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X-ray microtomography in herpetological research: a review

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Abstract. Herpetological research, like any other (palaeo)biological science, relies heavily on accurate data collection, particularly visualisation and quantification of anatomical features. While several high-resolution imaging methods are currently available, one technique in particular, x-ray microtomography or micro-computed tomography, is on the verge of revolutionising our understanding of the morphology of amphibians and reptiles. Here, we present a review on the prevalence and trends of x-ray microtomography in herpetological studies carried out over the last two decades. We describe its current use, provide practical guidelines for future research that focusses on the morphological study of reptiles and amphibians, and highlight emerging trends including soft-tissue and *in vivo* scanning. Furthermore, while x-ray microtomography is a rapidly evolving field with great potential, various important drawbacks are associated with its use, including sample size effect and measurement errors resulting from differences in spatial resolution and preparation techniques. By providing recommendations to overcome these hurdles, we ultimately aim to maximise the benefits of x-ray microtomography to herpetological research.

Keywords: μCT, anatomy, imaging technique, micro-computed tomography, micro-CT scanning, morphology, staining.

Introduction to x-ray microtomography

Recent advances in imaging techniques have greatly deepened our understanding of organismal morphology within a (palaeo)biological framework. Perhaps the most rapidly evolving development in the field of imaging technology relates to x-ray microtomography. Often referred to as high-resolution x-ray computed tomography (abbr. HRCT or HRXCT), or more commonly, micro-computed tomography (abbr. micro-CT or μCT), x-ray microtomography is becoming the leading method for visualising, describing and quantifying morphological structures with great detail and precision. To date, these technological advances have revolutionised (palaeo)biological research by providing novel taxonomic characters (e.g. Gauthier et al., 2012), facilitating systematic placement of extinct taxa (e.g. Bhullar, 2011; Müller et al., 2011; Dollion et al., 2015) and generating accurate input shapes for modelling and simulation methods (e.g. Kleinteich et al., 2008; McCurry et al., 2015; Jones et al., 2017), to name a few, and have played a particularly significant role in the study of reptiles and amphibians (Wake, 2012).

Computed tomography (CT), schematically illustrated in fig. 1, is defined as the acquisition process of two-dimensional (2D) projection images from different angles around an object by means of x-rays (Hsieh, 2015). These 2D projection images, in turn, can be used to reconstruct a three-dimensionally (3D) rendered volume to create a digital representation of that specific object. Conventional CT was originally popularised as a clinical diagnostic tool (Hounsfield, 1973) and is characterised by two important features that fulfil this purpose (1) the rotation of an x-ray source and imaging detector around a stationary sample (i.e. rotating gantry design geometry), and (2) restricted scanning parameters, including x-ray tube voltage and distance to the x-ray source, to maximise tissue penetration while minimizing exposure to ionising radiation.

Within two decades after the development of commercially available CT instruments,

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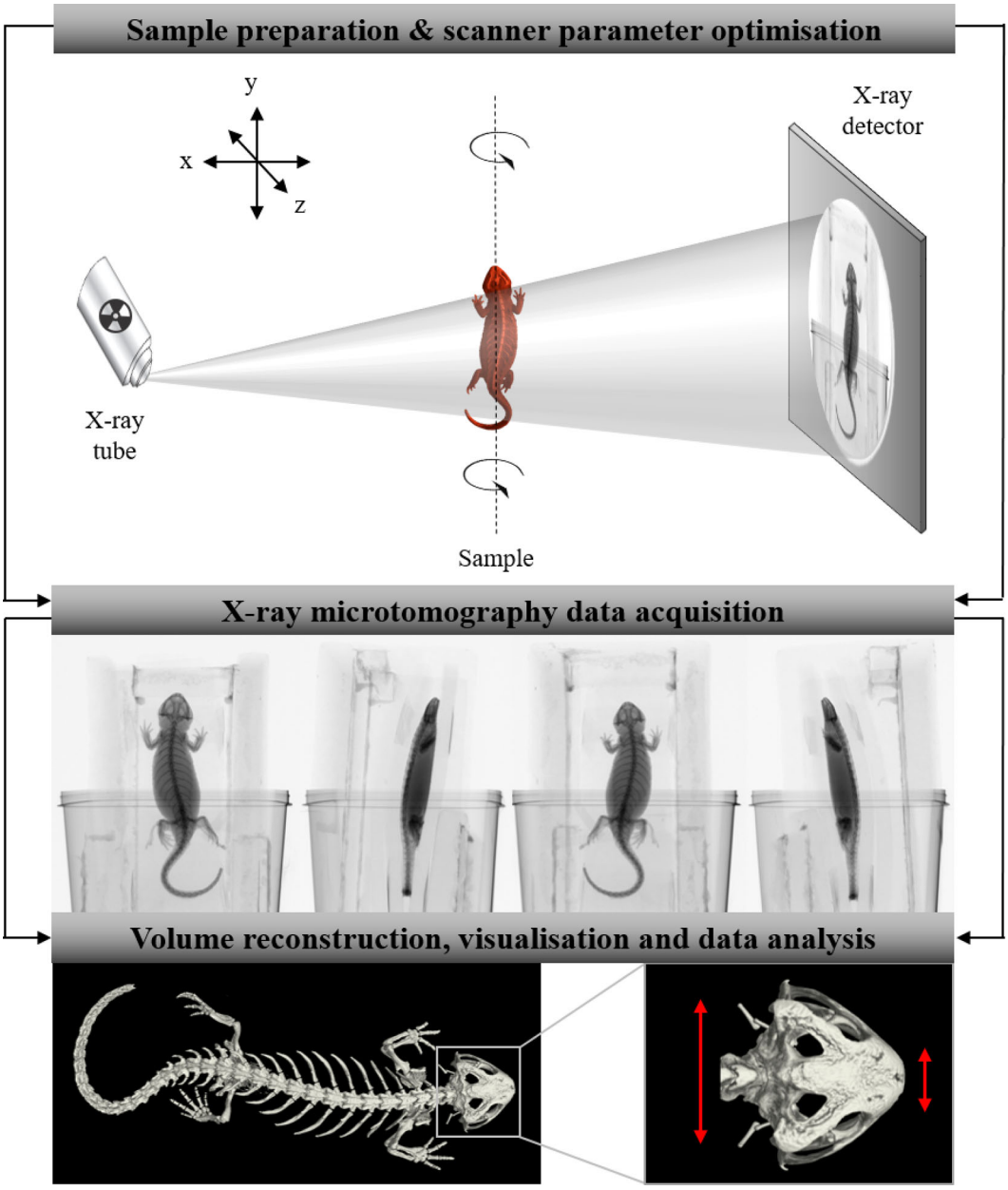

