 2011; Jordana & Köhler, 2011; Köhler et al., 2012; Marín-Moratalla et al., 2013; Moncunill-Solé et al., 2016; Nacarino-Meneses & Orlandi-Oliveras, 2019), specific mounting media for microscopy (like Eukitt®; Zedda et al., 2015, 2019) or epoxy resin (Kierdorf et al., 2013, 2019). The latter is here recommended, and it should be spread directly on the previously polished surface. The block is then placed on the frosted side of the slide, since it provides more adhesion surface, and a gentle pressure is applied to ensure total contact between the block and the slide. If possible, the use of a press (Figure 5e) is recommended to allow for a homogeneous pressure and prevent the block from slipping off the slide. If not, alternative solutions are provided by Lamm (2013) and Cerda et al. (2020). Although it does not always occur, bubbles may emerge between the slide and the block due to the porosity of the tissues. Under this scenario, it is recommended to repeat the process: detach the block, apply more adhesive and attach it to the slide once again. We recommend checking the mounted block a couple of times, ensuring the absence of bubbles between the slide and the block.

2.3 | Procedures for mounted block

This last macro-stage is divided into apical sectioning and grinding (Figure 5f), thin section preparation (Figure 5g) and curation. Most of the variables have already been considered. Accordingly, it is a quite mechanical and time-consuming part whose final product is a finished (paleo)histological slide ready for study.

2.3.1 | Apical sectioning and grinding

Many authors directly cut off a wafer from the block (Chinsamy & Raath, 1992; Cubo et al., 2012; De Margerie et al., 2004; Hayashi et al., 2023; Lamm, 2013; Montoya-Sanhueza et al., 2020; Schwartz et al., 2002; Schweitzer et al., 2007), which will be later polished on both sides and mounted on the slide before being ground to the desired thickness. However, we and some other authors (Cerda et al., 2020; Cerrito et al., 2021; García-Martínez et al., 2011; Iinuma et al., 2004; Jordana & Köhler, 2011; Köhler et al., 2012; Marín-Moratalla et al., 2013; Nacarino-Meneses et al., 2016, 2017; Zanolli et al., 2016; Zhao et al., 2013) section the block while it is mounted on the slide for several reasons. First, the slide facilitates the handling and positioning of the block during the cutting process, thus improving accuracy. Second, we can ensure parallelism between the two sides of the resulting thin section; particularly important when working with dentognathic remains since a slight tilt can preclude the visibility of the incremental structures. Third, it helps preserve the integrity of the embedded specimen/sample since a thinner slice is sectioned and excessive subsequent grinding is avoided. Accordingly, we suggest an initial thickness of 500 microns, since it allows correcting any eventual tilting in the slice with the subsequent grinding, carried out with a grinding wheel. Some devices, such as the manual thin section machine Petrothin, are equipped with both a diamond edge blade and a grinding wheel, while in others, the blade can be replaced. Once the 'thick' section is obtained, it is essential to check if any fracture occurred while sawing. If this is the case, apply extra resin to fill the fractures.

The next step is to grind the section to a specific thickness, it can be done using coarse grits discs (500p or 600p) on grinding/polishing machines and moving on to finer grits, however, we use the PetroThin® thin sectioning machine (Figure 5f), as it can serve both the purposes of sectioning and grinding. The primary concern with this machine is the speed at which the thickness is decreased, as a high pace can cause fractures. To address this, we recommend approaching the grinding as if divided into three steps (reduction, approximation, and refinement), lowering the speed from the first (grinding 10 microns at a time) to the last (grinding a few microns at a time) and frequently checking, with a micrometre, the thickness of the section. Additionally, to minimize the marks left by the grinding wheel, it is better to stop grinding about 40 microns before the desired thickness and finish it manually using grinding/polishing discs with finer grits (see Section 2.3.2). This last step is quite time-consuming and before undertaking it, it is recommended to check the thickness across the entire section, especially if working with old machinery or large slides, as the grinding might be slightly uneven. This is caused by the grinding disc being slightly crooked, and since we are working on a small scale, the bigger the slide, the greater the difference in thickness among the edges. To fix it, simply flip the slide and grind until the desired thickness is reached again.

