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# Flat does not mean 2D: Using X-ray microtomography to study insect wings in 3D as a model for comparative studies

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## Abstract

1. While not generally used for relatively flat structures, X-ray microtomography can provide decisive insights for comparative anatomy and evolutionary studies of flat structures, thereby solving long-standing ecological and evolutionary issues. This is particularly true for the study of insect wings, organs that have played a central role in the evolution and ecological success of insects, the first organisms to develop powered flight.
2. We present the challenges inherent in studying insect wings by microtomography, as well as solutions to overcome them. We highlight the impact of insect preparation (i.e. dry or ethanol-preserved specimens, complete specimens or only part of the wings, wings stained with iodine or with a 0.3% solution of phosphotungstic acid—PTA) and of the acquisition mode (phase contrast or not), on scan quality and ease of interpretation. We compare our results with those obtained by more 'traditional' techniques, based on examination of the wings with a binocular or SEM microscope.
3. We show that X-ray microtomography is effective in establishing vein homologies—a crucial step in many evolutionary studies in insects—by accessing the internal structures of the wing base itself, where veins originate. It can also be used for difficult tasks such as distinguishing between crossveins and main veins, establishing vein polarity (i.e. concave vs. convex veins) or vein fusions, situations that complicate the establishment of vein homology hypotheses and the completion of subsequent evolutionary studies. Unstained, ethanol-preserved specimens give the least conclusive results, while dry specimens and ethanol-preserved wings stained with PTA give the best results.
4. We show that 3D tools provide crucial insights for the study of insect wings, illustrating how CT-scan tools may contribute to a change in dimension, introducing quantification and functional approaches and hopefully stimulating new studies for other seemingly 2D organs in several other fields, including plant studies. We highlight, nonetheless, that this tool must be used in coordination with more

traditional observational techniques, at least for insect wings, to provide a more complete picture of these fundamental organs.

#### KEYWORDS

evolution, homologies, Insecta, microtomography, wing

## 1 | INTRODUCTION

### 1.1 | Why using microtomography for seemingly flat organs?

X-ray microtomography (micro-CT) is now a tool often used in comparative biology. It has provided many answers to various questions about evolution. Arguably, some of the most spectacular studies have allowed the observation of the inner anatomy and functioning of live insects (Poinapen et al., 2017). However, the full potential of microtomography, usually used for shapes well-developed in their three dimensions, remains to be discovered. While micro-CT observations of bulky organs have led to many advances, micro-CT acquisitions made on small and almost flat organs lag behind.

The main reason for this is apparently obvious, that is, the small size of the third dimension (i.e. the thickness) of such structures. Yet, a thorough or fresh scrutiny of poorly understood features, often but not exclusively thanks to technical advances, has sometimes led to invigorating reanalyses, revisiting of hypotheses or even the emergence of new disciplines (e.g. Miele et al., 2021; Peterson et al., 2007; Stuhr et al., 2018; Vinther, 2015). With recent advances in 3D imaging resolution, it is now possible to embrace the advantages of this technique, including for flat organs like insect wings and their veins. Furthermore, wings are attached to the thorax where important structures (known as bullae) are concealed and can only be accessed by real or virtual dissections, further justifying the use of microtomography rather than 'surface-3D methods', as well as confocal microscopy or laminography, which allow only incomplete acquisitions with limited angle rotation (Zuber et al., 2017).

Despite the gains in knowledge envisioned or already achieved (e.g. Desutter-Grandcolas et al., 2017; Jacquelin et al., 2018; Schubnel et al., 2020), the benefits of using microtomography for flat organs remain underappreciated. For insect wings, this is not only because these structures are flat but also because they are small and weakly contrasted under X-rays. This triple challenge requires specific expertise, which has motivated us to share our experience on the study of insect wings. Ultimately, we wish to encourage scholars to explore the advantages of 3D tools for equivalent, seemingly 2D structures, such as prothallium and leaves in plants, moth mines in leaves or animal skins.

### 1.2 | The wing: A 'must-see' for insects' evolutionary biology

Wings and their veins are crucial for studies in insect evolution and ecology. Wings have been pivotal organs since insects first

diversified during the Devonian-Early Carboniferous, enabling them to conquer new biotas and resources, escape predators, communicate or reproduce (Garrouste et al., 2016; Grimaldi & Engel, 2005; Schubnel et al., 2021; Sun et al., 2020). Insects were indeed the first to evolve powered flight, long before their followers, pterosaurs (Alexander, 2015). Because of their complexity and diversity, insect wings are a prominent source of data in reconstructing phylogenetic trees, which themselves support multifarious evolutionary studies (e.g. Huang et al., 2020; Li, Shih, et al., 2020). Moreover, the great majority of insect fossil remains are wings, which are used, relying on vein homology, to posit evolutionary hypotheses in the deep past (e.g. Bechly, 1996; Jouault et al., 2021). Likewise, studies in wing development, morphometrics, flight performances, or sound production—including those with biomimetic applications—need accurate homologies of the veins for comparative purposes (Blanke, 2018; Desutter-Grandcolas et al., 2005).

The importance of wings and their veins, together with the difficulty of their analysis, explain why several tools and criteria, briefly reviewed below, have been developed to observe wings and resolve vein homology issues in insects. Surprisingly, microtomography is not one of these tools and we argue here that, despite technical challenges, it should be added to the list of available tools. We focus on vein homology issues to illustrate our point.

An insect wing is made of two sheets of cuticle fused together, with tubes (veins) that transport the hemolymph and can contain (but not always) nerves and tracheae (transporting air into wings; Wootton, 2009). Three types of veins are distinguished: longitudinal main veins that emerge from the wing base and can be forked, crossveins, and intercalary longitudinal veins that are linked to main veins by crossveins. Each main vein emerges from a cavity (bulla basivenale) situated between the general cavity of the insect body and the vein itself at, or very close to, the wing base. Some veins are located on the dorsal sheet (named convex veins) while others are on the ventral one (concave veins; Kukalová-Peck, 1991; Salcedo et al., 2019).