Figure 1. Schematic overview of the x-ray microtomography process in which x-rays emitted by the x-ray tube pass through a rotating sample (here: *Tylototriton verrucosus*) and are recorded by an x-ray detector. The flexible position of the sample with respect to the x-ray source and detector allows for a greater spatial resolution compared to conventional (medical) CT scanners. The resulting projection images can be used to create a three-dimensionally rendered volume that can be visualised and from which data can be extracted.

a research-based approach was adopted with emphasis on increased spatial resolution, resulting in the development of x-ray microtomography (Sato et al., 1981; Elliott and Dover, 1982). In contrast to conventional CT, the majority of x-ray microtomography scanners are characterised by a rotating sample design in which the object rotates on a stage in the space between the x-ray tube and detector. The main advantage of this rotating sample design is the flexibility to scan relatively small samples at high resolution, but also to vary the resolution according to the size of the sample (du Plessis et al., 2017b). As a result, the spatial resolution generated by rotating sample geometry extends past the best resolution of all other gantry style instruments. To illustrate the different sample size and resolution capabilities: conventional (medical) CT provides the ability to scan larger objects with excellent contrast, but with a limited spatial resolution of 0.5-1 mm. The advantage of conventional CT is that it is extremely fast and widely available, therefore definitely useful for larger biological samples (e.g. crocodilians; Lauridsen et al., 2011; McCurry et al., 2017; Sellers et al., 2017). In contrast, typical x-ray microtomography systems allow for higher resolution imaging of samples generally ranging from 5-300 mm, with variable spatial resolutions from 300 μm to 5μm, or, when using transmission x-ray sources and appropriately accurate rotation hardware, down to 0.5 μm for very small samples. A more advanced variant of x-ray microtomography is represented by synchrotron radiation microtomography (abbr. SR-μCT or SRXTM) that uses highly monochromatic (parallel) x-ray beams. Not only can submicron resolution be achieved (up to 50-20 nm; Bartels et al., 2015), synchrotron radiation microtomography allows for high contrast images with easy material discrimination (even non- or weakly-absorbing tissues) and fast imaging due to the high flux (Westneat et al., 2008; Goyens et al., 2018). In addition, the imaging quality of very small samples, or those that are difficult to penetrate (e.g. fossils) can be greatly improved as a result of the monochromaticity and high intensity of the x-ray beams (Lak et al., 2008; Sutton, 2008; Galiová et al., 2010; Cunningham et al., 2014). Nevertheless, synchrotron radiation microtomography is rarely used in (palaeo)biological research because it is not easily accessible, the number of synchrotron facilities is limited compared to CT facilities and the operational cost is usually high. With the widespread availability of laboratory- or desktop-based x-ray microtomography systems, synchrotron radiation microtomography is only used when necessary due to its aforementioned advantages. For instance, to visualise soft tissues of very small samples, where submicron resolution and phase-contrast are required. Phase-contrast microtomography is a recent development is x-ray microtomography, which focusses on phase variations of the x-rays, and has been used to improve contrast in low-density materials, e.g. soft tissue, especially in synchrotron imaging beamlines (Núñez et al., 2017) but also lab-based x-ray sources (Larsson et al., 2016). We refer to Betz et al. (2007) for an overview of the (dis)advantages and applications of synchrotron radiation microtomography.

While conventional CT is still being used to investigate the internal anatomy of larger samples (Lauridsen et al., 2011; McCurry et al., 2017; Sellers et al., 2017), x-ray microtomography has allowed researchers to gain a more in-depth anatomical understanding of a broader range of taxa. Studies on reptiles (incl. squamates, rhynchocephalians, testudines and crocodilians) and amphibians (incl. caecilians, urodeles and anurans) have particularly benefitted from the advances in x-ray microtomography. Nevertheless, despite the increasing number of studies employing x-ray microtomography, the use of this technique in herpetological research has yet to reach its full potential. In this review paper, we present an overview of the current use of x-ray microtomography in herpetological research, provide details on the methodology including sample preparation, as well as recent advances in techniques, such as *in vivo* and soft-tissue scanning.