2.3.2 | Thin section preparation

Once the desired thickness is obtained, the next step is polishing. Bear in mind that although the main point is to polish the thin section, the beginning of this step involves a minor grinding in order to remove the previously formed scratches. We recommend avoiding the use of

abrasive powder such as carborundum as it might contaminate the thin section (Nakai & Boyde, 2024). Many authors prefer to polish using polishing/grinding machines. However, the use of paper grinding discs is also appropriate, as it provides better control over the slide and prevents intensive scratching. It is recommendable to work with a slide holder (Figure 5g), which ensures consistent/homogeneous pressure on the slide. Although there are different industry-produced slide holders available, we recommend 3D-printing your own holders. 3D-printing is currently widespread and many laboratories have access to a 3D printer. It is easily applied and provides cheap alternatives, moreover, it proves especially useful in those special circumstances for which industrial-produced equipment is not suitable. In both cases, this step consists of gradual polishing, starting with coarse discs (around p1000) and gradually moving to finer ones (up to p2500). Some authors refine the polishing by using cloth discs with diamond (Bromage et al., 2009; Chinsamy & Hurum, 2006) or aluminium oxide (Tafforeau et al., 2007). It is important to check the slide under the microscope due to the different wearing rates along the specimen/sample (Figure 6), especially concerning the edges of the slide and softer parts, such as dentine in dental thin sections. Additionally, distinct enamel microstructures exhibit different resistance to stress (Fortelius, 1985; Lucas et al., 2008), which likely affects their wear rate, although further studies are required to confirm it. As a result, the real thickness may be less than expected, especially considering that both sides of the section are polished and ground. Currently, there are limited solutions to address this issue. For instance, using a slide holder helps reduce uneven wear and, if needed, thicker areas can be manually ground with small pieces of paper grinding disks of different grits. Ultimately, the most effective solution is to frequently check the thin section under the microscope and stop before information is lost. In this regard, the final thickness may vary based on different factors such as the tissues targeted, the nature of the analyses, and the degree of fossilization. Additionally, when measuring with a micrometre, the measured thickness also includes the thickness of the substance used to mount the block onto the glass slide.

After polishing and reducing scratches to the minimum, if necessary, slides may be further subjected to cleaning. Many cleaning methods are available, such as ultrasonic cleaning (Schwartz et al., 2002, 2005; Zhao et al., 2013) or washing and alcohol dehydration followed by clearing with xylene (Zanolli et al., 2016) or Histo-Clear® (García-Martínez et al., 2011; Jordana & Köhler, 2011; Marín-Moratalla et al., 2013; Nacarino-Meneses et al., 2017; Orlandi-Oliveras et al., 2019; Schwartz et al., 2002; Schwartz et al., 2005). However, we recommend avoiding ultrasonic cleaning when processing fossil thin sections since, in the case of poorly preserved and/or especially fragile material (such as fossil dentition), it might be detrimental.

Once the thin section is ready, it can be decided whether a glass coverslip is needed. We recommend covering at least extant tissues since they are prone to temperature and humidity variations which can damage their original features (Figure 7). Fossil tissues can also be covered, but it should be taken into account that covering is usually an irreversible process, albeit in practice some types of glue, such as Poly-Mount (Lamm, 2013), are possible to remove. Covering, apart from protection and support, is also useful for eliminating artificial scratches that form during thin section processing since the substance used to glue the specimen/sample fills the scratches, allowing for a clearer vision. The most used substances are DPX mountants (Nacarino-Meneses et al., 2017; Schwartz et al., 2002, 2005; Zanolli et al., 2016); however, some authors use Poly-Mount, a liquid plastic adhesive (Lamm, 2013). We also recommend the use of Loctite 350, a UV curing adhesive, which has proven to not react with the specimen/sample and provides a clear vision of the specimen.