Since the second half of the 19th century, entomologists have tried to solve the problem of identifying and naming insect veins, but they have failed to reach a consensus (for a list of the different paradigms see Schubnel et al., 2020). This failure stems from the different criteria used for vein identification, which are all imperfect and suffer from exceptions, such as the tracheae or polarity criteria (e.g. Li et al., 2018 *contra* Desutter-Grandcolas et al., 2017; Fraser, 1938; Needham, 1907; Schubnel et al., 2020). Snodgrass (1909) proposed to determine the exact origins of the main veins by tracking them to their extreme bases (i.e. to the bullae), but at the time there was no efficient tool to see inside the sclerotized wing base. The bullae are generally concealed

by sclerites involved in the articulation of the wing with the body, so that he and other authors have confused the bullae with these sclerites (Hamilton, 1971, 1972; Kukalová-Peck, 1991; Kukalová-Peck & Brauckmann, 1990). Microtomography, allowing a 3D view of hidden small structures, is the tool Snodgrass lacked.

### 1.3 | Objectives

Microtomography has already been applied to observe insects and most of their body parts (e.g. Betz et al., 2007; Mensa et al., 2022; Schoborg, 2020), but its application on wings is quite recent and uncommon. Although the use of microtomography for insect wings may open up new avenues, it remains unappreciated, as certain specificities of the wings (i.e. flat, small, weakly contrasted because of the constitution of the organic cuticle and organs like tracheae) make it challenging (see problems with membrane acquisition in Stelzer & Krenkel, 2022). In recent years, we have applied microtomography to scan the bases of the wings and the basal portions of the veins in several insect orders (Desutter-Grandcolas et al., 2017; Jacquelin et al., 2018; Schubnel et al., 2020), progressively developing and improving our protocol to overcome the challenges posed by the specificities of insect wings. Here, we explain the methods we have developed to obtain high-contrast 3D images of insect wings. Our goal is neither to provide a best practice guide (see for instance Betz et al., 2007) nor to simply add a new application for insects (e.g. Donato et al., 2021; Fidalgo et al., 2020; Schoborg, 2020). Instead, we wish to highlight the difficulties encountered, contrast different solutions, and give a specific protocol for the preparation, scanning and reconstruction of insect wings. Our protocol is especially indicated for desktop micro-CT systems and might be applied to any organic microstructure. We show that the reconstructions obtained can support hypotheses of vein homologies within evolutionary lineages. We illustrate our technical and scientific advances with real examples showing how wing vein features can be identified, even in tricky situations, and how this contributes to a better understanding of insect evolution. Overall, we hope that this experience sharing will inspire researchers to use micro-CT for other challenging structures and ultimately open new avenues of research.

## 2 | MATERIALS AND METHODS

### 2.1 | Specimen selection

Several criteria must be considered before using microtomography on insect wings. From our failures and successful experiences on different winged insect orders (this paper, but also Desutter-Grandcolas et al., 2017; Jacquelin et al., 2018; Schubnel et al., 2020; and unpublished scans; we have tested 19 orders of winged insects and have so far successfully reconstructed the wing venation patterns for 16 of them), we can tell that the most important ones are the size of the wings, whether wings can be cut or not and their preservation medium.

#### 2.1.1 | Wing size

Generally, microtomography acquisitions have a higher quality for large wings, because large specimens are often more robust and more sclerotized than smaller ones, resulting in higher contrast images. They also have larger veins, are easier to interpret and exhibit more complete venation patterns. The smaller the wing, the harder it is to get clear scans. In a conventional cone-beam micro-CT system, high resolution (i.e. with small voxel size, and thus a high level of detail) requires working with the smallest possible focal spot size to limit the geometric blurriness. Indeed, considering the geometrical un-sharpness equation— $U_g = F(M - 1)$ , with  $F$ : the focal spot size and  $M$ : the projection magnification defined as the ratio of the source-detector distance (SDD) to the source-object distance (SOD)—it is obvious that a magnification without loss of sharpness is achievable by reducing the focal spot size. Then, for a given resolution, it is important to control this parameter and to adapt it consequently (Flynn et al., 1994). In our case (see Section 2.3), we referred to the nanofocus X-ray source manufacturer chart (i.e. Hamamatsu) which evaluates the focal spot size as a function of tube voltage and focusing mode for a fine cathode filament (LaB6). However, reducing the size of the spot requires restraining the tube power to withstand heat loading without melting the anode target. This means, for a given voltage, to limit the X-ray intensity, which governs the total numbers of photon for each projection, and then lower the signal to noise ratio (SNR). Decreasing the voxel size by a factor of two requires the collection of  $2^4$  more photons to obtain the same SNR (Stock, 2019). This contrast alteration can be mitigated by increasing scanning time (exposure time, averaging more frames per image, increasing the number of projections, preferably in that order), by shortening the SDD to increase the solid angle of the X-rays the detector can capture, which means positioning the sample as close to the source as possible, or by adding a contrast agent (e.g. Metscher, 2009a, 2009b; Pauwels et al., 2013). Binning, which means to combine pixels of the detector to create one larger pixel, could be an option to increase SNR, contrast sensitivity and reduce acquisition times. However, as it decreases the spatial resolution or the field of view, we do not find this practical for insect wing analyses.

On the other hand, very wide wings can be problematic because their size impacts the distance between the sample and the X-ray source (for conventional cone-beam micro-CT systems, magnification and therefore spatial resolution are maximized by minimizing the source-to-object distance). Consequently, large wings cannot be scanned in their entirety with high precision in most micro-CT systems. Depending on the sample and the purpose of the scans, removing the wings from the specimen might be an option. Note that, while broad wings are problematic, long wings are less an issue because they can be studied with a vertical stack of successive scans over the different heights of the wing.

#### 2.1.2 | Wing removal

Better results for wing scans are obtained from dissected specimens, when wings and their bases are separated from the bodies, and only

the region of interest (ROI) is scanned. This is a suitable solution for wing vein issues: one can focus on the base of a wing and scan the articulation zone because of the relative stability of the wing base, which proved constant in large clades. But this solution might not be adequate for other purposes or for specimens that should not be damaged, such as rare specimens or holotypes. In this regard, it should also be kept in mind that microtomography methods are not entirely non-destructive, because X-rays may damage DNA, polymerize some materials, and alter organic molecules (Immel et al., 2016; Sumner-Rooney et al., 2019).