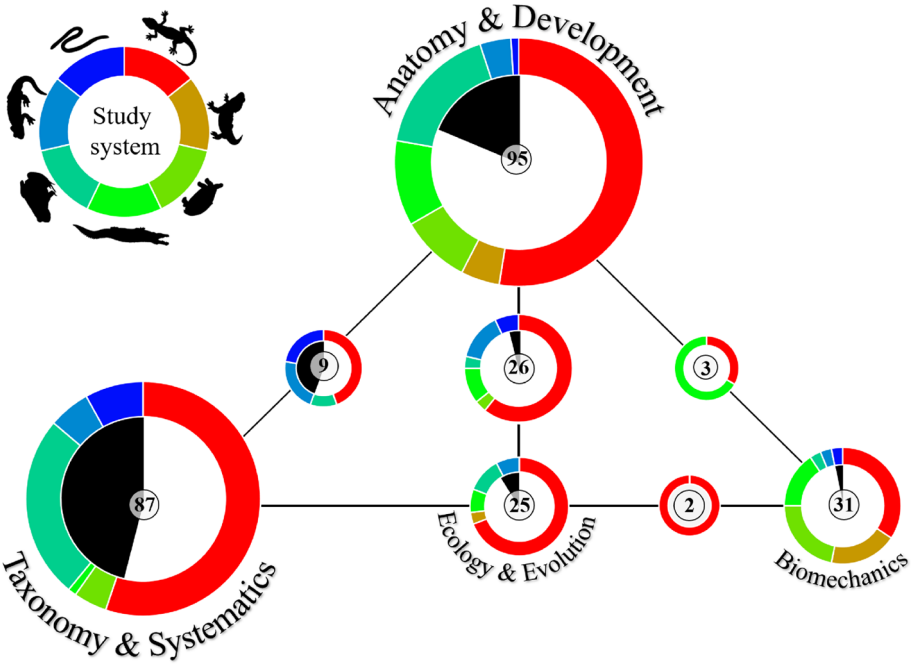

Figure 2. Diagram showing the use of x-ray microtomography in herpetological research. The circles represent the number of studies classified as one of four main topics (i.e. *anatomy and development*, *taxonomy and systematics*, *ecology and evolution* and *biomechanics*) or a combination of two main topics. The inner pie chart indicates the proportion of studies that use fossil material (black), whereas the outer pie chart indicates the study group. From top to bottom (in a clockwise direction): squamates (red), rhynchocephalians (orange), testudines (light green), crocodilians (dark green), anurans (turquoise), urodeles (light blue), caecilians (dark blue).

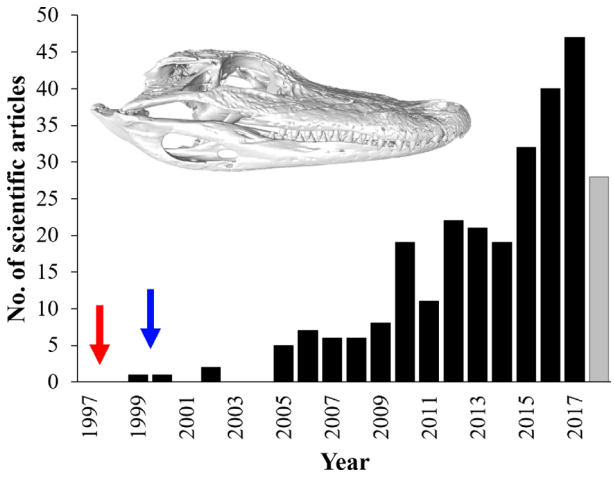

Figure 3. Historical analysis of the use of x-ray microtomography in herpetological research from 1997-2018. While the use of x-ray microtomography has increased rapidly since the mid-2000s, studies are accumulating exponentially in recent years (the grey bar represents studies from January to July 2018). The red and blue arrows indicate the first unpublished and published records of tomography scans, respectively. The three-dimensionally rendered skull of *Alligator mississippiensis*, based on data from Rowe et al. (1999), provides an early example hereof.

and discuss current considerations and future directions. By doing so, we aim to provide a reference source, not only for future herpetological studies, but also for curators of zoological collections and staff of CT-scanning facilities involved in these studies.

Current applications in herpetological research

A literature search was conducted using electronic databases, including Web of Science, Scopus and Google Scholar, with primary search terms "computed tomography", "HRCT", "HRXCT", "Micro-CT" or "μCT" on the one hand, and "reptile", "amphibian", "frog", "salamander", "caecilian", "lizard", "snake", "tuatara", "crocodile" or "turtle" on the other hand. We included studies that make use of both extant and extinct taxa, but the latter were restricted to those concerning taxa that share an immediate relationship with the extant amphibian/reptilian groups. For each study, we recorded the main research topic(s) (i.e. *anatomy and development*, *taxonomy and systematics*, *ecology and evolution* and/or *biomechanics*), the study group(s) (i.e. *squamates*, *rhynchocephalians*, *testudines*, *crocodilians*, *anurans*, *urodeles* and/or *caecilians*), and noted whether or not fossil samples were included. We subsequently visualised this information in a network map (fig. 2). In addition, we described the type of data obtained from the scans (i.e. *descriptive*, *measurement*, *geometric morphometrics*, and/or *simulation/modelling*), recorded detailed information on data collection including the origin of the scans (i.e. *authors*, *previously-published* and/or *database*), the type of scanner, the scanning parameters (i.e. voltage, current, spatial resolution, exposure time, scan time), the number, type and preparation of samples and whether staining was used. A total of 278 studies, ranging from 1999 till July 2018, were eligible for inclusion. Purely-methodological studies were excluded from the data analysis, but instead form an integral part of the review. Although the list is not exhaustive, we believe it to be a thorough and insightful representation of the use of x-ray microtomography in herpetological research. The complete list of studies included is available as supplementary datafile S1.

Historical background

The first x-ray microtomography scans were conducted on Arizona night lizards (*Xantusia arizonae*) and on amber-preserved geckos of the genus *Sphaerodactylus* in 1997 and 1998, respectively (Richard A. Ketcham, pers. comm.), with the latter being published by Grimaldi and colleagues in 2000. Prior to that time, the cranial anatomy of the American alligator (*Alligator mississippiensis*; TMM M-983; http://www.digimorph.org/specimens/Alligator_mississippiensis/adult/) was examined in 1995 using an industrial CT scanner (SMS 101, Scientific Measurement Systems Inc., Austin, Texas) and became available as the "Introduction to *Alligator*: *Digital Atlas of the Skull*" by Rowe et al. (1999). Despite the considerable amount of effort undertaken by the University of Texas High-Resolution X-ray CT Facility to digitise various reptilian and amphibian taxa in the early 2000s (http://digimorph.org/), it was not until 2005 that x-ray microtomography became regularly used in herpetological studies, with an exponential increase in use since 2015 (fig. 3).