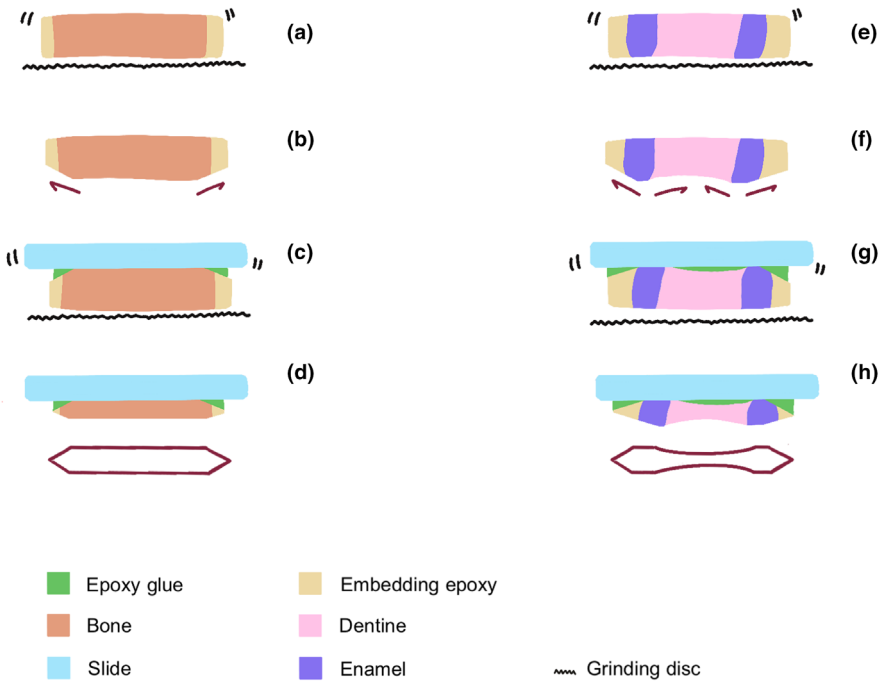

FIGURE 6 Different wearing rates on a transversal section of a thin section of bones (a–d) and teeth (e–h). The surface is polished/ground (a, e); The edges of the specimen/sample tend to wear more (b) as well as the dentine in dental thin sections (f); The specimen/sample is mounted on a slide and the empty space is filled by the mounting medium (c, g) then it is ground; The thin section is thinner than expected and information is lost at the edges and/or in softer areas (d, h). The motion lines in (a), (e), (c) and (g) represent the thin section being ground. The arrows mark the direction of the slope due to different wearing rates. The shown wearing rates are exaggerated in order to explain the phenomenon.

FIGURE 7 Thin section of a first lower molar of *Capreolus capreolus*, transversal section of metaconid (right) and protoconid (left), 25× magnified thin section. The thin section was not covered causing lifting (highlighted in red) and negating a precise observation of the microstructures.

2.3.3 | Curation

Last but not least, it is imperative to safely store all the material as well as all the information that it carries. Quantitative and qualitative data should be archived in a spreadsheet-like program (Microsoft Excel proves convenient for this purpose) and any multimedia file (photos, photogrammetry, etc.) should be catalogued consequently. However, it is important to mention that file formats may become obsolete over time, especially if they are proprietary formats. To ensure future data accessibility, particular attention should be paid to saving files in alternative formats (Excel spreadsheets should also be saved as .csv and .txt). While block and slide labels should also include some information in order to prevent confusion, the primary data repository remains the spreadsheet. Accordingly, slides and blocks are labelled with the most important information like original specimen ID number, anatomical element, side and, in the case of thin sections, a progressive number (as multiple thin sections can be derived from the same block).

The spreadsheet file is recommended to include a section for the thin sections and another for the blocks. Most of the information is shared from both sections (information redundancy helps to detect eventual errors), however, each one requires section-specific data. Common data should comprehend ID number, species, sex, age of the individual, anatomical element, side, institution/collection of provenance, locality, geological age and any other information (pathologies, taphonomy, geology, etc.) that can be relevant for (paleo) histological research. The thin section-specific attributes are thin section label, orientation, notes on thin section preparation, anatomical cut, last registered thickness, covering and observed microstructures. Whereas for blocks it is important to keep note of the block label, anatomical cut(s), block preparation, associated material, the number of derived thin sections, and the associated projects or research papers. Regardless of what attributes are included, the utmost importance must be given to standardization as consistency is necessary for the creation of a (paleo)histological collection. Standardized data are useful for more than one reason: they allow us to organize the collection and easily carry out research projects, permit to avoid the loss of important information and promote collaborations and reproducibility of research (Wilson & De Boef Miara, 2013). Finally, storing information on specimen preparation consents the evaluation of the long-term effects of the techniques on the material. In this regard, it is important to adequately store (paleo)histological thin sections and keep them under a controlled environment to prevent damage and maintain the original features. Lamm (2013) suggest using a constant temperature and humidity of, respectively, 21°C and 48%. Variations in these parameters cause the most damage to the collection. Also, it is important to keep the thin sections out of direct sunlight to prevent damage caused by photodegradation.