### 2.1.3 | Preservation medium

Most insects are stored in one of the three following ways: dried pinned specimens preserved in air, specimens preserved in ethanol, or specimens mounted on microscope slides. The glass of microscope slides complicates the use of microtomography—glass is often too attenuating to scan organic matters. Both dried and ethanol-preserved specimens can be used, but the preservation medium influences the state of the specimens and the quality of the resulting scans, notably the contrast between the object and the medium.

## 2.2 | Specimen preparation

Whatever the size of the studied specimen, a scan performed with the entire specimen will often have a coarser (i.e. with larger volume voxels, and thus a lower level of detail) resolution than a scan using a wing separated from the insect body: the entire insect will often be wider than the isolated wing, which can impact the source-to-object distance. The best results are achieved when the wing is still attached to the half thoracic segment, thus preserving the articulation sclerites and the bullae. Muscles do not interfere with the reconstruction so that cleaning the sclerites, as done for example for electronic microscopy, is unnecessary. For our purpose (i.e. wing vein homology), the ROI is represented in [Figure S1A](#).

For a dried specimen, the scan can be done without any further preparation. To avoid metal artefacts in the reconstruction, it is often safer to remove pins from pinned specimens, as a pin is almost always placed between the wings. As microtomography needs motionless and stable samples, the distal part of the sample can be maintained between two layers of polystyrene or polyethylene foam, and the whole sample placed in a plastic tube (glass, more attenuating, must be avoided) as thin as possible ([Figure S1B](#)). The specimen or the ROI can also be glued (e.g. universal glue) to cardboard.

For ethanol-preserved specimens, drying them must be avoided because this can damage the specimens, especially the delicate wings, while critical point drying is unsuitable for large insect wings. When a ROI has been dissected, we recommend to soak it in a solution with a contrast agent. Two contrast agents were initially tested, iodine (Metscher, 2009a) and a 0.3% solution of phosphotungstic acid (PTA; Metscher, 2009a, 2009b; see also results). As the first

tests with iodine were not conclusive, only the PTA solution was tested and used. For wings measuring more than a centimetre, staining should last several weeks to produce good results, with a very variable speed of penetration from one wing to another, down to 0.2mm/day through the veins and even slower through the membrane. Due to the low penetration rate of the PTA, we recommend cutting the wing apex so that the PTA can soak into the wing at both ends. The stained specimen/ROI is placed in ethanol (70% or 95%) inside a thin plastic tube. We recommend enveloping the specimen/ROI with as much polyethylene foam (wherein more than 90% of the volume is air) as possible to stabilize it and limit the quantity of ethanol in the tube, ethanol increasing X-ray attenuation comparatively to the air. This allows maintaining a relatively good transmission rate, resulting in a better SNR.

Several specimens were prepared in one of those three ways (dry, ethanol-preserved stained or not) and scans were obtained using similar conditions of acquisition—a few characteristics (voltage, resolution, etc.) had to be optimized to accommodate size differences between samples ([Table S1](#)). Among those, the scan of a reticulated beetle *Cupes raffrayi* Fairmaire, 1885 (Coleoptera) was particularly illuminating, as discussed below. We used it as an additional example to show how microtomography contributes to a better understanding of insect wing venation and evolution.

## 2.3 | Microtomography acquisition

Insects are mainly composed of organic materials, which have relatively low density and X-ray absorption. Thus, X-rays of relatively low energy are sufficient to obtain good results (60–150kV), as noted long ago by Goby (1912, 1914) who experimented with insect radiography. Those energies can easily be obtained with a laboratory micro-CT-scan or a setup using synchrotron radiation. Insects are small animals and their wings are even smaller (from 0.2 to 140mm, generally less than 30mm), which therefore requires a fine resolution. For the wings we have scanned so far, we mostly used a resolution between 0.5 and 10  $\mu\text{m}$ , depending on the size of the sample.

The scans obtained at the University of Poitiers were conducted with an *EasyTom XL Duo* (RX-Solutions) and its *cooled transmission nanofocus X-ray source* (L10711 with a LaB6 cathode and W target with a beryllium or diamond output window, Hamamatsu) coupled with a flat panel detector (*PaxScan 2520DX*, Varian; pixel size: 127  $\times$  127  $\mu\text{m}$ ). At the end of each acquisition, additional reference projections were recorded to correct possible spot drift. Image reconstructions were done with Xact (RxSolutions) using a classical filtered back projection algorithm based on the Feldkamp method for cone beam geometry and with a Tukey border filter (Kak & Slaney, 2001).

We also experimented with phase contrast on several insects at the beamline PSICHÉ of SOLEIL, the French synchrotron radiation facility (Gif sur Yvette, France; [Table S1](#)). Phase contrast is known to greatly improve the quality of scans done on low-density objects such as soft tissues, which seems ideal for insect wings.



At the MNHN X-ray tomography imaging platform, AST-RX, we used a GE Sensing & Inspection Technologies Phoenix X|ray v|tome|x 240–180L with the GE nanofocus X-ray 180kV/15W tube coupled with a 400×400mm detector of 20,242 pixels matrix (pixel size: 200×200 μm).

## 2.4 | Venation reconstruction

To study venation homology, we used manual segmentation to reconstruct insect wing venation. This was performed with Avizo 9.5.0 (Thermo Fisher Scientific). We observed that the veins, membrane, and the rest of the insect cuticle share a similar level of absorption—and thus have similar grey values in the final scan—which limits the possibility of automatic segmentation. We explain below how we proceeded with manual segmentation, while noting that segmentation highly depends on scientific objectives.