Anatomy and developmental biology

The study of the internal anatomy of an animal's body is frequently preceded by destructive methodologies such as dissection or excision of tissues, ultimately leading to the destruction of the sample or specimen. X-ray microtomography, on the contrary, provides morphological information in a relatively non-invasive manner. It is thus no surprise that technological advances in the development of x-ray microtomography, like medical CT, have been expedited by preclinical research studies in which

the internal anatomy of classical experimental models (e.g. mouse, rat, rabbit) is studied (reviewed in Holdsworth and Thornton, 2002; Ritman, 2004). Indeed, the study of the skeleton and bone architecture appears to have been the driving force behind the early advancement of x-ray microtomography systems (Feldkamp et al., 1989), due to the contrast between soft tissue and bone. Likewise, in herpetological studies, x-ray microtomography is being frequently used to describe skeletal morphology, particularly cranial anatomy (e.g. Palci et al., 2016; Ollonen et al., 2018). In addition, x-ray microtomography is recurrently used to investigate skeletal and extra-skeletal elements that have no reliable proxies that can be measured externally (e.g. osteoderms; Broeckhoven et al., 2017a, b; 2018a, b, c). Unsurprisingly, 48.2% of the herpetological studies that use x-ray microtomography encompass a purely anatomical and/or developmental component. Nevertheless, skeletal features only represent one aspect of anatomy. Hence, in recent years, soft-tissue scanning has become increasingly popular in herpetological research. The availability of protocols describing the appropriate use of staining agents (Gignac and Kley, 2014; de Souza e Silva, 2015; Gignac et al., 2016; see below) facilitated not only the visualisation of the architecture of calcified structures, but also of organs, tissues and vasculature (e.g. Porter and Witmer, 2015; Kleinteich and Gorb, 2016; Heiss et al., 2017; Kleinteich and Gorb, 2017; Krings et al., 2017a; Molnar et al., 2017; fig. 4). Soft-tissue staining thus allows for digital dissections in which internal organs can be observed in their natural anatomical context. For example, Porro and Richards (2017) used soft-tissue staining techniques and x-ray microtomography to demonstrate topological relationships between the soft-tissue structures and skeleton in *Xenopus laevis*, one of the most commonly used model organisms in biological research.

Besides visualisation, another advantage of x-ray microtomography over traditional methodologies is that highly accurate *in situ* measurements can be obtained. These include measurements of structures with well-defined edges (e.g. bone) in a three-dimensional space with high accuracy (e.g. Broeckhoven et al., 2016) and extraction of volumetric information from structures of interest such as the brain, vestibular system and vascular canals (e.g. Boistel et al., 2011; Baeckens et al., 2017; Broeckhoven et al., 2017a; Palci et al., 2017). However, despite this potential, 70.1% of the herpetological studies use x-ray microtomography merely for descriptive purposes. Scan-based measurements, either linear or volumetric, characterise 16.2% of the studies, whereas geometric morphometrics are limited to 9% of the studies. The limited extraction of measurements from scans is likely the result of the relatively low number of scans typically used by herpetological studies employing x-ray microtomography (see below).

Taxonomy and systematics

Despite rapid advances in genetic techniques and the ease at which sequence data can be generated, formal morphological descriptions remain pivotal in systematic and taxonomic research. Given the time-consuming nature of the latter, many genetically delineated species lack formal morphological descriptions, as mentioned by Faulwetter et al. (2013). Firstly, species descriptions are complicated by the fact that the morphological study is often restricted to describing external characteristics, in many cases due to the importance of the type specimen(s). It is therefore no surprise that x-ray microtomography has become a highly useful tool for taxonomy and systematics, with recorded use in 34.5% of all herpetological studies that employ x-ray microtomography and focus predominantly on anurans and squamates (fig. 2). A significant contributor to the increase in use of x-ray microtomography is the ability to uncover unprecedented phylogenetic characters, which may, or may not, provide novel in-

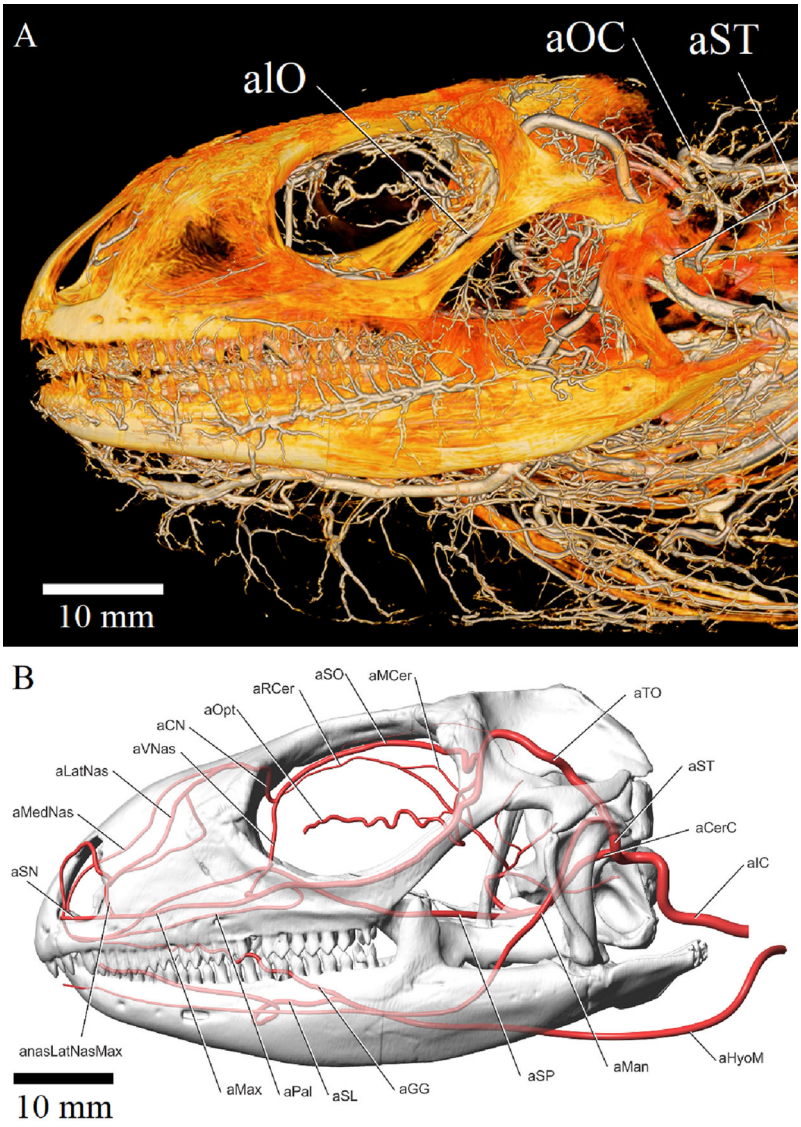

Figure 4. A. Three-dimensionally rendered image of the head of an *Iguana iguana* revealing the blood vessels after arterial and venous injection with a solution of coloured latex and barium. B. Schematic representation of the arteries. From Porter and Witmer (2015) under a CC-BY 4.0 license.

sights into amphibian and reptile phylogenies (Gauthier et al., 2012), or be used to discriminate closely related taxa (Prötzel et al., 2015). To illustrate, Prötzel et al. (2015) use iodine stained hemipenes of the Malagasy chameleons of the genus *Calumma* for species delimitation purposes (fig. 5) and highlight that misinterpretation of three-dimensional structures such as hemipenes is much lower when using x-ray microtomography, compared to light microscopy.