3 | DISCUSSION AND RECOMMENDED PROCEDURES FOR COMMON METHODOLOGICAL ISSUES

Our exhaustive review of the main methodological papers in histology of the last decades shows that the work of Lamm (2013) provides the most complete guide for hard tissue thin-sectioning techniques. Also worth mentioning is the work of Cerda et al. (2020), which describes useful protocols especially intended for laboratories with limited funding. Although the methods described there have greatly contributed to our current understanding of (paleo)histology, these are specifically intended for use on bones and focus in particular on dinosaurs and other large archosaurs—while methods based on teeth among other groups of vertebrates, both extant and extinct, have been little mentioned or overlooked. Although thin section techniques share several core steps in common irrespective of the study group, there are also some important differences. As such, a concise description of the different processes used to prepare thin sections from teeth in other groups, and in particular mammals that show considerable differences in size and mineralization compared to dinosaurs, is paramount.

Most of the techniques (some of them new) thoroughly described above with well-illustrated examples have been selected and designed due to their direct application to relatively small specimens

with specific necessities, as well as to extant material. In the case of mammals, their dental (paleo)histology is instrumental in providing a wealth of information that can be used to answer important questions about growth, life history, metabolic regimes and physiology. Procedures for preparing, embedding and sectioning dentognathic specimens from mammals can be challenging, particularly because of their small scale, extremely irregular shape and high fragility. The following section describes the most frequently encountered problems in histological research and provides an outline of effective solutions adapted to challenging specimens, which have proven very effective in our lab.

Extant or not fully fossilized specimens – Working with non-fossil specimens involves a series of differences that, if overlooked, might lead to poor results. Extant or not fully mineralized specimens imply different compositions, the presence of organic material, and a relatively higher fragility of the specimens (such as in the case of teeth). All these differences must be addressed specifically depending on the specimen and desired outcomes; however, some common practices are hardly unavoidable. These practices include embedding in resin, degrease, dehydration and, less frequently, stabilization. Resin embedding, especially with epoxy resins, is one of the most effective as it does not require partial decalcification of the specimen. Degrease and dehydration in alcohol are also recommended for extant specimens, as water and organic compounds could interfere with the embedding process. Stabilization is another step often required as the specimen might break during extraction, especially due to the unfilled cavities. Apart from the many processes applied during the preparation of such specimens, covering their thin sections is also highly recommended as it helps reduce the risk of curling or detaching due to temperature and humidity variations.

Embedding resin – The importance of the embedding resin is often overlooked since the reason for which one type is selected over another is usually not clarified. Specific parameters (e.g. viscosity and refraction index) to take into consideration when selecting a resin have been discussed, which are especially useful for laboratories with limited resources. After testing different options, we found that the most effective resins are epoxy resins with low viscosity and low refraction index (e.g. Araldite, Epo-tek, Epofix) although some cheaper alternatives can also be considered (e.g. Epo-150), especially in laboratories with limited funding.

Preventing yellowing – Although yellowing naturally occurs over the long term (as a result of oxidation reactions), sunlight and thermic variation can accelerate and worsen the process. This can dramatically decrease the visibility, negatively influencing our ability to precisely locate and section the specimen/sample and complicating subsequent stages of preparation. It has been described as related to curing heat and, to address it, pouring a small amount of resin and catalyst (100 g in total) and letting it cure is recommended before pouring another one until the specimen/sample is completely embedded. We recommend curing the resin in a refrigerator since, albeit it may lengthen the curing time, it is useful for preventing yellowing. Furthermore, it has proven to work with approximately 135 g of resin/catalyst, although further testing may be required for larger quantities.

Sectioning small specimens – Cutting small specimens is the most challenging stage through the entire process in (paleo)histology of mammals, and apart from being more fragile and inclined to chipping, they are also more affected by kerf loss as the blade thickness represents a larger percentage of the specimen size. To address the issue, the most effective technique is to fully embed the specimen. In case of the specimen being particularly small avoiding the apical cut and directly proceeding with the grinding can be considered. Although more attention has to be paid during the grinding and polishing steps, most small specimens are suitable for an apical cut. In this regard, fragile specimens, such as dentognathic remains, need to be frequently checked for fractures after being sectioned and, if needed, stabilized by pouring resin.