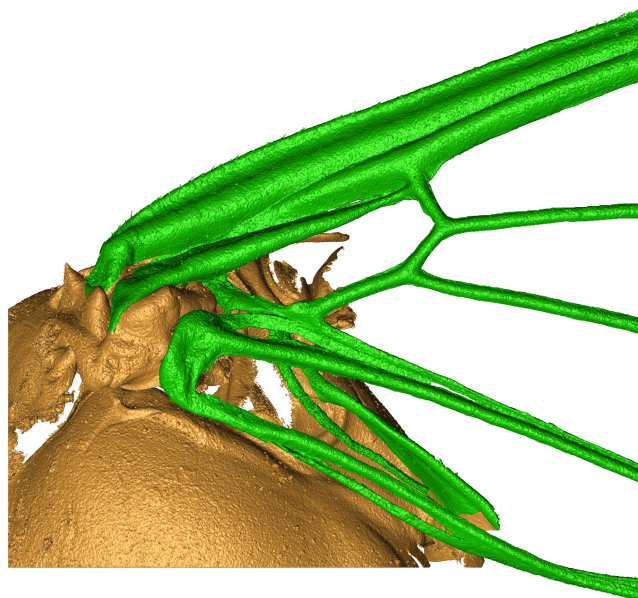
Vein segmentation was performed from transversal views of the veins. We start by manually highlighting a zone, usually circular, containing the vein on a slice. We repeat this operation every 5 to 50 slices, depending on the straightness of the vein, that is with a larger step when the vein is straighter and thus easier to follow. Obviously, a slice-by-slice segmentation could be performed but it is time consuming and would not significantly improve the reconstruction. Next, we use the selection interpolation tool to highlight the regions between the manually segmented slices. The resulting selection is a cylinder containing both the vein, its immediate surrounding and, within the vein, the medium. This selection is stored in a temporary material. Then, with the magic wand tool (region growing selection), we select the voxels with vein's gray values inside the cylindrical material. This allows us to isolate the veins from the surrounding medium.

## 3 | RESULTS

### 3.1 | Observations of challenging and essential features of wing venation

An advantage of using microtomography on insect wing venation is the possibility to observe hidden bullae. Tracking the veins to their very bases has always been a barrier that microtomography can now overcome. Veins can easily be followed to the extreme wing base where they originate, and bullae identified despite the overarching sclerites (Figure 1). Each main vein can then be followed from its extreme base in its bulla.

In addition, features classically used to assess vein morphology and anatomy—for example to establish vein homology—can be observed with micro-CT-scans; the adequacy of those features for vein identification can then be checked. Those features are: vein polarity (relative convexities), vein fusion and tracheae. Veins are tubes whose cross-sections appear as hollow spaces, rounded or diversely oblique depending on the orientation of the veins relative to



**FIGURE 1** Reconstructed scan of a cicada showing how bullae could be differentiated from sclerites. Veins and bullae are in green, while the rest of the thorax is in bronze. Note the granular aspect of the thorax linked to the low contrast and signal to noise ratio: this is one of the few unstained in ethanol scan we were able to reconstruct.

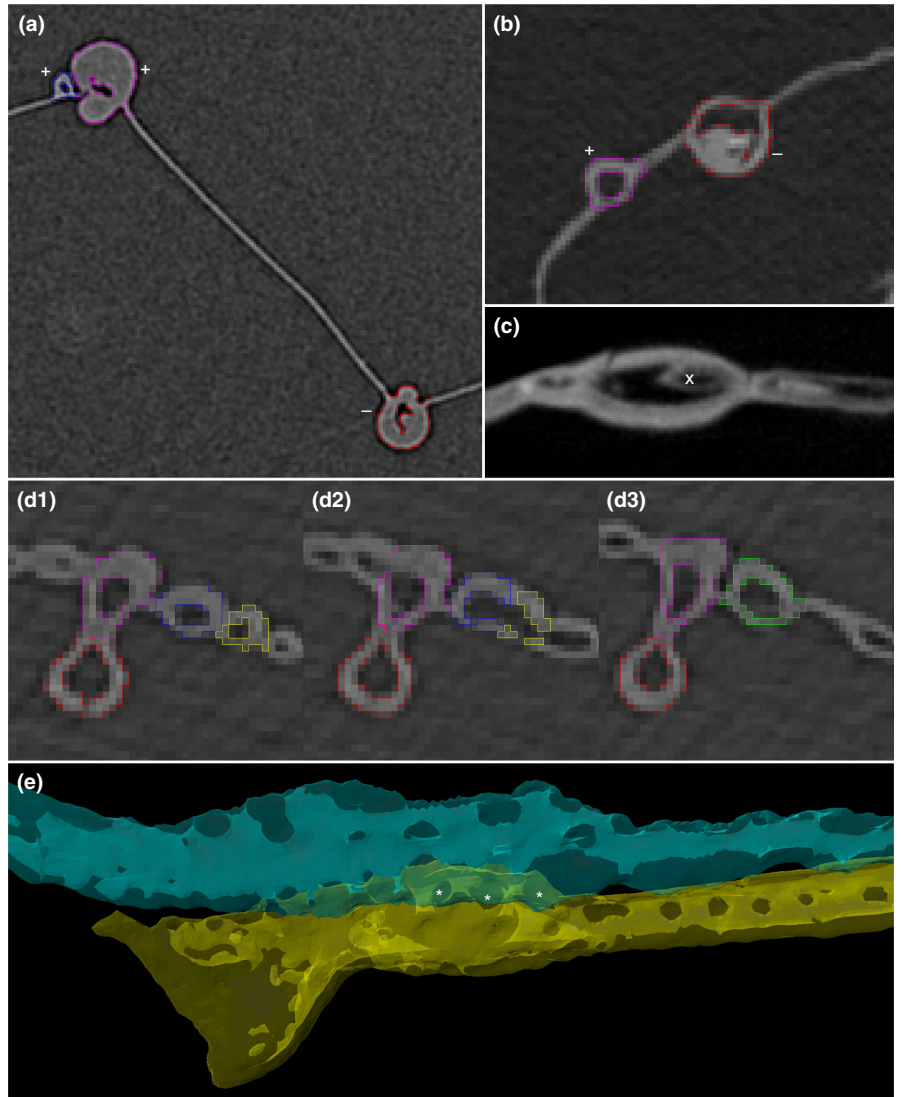
the scan plane; their polarity is easily identified from the position of those hollow spaces above or below the wing plane. An off-centered vein above the wing plane is convex, while an off-centered vein below the wing plane is concave. This true polarity might differ from the apparent polarity (Figure 2a,b). Delineating the ROI with the wing base and thoracic sclerites helps to interpret wing orientation and vein polarity. Tracheae are also easily identified through micro-CT. When they run within veins, they appear in cross-section as a circle within the vein, i.e. as a tube within a tube (Figure 2c). Delineating vein fusion is challenging with microscopic observations: when two veins converge, it can be difficult to tell whether they are fused or simply running one next to the other. With CT-scans, it is a fairly straightforward task: with sufficient resolution, one can actually see whether the veins are fused (i.e. two tubular structures become one after fusion; Figure 2d) or whether a membrane separates the veins (i.e. the contours of the veins run separately), with or without crossveins (Figure 2e).