A second concern regarding morphological description is that obtaining comparative material for systematic revision through loans from museums can be difficult or nearly impossible, particularly when it comes to holo- and paratypes. Transportation of specimens requires significant effort and may be costly, besides the risk of losing irreplaceable specimens. In light of this, the establishment of virtual copy or 'cybertype' databases could provide the re-

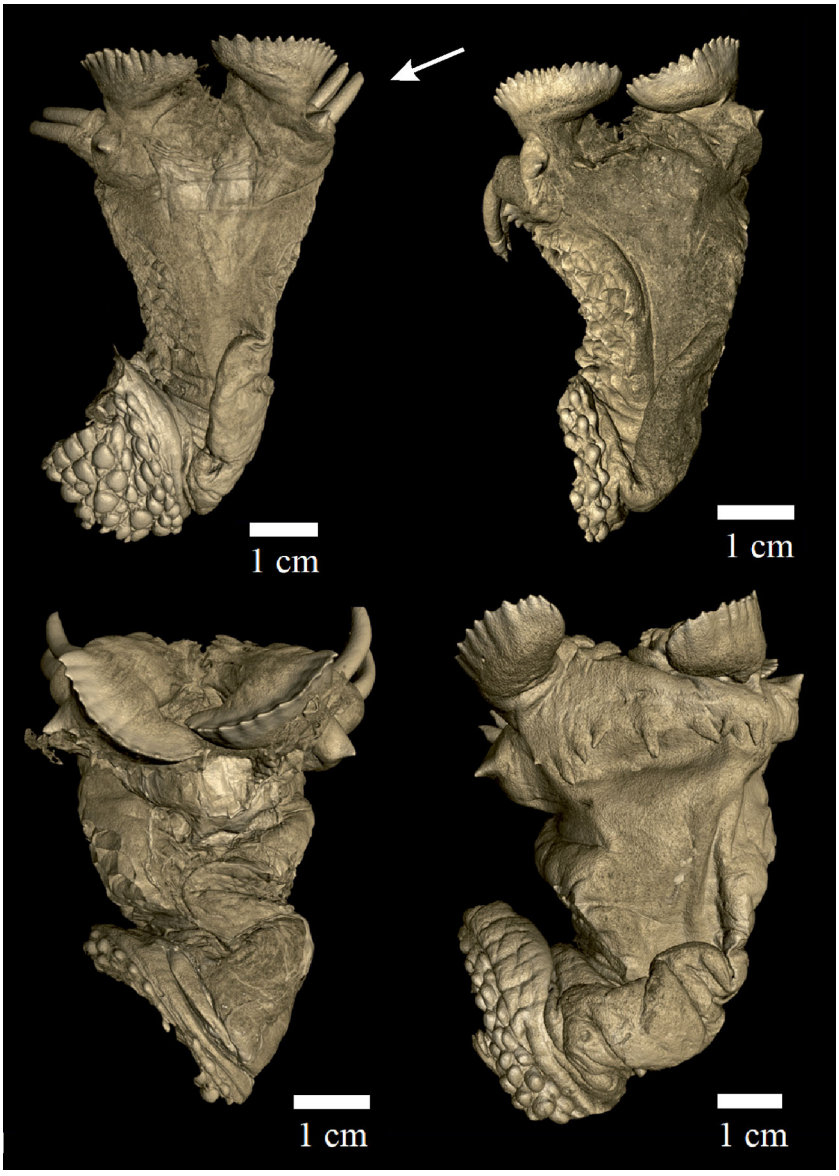

Figure 5. Microtomography scans of hemipenes of the Malagasy chameleon genus *Calumma* in sulcal view. The top images represent intraspecific variation in hemipenis morphology in *C. boettgeri*, whereas the bottom images show intraspecific variation in *C. linotum*. The papillae (indicated by the arrow) can be entirely everted or retracted, which might make morphological description using more traditional imaging techniques (e.g. light microscopy) prone to misinterpretation. This example illustrates how previously overlooked taxonomic characters can be visualised using a combination of soft-tissue staining and x-ray microtomography, and be used for species delimitation purposes. From Prötzel et al. (2015). Copyright © 2015 Magnolia Press.

search community access to morphological information of reference specimens with known identity (see Faulwetter et al., 2013 for a discussion on 'cybertaxonomy'; see below). In most cases, these publicly-available cybertypes will be suitable to confirm species identification without using material from natural history collections, thereby providing a more time- and cost-effective alternative for species examination (Landschoff et al., 2018).

Ecology and evolution

Reptiles and amphibians have a long history of serving as model study organisms in an array of ecological and evolutionary disciplines. Herpetological studies that attempt to disentangle the effects of natural and/or sexual selection on phenotypic traits have benefitted greatly from x-ray microtomography (e.g. Houssaye et al., 2013; Sanger et al., 2013). For example, x-ray microtomography has provided new insight into the ecological drivers of defensive morphologies (Broeckhoven et al., 2018b, c), unravelled the evolutionary history of modern snakes (Yi and Norell, 2015; Da Silva et al., 2018; fig. 6) and ecomorphological relationships among chameleons (Dollion et al., 2017). In addition, x-ray microtomography has allowed researchers to analyse gut content and infer trophic relationships among taxa, both extant (e.g. Bochaton et al., 2015; Ribeiro et al., 2017; fig. 7) and extinct (e.g. Smith and Scanferla, 2016). The proportion of herpetological studies that employ x-ray microtomography to examine aspects of ecology and evolution is 19.1%, of which 73.6% focus on squamates (fig. 2). The relatively low number of studies could be attributed to large sample sizes typically required for ecological and evolutionary research, particularly comparative studies (see below).