Cutting liquids – Another poorly considered aspect of sectioning is the cutting liquid used in the process, as it can occasionally damage both the machinery and the studied material. To address this, avoid the use of water to prevent the machinery oxidation and use instead ethylene glycol, which has proven to not affect our specimens.

Addressing different wearing rates – Although the different wearing rates within the thin section may cause a loss of information, Nacarino-Meneses et al., 2017 is only briefly mentioned in the literature. We have included a detailed and well-illustrated scheme (Figure 6) to help explain this phenomenon, often found during dental thin section processing.

Marked section plane – Bone (paleo)histology often involves sectioning a non-extremely accurate area such as the mid-shaft in long bones, whereas dental (paleo)histology demands much more precision to obtain an exact section of the tooth cusp. To address this, we have developed a technique that consists of marking the section plane on the resin-cast base on which the specimen/sample is fixed before embedding. This marked line remains visible once the resin block cures and allows for the production of precise sections by reducing refraction misplacement during sectioning.

Engraving – The tensile strength exerted by the mounted resin on the slide can potentially lead to slide breakage. To prevent it we recommend carving linear grooves in the resin surrounding the specimen/sample before mounting the block (engraving). The tensile strength is generated due to temperature and humidity variations as the resin expands and shrinks. In this sense, the grooves reduce the grip area and consequently lessen the force applied to the slide.

Sectioning a mounted block – Cutting a "wafer" directly from a cured resin block is a methodology followed by numerous authors. While this technique works well with large fossil bones, it might not be the best option for small and/or fragile specimens, especially if considering that both sides of the wafer need to be polished. Moreover, directly sectioning a wafer from the block requires the former to be thick enough to be manipulated and glued onto the slide, which implies a loss of material due to the subsequent grinding. As an alternative method, also applied by numerous authors, mounting the block between the two cutting phases results in easier manipulation,

greater precision and finer slicing which translates into a better preservation of the material.

3D-printed accessories – 3D printers have recently emerged as a valuable tool in many laboratories. They are suitable for not only producing high-quality replicas of the original specimens for dissemination and storage purposes but also common accessories (such as slide holders for grinding/polishing and slide racks for curation) that facilitate some (paleo)histological protocols.

4 | CONCLUSIONS

Paleohistology has recently gained increased popularity and, as a result of such 'golden age', its methods have spread and developed more or less independently across the scientific community. Few methodological works constitute the foundations of most research and, although the main passages are shared by most methodologies, an overview of the state of the art is necessary.

With this work, we provide a comprehensive and up-to-date review and summary of the current methods in paleohistology. Apart from summarizing the traditional paleohistological techniques, we also designed and included new methods, among which the most important focus on the processing of small specimens, both extant and fossil. These techniques have proven effective for the correct elaboration of dentognathic remains and we consider them a valuable contribution to the current methodologies. In this regard, the thin sections produced at our laboratory, with the techniques here described, have consistently proved of great quality, allowing for the identification of hard tissue microstructure utilized in both histological and paleohistological research. As such, this work offers a valuable starting point for beginners as well as a practical tool for experienced technicians and researchers.

The standardization of paleohistological methods, intended as the reduction to a single model of all the different techniques, is one of the crucial objectives of this methodological work. Despite different processes being applied depending on specimens and objectives, it is essential to standardize the techniques and flatten all the differences to a reasonable amount useful to appropriately process most of the material. Furthermore, standardization encourages the progress and emergence of new techniques by nourishing awareness and providing a sturdy basis from which to start. This is especially true for users whose primary proficiency does not fall within the production of such thin sections. In this regard, with this guide we address the standardization of such processes, promoting the accessibility and application of these techniques in modern paleohistological research.

AUTHOR CONTRIBUTIONS

Andrea Cuccu, Beatriz Azanza and Daniel DeMiguel conceived the manuscript; Andrea Cuccu and Teresa Calderón designed the methodology and experiments; Andrea Cuccu and Daniel DeMiguel wrote the manuscript with input from all authors after reviewing the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests to declare that are relevant to the content of this article.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

This manuscript does not contain any data/code.

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