The scan of the 'reticulated beetle' *Cupes raffrayi* illustrates these advantages. While some veins are indistinguishable at the wing base (see Figure 3a–c), microtomography allows them to be easily separated (Figure 3d).

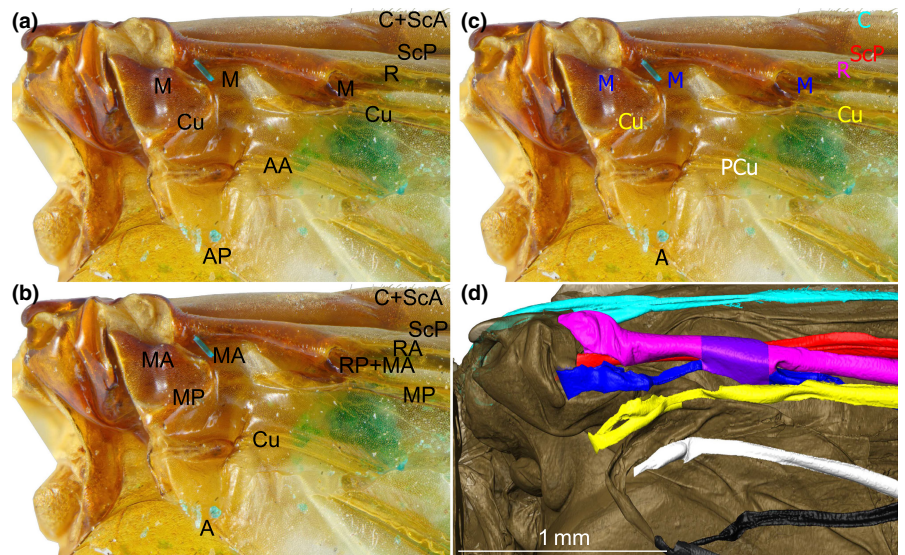
### 3.2 | Tests of different preparations for microtomography acquisition

Among the specimens used, we had three specimens of closely related mayflies (Palaeoptera, Ephemeroptera, *Ephemera* Linnaeus,

**FIGURE 2** Essential features of wing venation that are often used to assess vein homology hypotheses. (a) Polarity of veins and two joined veins (in magenta and blue) in a *Zygoptera* (convex veins identified with sign +, concave with -). (b) Polarity of veins in a *Phasmidae*; the red vein seemed convex in direct observation but is actually concave (convex veins with +, concave with -). (c) A trachea (symbol x) enclosed in a vein of a *Phyllidae*. (d) Succession of three slices showing the fusion of two veins (blue and yellow to green) in a wing of a *Trigonopterygidae*. (e) Intercalary veins (identified with the symbol \*) between two joined, but well separated, bullae in a wing of a *Trigonopterygidae*.