Biomechanics

Vertebrate biomechanics often rely on finite-element analysis or modelling to reconstruct stress, strain and deformation, explore fluid dynamics and identify patterns of heat transfer (reviewed in Rayfield, 2007). Examples hereof include the mechanical strength of turtle shell shapes (Rivera and Stayton, 2011; Stayton, 2011), venom flow through snake fang canal (Triep et al., 2013) and the thermoregulatory capacity of lizard and crocodilian osteoderms (Broeckhoven et al., 2017a; Clarac et al., 2017). Scans obtained through x-ray microtomography can be readily converted to (mesh-based) models and provide a significant advantage over more traditional methods, such as three-dimensional laser surface scanning, because internal voids and spaces can be easily incorporated. For instance, the mechanical behaviour of snake fang phenotypes with venom-conducting canals (Broeckhoven and du Plessis, 2017; du Plessis et al., 2017a; fig. 8A) or osteoderms with a high degree of vascularisation (Broeckhoven et al., 2017a; fig. 8B) can be readily investigated using simulations conducted directly on high-resolution microtomography scans. Likewise, multi-body models can be used to study the mechanics of complex, integrated functional systems, such as those involved in vertebrate locomotion and feeding (Gröning et al., 2013). For example, three-dimensional models are frequently used to infer *in vivo* bite forces from extinct and extant taxa including rhynchocephalians (Curtis et al., 2010) and lizards (Gröning et al., 2013). Nevertheless, the accuracy of model predictions appears to be highly dependent on the musculature (Gröning et al., 2013). More detailed information on muscle architecture and size, which can be easily obtained by using staining techniques (see below), might provide much improved models for simulation analyses. The percentage of herpetological studies using x-ray microtomography to gain insight into biomechanics is relatively low, only 12.9%, with studies focussing mainly on reptiles (94.4%; fig. 2).

Palaeobiology

In palaeobiology, x-ray microtomography can be used to examine the morphology of fossilised taxa without mechanical removal of the surrounding matrix (reviewed in Sutton, 2008; Cunningham et al., 2014). By doing so, damage to delicate structures can be avoided and well-preserved soft tissues can be examined. X-ray microtomography is not only used to provide

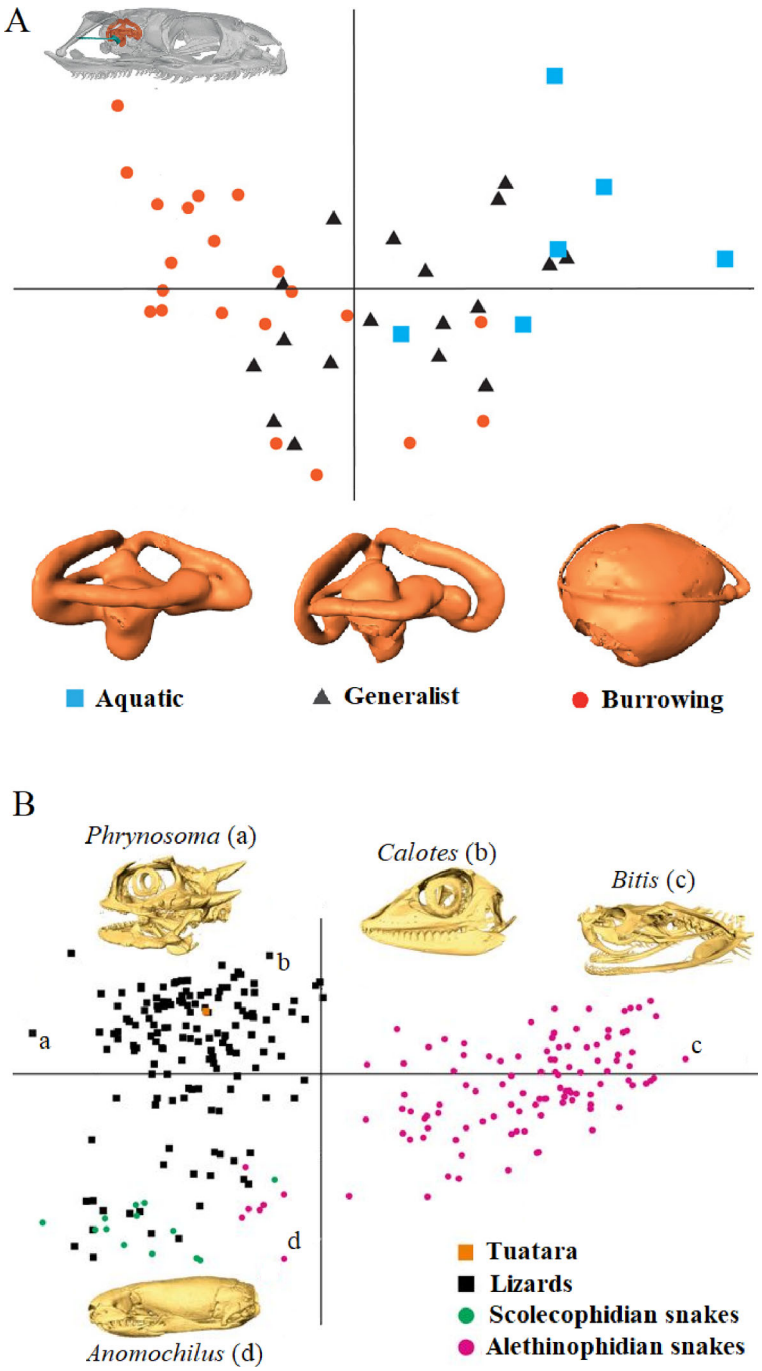

Figure 6. Graphs showing morphological variation in squamate vestibular (A) and skull shape (B), respectively, based on geometric morphometric analysis of microtomography scans. Accurate morphological information combined with large sample sizes can provide answers to evolutionary questions, such as the origin of modern snakes. Modified from Yi and Norell (2015) and Da Silva et al. (2018) under CC-BY 4.0 licenses.