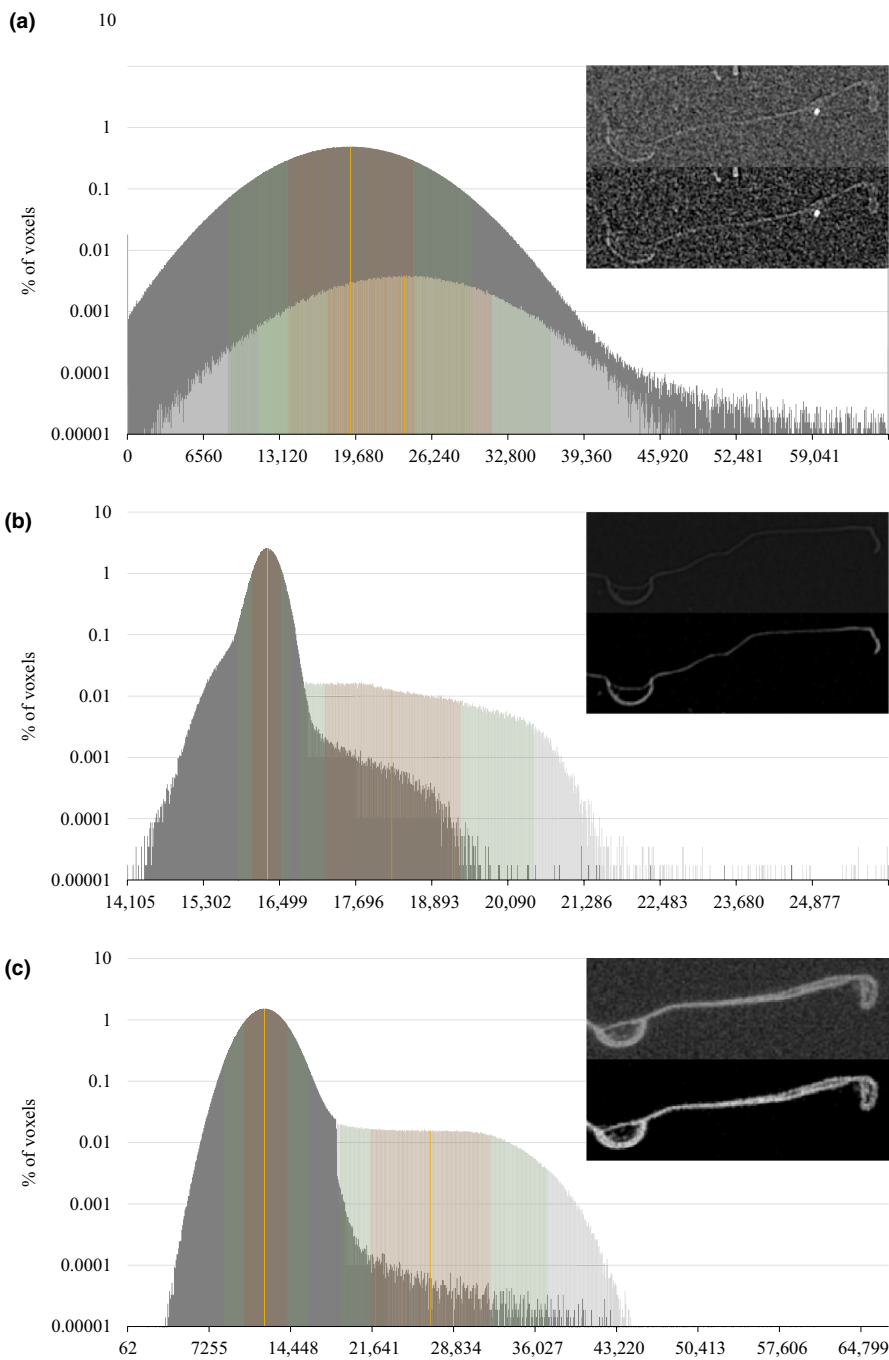


**FIGURE 3** Comparison between an optical view and a microtomography reconstruction of the wing base of a *Cupes raffrayi*. (a) Optical view, names of veins after Kukulová-Peck and Lawrence (1993). (b) Optical view, names of veins after Fedorenko (2009). (c) Optical view, veins after our microtomography reconstruction. (d) Our microtomography reconstruction (veins colours correspond to their homologies given in (c)). Note that the bases of M and Cu are almost indistinguishable in optical view.



1758) with similar wing base morphology, which allowed us to compare the impact of the preparation methods on the resulting scans (i.e. dried, ethanol-preserved, ethanol-preserved and stained).

The histograms contrasting the ROI and their medium are shown in Figure 4. The scan of the specimen in ethanol without staining (Figure 4a) gave poorly contrasting results (i.e. highly overlapping



**FIGURE 4** Impact of different preparations of *Ephemera* specimens on final scans shown with histograms of selected ROI. (a) In ethanol. (b) Dried. (c) In ethanol and PTA stained. The latter preparation provides the best results. Medium (ethanol or air) in dark grey, wings in light grey. Mean values: yellow lines, deviations in red, and 95% of values intervals in green. An example slice (top right of panels) is given for each kind of preparation in its original contrast (top) and after adjustment of the grey level dynamic range (bottom). Scans were done with EasyTom XL Duo (RX-Solutions) at University of Poitiers: see [Table S1](#) for detailed technical information.

light and dark grey histograms). It was not possible to correctly differentiate the wing from the medium because of a low SNR. This resulted in a loss of the fine details generally needed to make good reconstructions. Two scans of other insects were performed in ethanol without staining (list given in [Table S1](#)). Both showed poor contrast and were difficult to reconstruct, although a bit easier for the large and highly sclerotized wing of the cicada.

The scan of the dried *Ephemera* specimen ([Figure 4b](#)) showed better contrast between the air and the wing, sufficient to differentiate them easily. However, damage was visible for the smallest and thinnest veins: the margins of those veins did not appear tubular, contrary to both in ethanol scans, but were restricted to thin, sometimes inconspicuous, folds ([Figure 4b vs. c](#)). Those veins are

sometimes completely collapsed, a phenomenon we observed in the majority of scans of dried specimens. This does not occur with large and sclerotized veins or with wing bases, which are reinforced, so that reconstructions in the proximal area of dried wings are more accurate.

The scan of the *Ephemera* specimen soaked with PTA in ethanol ([Figure 4c](#)) gave the best results. It was at least as contrasted as the scan in air, and had a much higher mean wing-to-medium ratio (2.19 vs. 1.12). Furthermore, a long preservation time in ethanol—in this case more than 175 years—did not damage the veins, contrary to the desiccation seen with air preservation. The presence of ethanol also preserves the wing from possible drying due to the heat released during the scan, which, at fine resolution, lasts several hours ([Table S1](#)).



## 4 | DISCUSSION

### 4.1 | Wing vein homology and insect evolution: The added-value of microtomography

Insect wings are the focus of many evolutionary studies, whose hypotheses require accurate identification of wing veins. Yet, despite centuries of investigation, wing vein homology remains controversial, mainly because of flawed homology criteria coupled with observational challenges (Hamilton, 1971, 1972; Kukulová-Peck, 1991; Needham, 1907; Snodgrass, 1909). The difficulty arises from the small size of most insects, and the even more extreme small size of their veins. Further adding to the problem is the great diversity of venation plans in insects, and the locations of the bases of the veins, hidden under the thoracic sclerites. Here we show how microtomography, a 3D tool, contributes to a breakthrough in the study of flat insect wings: it allows traditional homology criteria of veins to be assessed, and also access to the very base of the wing where crucial structures for vein identification, the bullae, are concealed.

As the main veins originate from bullae, identifying those veins requires locating the bullae and investigating their morphology. We have shown elsewhere, by solving the long-running uncertainty concerning the post-cubital vein (e.g. Comstock & Needham, 1898 *contra* Snodgrass, 1935 *contra* Kukulová-Peck, 1991), how enlightening the search for bullae can be for our understanding of vein homology and its evolutionary aftermath: with rare exceptions, the post-cubital vein had previously been accounted for only in Hemiptera, but we have proven its existence in all neopteran insects (Cryan & Urban, 2012; Schubnel et al., 2020).

We reached this conclusion by combining microtomography, on extant and extinct taxa, with more traditional observation techniques. Microtomography can reveal bullae, allowing the main veins to be identified. It can also be used with high accuracy (as long as scan resolution is adequate) for observing features used as traditional criteria of vein identification: vein polarity, vein fusions, and the presence of tracheae. As powerful a tool as it may be, X-ray microtomography is however blind to several vein details such as colours or textures, setae, and spines if the resolution is not sufficient. Those details can be important for vein homology (as a 'special quality criterion' of Remane, 1952) or for studies on wing evolution. For instance, the presence of the same spines on two distinct veins provides information on how wing venation has evolved in Odonoptera (Nel et al., 1993); these changes would have been difficult to track from micro-CT scans alone. Similarly, some veins, especially those that are very close to each other in young imagoes (see Banerjee, 1988), might be hard to interpret from micro-CT scans. Extra-features such as the colour of veins might help in this interpretation. In addition, microtomography has a cost (in time, data storage, and money) that might be difficult to circumvent: depending on the scan quality, manual segmentation may take several days or weeks to complete. Thus, microtomography provides a global view of venation that should be contrasted to and complemented by more traditional observation techniques.

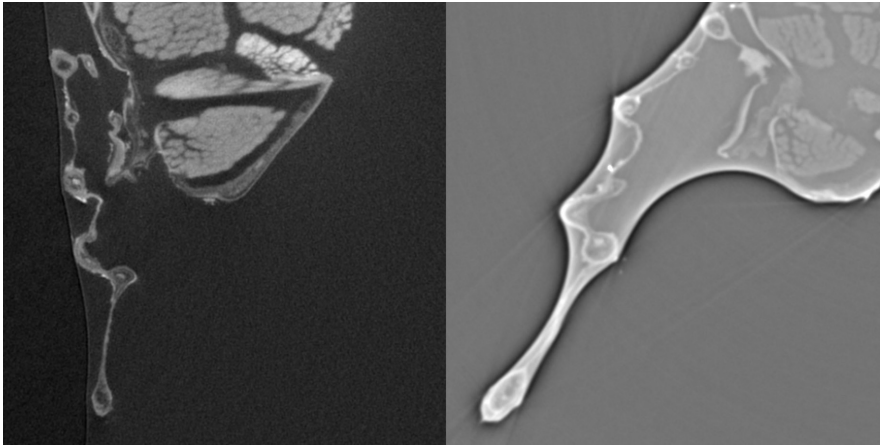
The reconstruction of the scan of a reticulated beetle (Cupedidae) sheds additional light on the benefits of microtomography. Beetles have modified hind wings, which are typically folded to be covered by the elytra. Those folds imply a strongly modified venation, for which it is difficult to find homology with those of other insects. Kukulová-Peck and Lawrence (1993: figure 12, 2004) considered that the R is basally divided in a RA and a RP, whereas Fedorenko (2009, 2015: figure 1) considered these veins to be simple R and M in contact near the base of the wing. This disagreement has remained unsettled. Our micro-CT-scan reconstruction shows that the R is not divided at the base and that the alleged 'RA' and 'RP' veins observed by Kukulová-Peck and Lawrence (1993) come from a vein that is itself the short fusion of two veins very close to the base of the wing. These two veins have clearly separated bases and bullae, thus the anterior one is the true radius and the posterior one the median vein. Then it is highly likely that the two apical veins are also R and M that re-separate, in accordance with the hypothesis of Fedorenko (2015: figure 1). This has important consequences for the homologies of the next main longitudinal vein emerging from a bulla separate from those of R and M, which Kukulová-Peck and Lawrence (1993, 2004) considered to be the M but that actually corresponds to the Cu, also in agreement with Fedorenko (2009). Nevertheless, this last author considered that M is completely fused with R basally, and figured a single stem for both veins, which is not the case. Thus, the micro-CT scan tool has allowed us to choose between different hypotheses and also to refine the patterns of venation.

### 4.2 | Test of different preparations for microtomography acquisition

Three issues limit the effectiveness of microtomography in the study of wing venation: specimen size, the shape of structures, and the contrast obtained in microtomographs. It is the need to address these three aspects simultaneously that presents the greatest challenge. Essentially flat, wings have been mistakenly considered simply two-dimensional—and thus virtually ignored by 3D approaches. Weakly contrasted wing structures might be difficult to reconstruct, especially when wings are preserved in ethanol. Furthermore, scanning insect wings requires fine resolution, which comes at the cost of the scan field of view when using a cone-beam X-ray source (Betz et al., 2007).

Insect specimen preservation techniques add extra difficulties for successful scans of wings and their veins. Dried specimens often have collapsed veins, the smallest ones being the most impacted. This kind of damage likely results from a pressure decrease that occurs inside the veins when the specimen dries after its death. There is no difference in compositions between the veins and membrane. Therefore, the only way to differentiate them is to search the hollow structure of the veins, an impossible task if they have shrunk significantly. Although this rarely affects the main veins, where only the thinnest of dorsal or ventral part is slightly collapsed, such damage remains a problem because it can potentially hide subtle but important details.

Ethanol-preserved specimens do not have this problem but present their own issues: a contrast that is far too weak, and a low SNR.



**FIGURE 5** Wing base of two specimens of *Merope tuber*. Left: phosphotungstic acid (PTA) stained, scanned in Poitiers. Right: Not stained, phase contrasted, scanned in Psiche beamline, Soleil Synchrotron. PTA staining allows easy differentiation of air, ethanol and wing. Phase contrast also helps to see the specimen, but adds a white border between ethanol and air, hiding some edges of the wing.

This might be solved by staining of the specimen/ROI before the scan (Metscher, 2009b). We highly recommend avoiding using unstained ethanol specimens, as the scans would likely be unusable. It should be noted that it is not practical to stain entire specimens with PTA, as the dye does not readily pass through the cuticle.

Using phase contrast in a synchrotron micro-CT is another option (Mayo et al., 2003) but it can produce either positive or negative effects. In unstained ethanol-preserved insects, phase contrast greatly enhances the contrast between the insect and the medium, and allows delineating fine details. However, it also increases contrast between air and ethanol in bubbles, which complicates the reconstruction, as ethanol tends to form thin layers between wings and bubbles, which are difficult to differentiate from the wing itself. Air bubbles must be removed or moved as far as possible from the wing, a delicate procedure that must be undertaken correctly before conducting the scan. Moreover, phase contrast does not add as much contrast to wing veins as does PTA staining (Figure 5). In dried insect wings, for which contrast is not an issue, using phase contrast can have detrimental effects. Because the difference of density between the wing and the air is very high, an incorrectly adjusted phase contrast could be too strong and ‘burn’ (in its photographic meaning, that is saturated grey levels with completely white voxels) the whole wing. This results in a tremendous loss of details, especially within veins. Worse, such phase contrast also tends to increase some artefacts, such as reflection artefacts, leading to further loss of details. Of course algorithms (i.e. Paganin algorithms that are commonly used when phase contrast occurs in micro-CT devices) can limit this problem, but they can add their own artefacts.

Overall, phase contrast highlights interfaces, which is beneficial when contrast is very low, but also tends to complicate the reconstruction as it enlarges the range of values taken by the whole wing. Thus, in our experience, phase contrast is mostly inadequate, except maybe for scanning ethanol-preserved wings that cannot be stained.