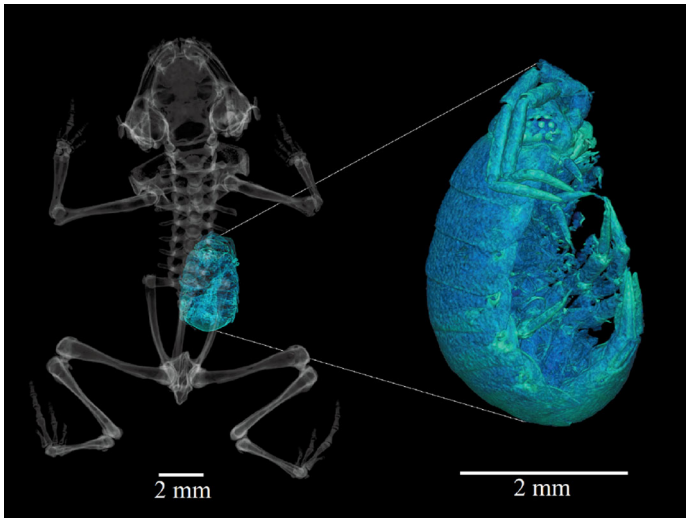

Figure 7. Three-dimensionally rendered image of a *Brachycephalus curupira* paratype with isopod present in the stomach showing how x-ray microtomography can be used to obtain dietary information from valuable or rare specimens. From Ribeiro et al. (2017) under a CC-BY 4.0 license.

detailed anatomical information for newly described fossils (e.g. Xing et al., 2018b) but has also provided significant insight into the evolution of structures (e.g. crocodilian brain; Witmer et al., 2008), and can be used to assess and quantify differences between extinct and extant taxa (e.g. Dollion et al., 2015; Matthews and du Plessis, 2016). Nevertheless, the technique might not be suitable for all fossils, because successful acquisition of data is limited mainly by the size of the object and density of the surrounding matrix. Firstly, scanners must be sufficiently large to accommodate the specimens and the voltage of the x-ray tube must be powerful enough to allow for penetration of the material (Abel et al., 2012; see below). Secondly, sufficient density contrast must be present otherwise the voxel grey values of the fossil are impossible to distinguish from those of the surrounding matrix (Abel et al., 2012). Despite the aforementioned hurdles, 24.1% of all herpetological studies that employ x-ray microtomography include or are based on fossil material (fig. 2). Fossils embedded in amber are particularly suited for x-ray microtomography because of their size and composition (Dierick et al., 2007), with numerous examples present in herpetological literature (Grimaldi et al., 2000; Polcyn et al., 2002; Daza and Bauer, 2012; del Rosario Castañeda et al., 2014; Sherratt et al., 2015; Fontanarrosa et al., 2018; Xing et al., 2018a, b; fig. 9).

Methodological approaches and techniques

Sample preparation

Sample preparation for x-ray microtomography requires little time and/or technical expertise and can be adjusted based on individual preferences, guidelines provided by the scanning facility or guidelines stated in agreements with research institutions (i.e. museums). However, proper sample preparation (fixation) is pivotal to avoid changes in physical characteristics of the sample over time (i.e. shrinkage) and to avoid movement of the sample relative to its original position. The latter is more important for x-ray microtomography systems in which a rotating sample design is used than for systems with a rotating gantry design (e.g. medical CT-scanners, *in vivo* micro-CT scanners). Sample preparation consist of (1) pre-scan fixation and

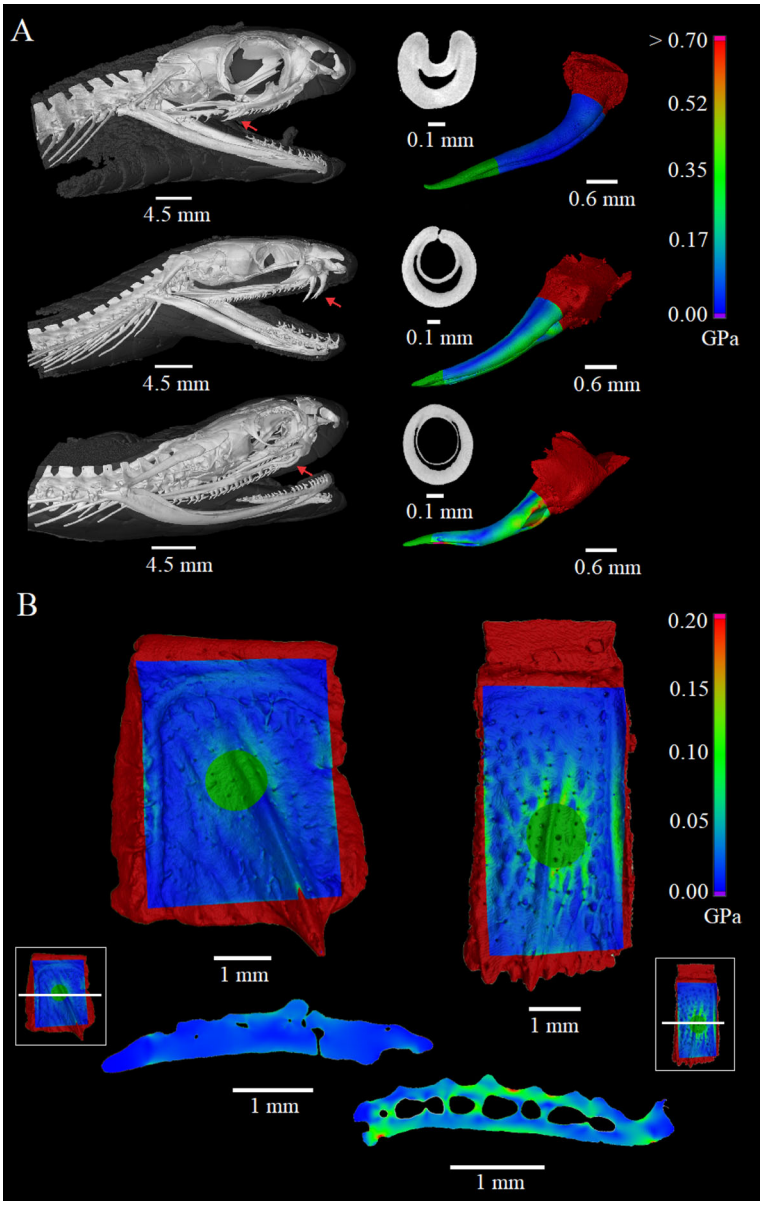

Figure 8. Three-dimensionaly rendered images showing the stress distribution (in GPa) in venomous snake fangs (A) and cordyline lizard osteoderms (B) after a load is applied. Modified from Broeckhoven and du Plessis (2017b) and Broeckhoven et al. (2017a) with permission.