#### 4.3 | Microtomography versus (serial) microscopic section

The main, if not only, alternative to study a wing in 3D, while keeping the possibility to observe the interior of the structures, is to

obtain microscopic sections. This method has been used to address many questions about wings, such as the organization of the cuticle (Banerjee, 1988) or wing veins homologies (Franielczyk-Pietryra & Wegierek, 2019). Although microtomography seems unsuitable to study wing ultrastructures like cuticle organization, as such details do not appear in microtomography acquisitions, it also has a few advantages, which justify, for instance, its use for wing veins homologies. The most important one is that microtomography allows continuous data to be obtained, while only a few microscopic sections are generally done, leading to non-continuous data and a potential loss of information. Although it is possible to do serial microscopic sections on an entire wing or a precise ROI, this is a very laborious task, and microtomography is clearly simpler. Another important difference is that, in microtomography, voxels are cubic, while the sectioning dimension is generally far larger than the two others in serial section. This can either be an advantage or a drawback, depending on the objective. Overall, serial sectioning implies a tremendous loss of detail in the sectioning dimension, but it also allows obtaining a far finer resolution in the two other dimensions—even better than what could be obtained in microtomography for a similar width—and could circumvent the potentially problematic width versus resolution trade-off of microtomography. This can be very useful for some specific issues: for example, we observed that, especially in large wings, the membrane is often so thin that, to acquire the full width of the wing, the resolution is too coarse to allow a precise measurement of the membrane thickness, as it can be represented by only a few voxels in its thinnest parts.

#### 4.4 | What next: Changing dimensions through 3D methods, a new challenge in natural history and comparative biology for organic microstructures

The originality of our approach, made possible by the use of the modern tomographic tools and its adaptation to the context of organic 2D structures, lies in four principles and results applicable to a large number of experimental situations of biological studies:

1. Ontogenetic implications, here looking at the topological origin of structures is often pertinent: in the case of insects, the

ontogeny of the internal structures of the wing has, till now, been studied through 2D observations.

2. Organic composition is a recurrent problem in natural history structures studies, and often in histology and many organic microstructures: in adapted and combined use of easy-to-use contrast agents to compensate for the poor performance of X-ray observations of organic materials, without mineral chemical elements, is a transposable study model.
3. Changing the scale of observation (e.g. nano-scan) to gain access not only to information from the base of the wing but also to details of parts of the veins to detect captures, anastomoses, hidden structures and understand the functions of the veins. For instance, the 3D structure of the trachea in the wing pads of the insect nymphs remains to be studied.
4. The change of scale and the focus on these precise aspects of wing morphology lead to a change of dimension, from 2D to 3D. This is also what can be called a fractal dimension, which can imply a denomination including decimals to the observation dimension (e.g. 2.5D at an intermediate scale) as a quantity that is intended to translate the way in which a fractal set fills space, at all scales. In the case of fractals, it is non-integer and greater than the topological dimension.

These points will have to be taken into account for physiological studies (or any quantification), for example on the functioning of the wings or any organic structure.

## 5 | CONCLUSION

While microtomography was initially developed about 40 years ago (Elliott & Dover, 1982), it has only recently been applied to biological research, rapidly leading to spectacular progress (Ritman, 2004). Examples are many and include studies on living animals and their tissues (e.g. Broeckhoven et al., 2017; Ostwald et al., 2020; Poinapen et al., 2017), plants (e.g. Panarese et al., 2016; Polo et al., 2020), or fossils (e.g. Kouraiss et al., 2019; Tafforeau et al., 2006). Here, we have illustrated an overlooked and challenging situation wherein microtomography brings decisive insights by a combination of methodologies and new approaches. After refining our protocols, we have performed micro-CT scans on 19/23 orders of winged insects. For 16 of them (i.e. 84%), we succeeded in reconstructing their wing venation patterns, showing that microtomography can be widely applied to the study of insect wings. To share this experience, we have provided a specific protocol and guidelines to analyse insect wings and identify their veins, structures that are tiny and weakly contrasted. Most importantly, although wings are mostly flat, we have shown that X-ray microtomography provides an innovative and fresh look at those structures, which are too often mistakenly considered as 2D. With the use of X-ray tomography, we expect dramatic advances in our understanding of the anatomy, functioning and evolution of flat structures, be they insect wings, plant leaves or other structures (Li, Delzon, et al. 2020). Such progress will undoubtedly benefit from

technical advances in scientific imagery, developments that continue to push the limits of this field and its techniques (resolution, acquisition time, signal-to-noise ratio, contrast, automatic reconstruction). Nanotomography, for instance, would allow observing details of the wings too small to be observed with microtomography, such as the organization of the cuticle, nerves or small setae.

The change of scale and dimension of our observations, including the fractal version, allows us to consider serving as models for all biological structures, especially those where mineral structures do not appear. This change of observation methods is universal and relevant to take up a certain number of anatomo-morphological or functional approaches. For instance, leaves and leafy plant organs, thallus (Reeb et al., 2018), seed germination, including those in collections (agronomy and conservation of rare species), root development, in nature and under experimental conditions, the micromorphology of embryonic development of arthropods, any morphological element that requires a change of scale of observation to describe its precise morphology and involving a continuous fractal variation in dimension.

Besides stimulating research on insect wings, we hope our study will encourage scholars to develop such use of microtomography as a model of comparative approaches for other structures that, at first glance, might have been ignored or overlooked because of their seemingly 2D appearance and known to be poorly observable under X-ray.

## AUTHOR CONTRIBUTIONS

Thomas Schubnel, André Nel and Romain Garrouste conceived the ideas, and Thomas Schubnel, Arnaud Mazurier, André Nel and Romain Garrouste designed the methodology. Thomas Schubnel conducted the data analysis and wrote the first draft of the manuscript. Thomas Schubnel, Phillipe Grandcolas, Laure Desutter-Grandcolas, Frédéric Legendre, André Nel, and Romain Garrouste wrote the manuscript. All the authors contributed significantly to the drafts and gave final approval for publication.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

All data, including Supporting Information figures and material, are available on Zenodo (European Organization for Nuclear Research & OpenAIRE, 2013): <https://doi.org/10.5281/zenodo.7194658>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1.** *Seμβlis phalaenoides* caddisfly (Trichoptera) with the 'region of interest' shaded in blue.

**Table S1.** List of the scans done with their mains characteristics.