(2) sample mounting. While little fixation is required for samples like fossils or dried skeletal material, the majority of specimens need to be fixed in formalin or Bouin's solution, preserved in ethanol, flash-frozen in liquid nitrogen, dried by means of air, chemicals or using critical-point drying, or a combination of techniques. Gutiérrez et al. (2018) found that (air) drying samples (here: crickets) provides the best result, with formalin-fixation in combination with air- or critical-point drying resulting in a more natural tissue appearance with less artifacts compared to ethanol or fixation using Bouin's fluid (but see Sombke et al., 2015). Critical-point drying has been successfully used for herpetological samples (i.e. tadpoles) and

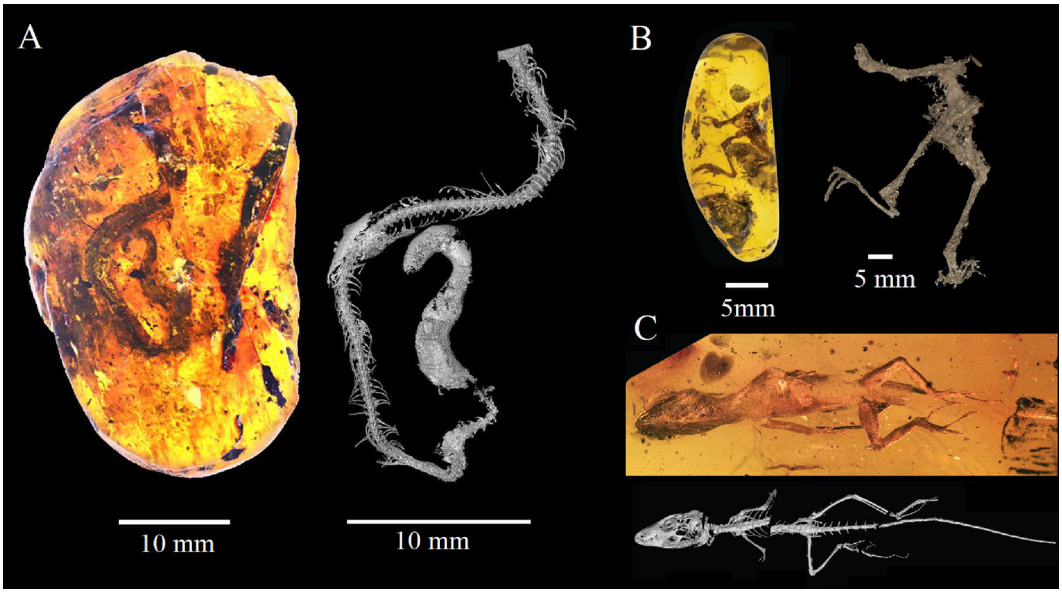

Figure 9. X-ray microtomography is particularly suitable for amber fossils with numerous examples present in literature including snakes (A), frogs (B) and lizards (C). Images (A) and (B) are modified from Xing et al. (2018a, b), whereas image (C) is taken from Morphobank (http://morphobank.org/permalink/?P1108; based on Castañeda et al., 2014) under CC BY and CC BY-NC 4.0 licences.

even allows for the discrimination and identification of softer tissues (Krings et al., 2017b). While the majority of fixation techniques have limited invasive effect on anatomical structures besides shrinkage (see below), Shu et al. (2018) reported that the skulls of tadpoles could hardly be discerned by x-ray microtomography after formalin fixation, due to its decalcifying capacity. Hence, formalin solution used for fixation should be kept neutral (buffered) to reduce decalcification (Shu et al., 2018). It must be noted that the choice of pre-scan fixation will be largely determined by the ultimate purpose of the specimen and that fixation techniques are not necessarily selected in function of image quality optimisation. For example, museum collections generally consist of formalin-fixed, ethanol-preserved specimens. Given the importance of these specimens, (air) drying might not be suitable given its potentially invasive and lasting effect on skin morphology, particularly in amphibians.

Sample mounting requires the use of low-density materials (e.g., agarose, floral foam, polystyrene, plastic, cardboard) to separate the sample from the high-density hardware and, more importantly, avoid movement of the sample during stepwise rotation (du Plessis et al., 2017b; Keklikoglou et al., 2016a; supplementary fig. S1). Furthermore, to avoid dehydration and consequently shrinkage during the scanning, the sample can be wrapped in a cloth drenched in fluid prior to mounting, or covered with plastic film (e.g. Parafilm®) (e.g. Kurosaka et al., 2008; Scherz et al., 2014, 2015). Alternatively, specimens can be scanned inside liquid-filled tubes (e.g. Kleinteich and Gorb, 2015; Lukanov et al., 2016). In the latter case, care should be taken that the specimen does not contact the edges of the tube as this could complicate the image processing steps (du Plessis et al., 2017b). Ethanol provides a greater tissue contrast than water, because of its lower density (Metscher, 2009). Lastly, samples should not be mounted vertically but tilted at a slight angle to minimise parallel surfaces to the x-ray beam and consequently reduce image artifacts (du Plessis et al., 2017b; supplementary fig. S1).

As mentioned before, pre-fixation and mounting techniques might affect the physical characteristics of the sample. Drying samples, for example, might not cause substantial shrinkage in skeletal features, but could shrink and even damage soft tissues (Faulwetter et al., 2013; Krings et al., 2017) and might therefore be unsuitable for reference (museum) specimens. Shrinkage has got a crucial influence on morphological analysis, particularly with regards to quantitative measurements, and could complicate subsequent data analysis across studies. Surprisingly, 33.5% of herpetological studies that employ x-ray microtomography and make use of extant material do not provide any information on the condition of the sample (e.g. preserved, fixed). Moreover, only 13.3% of the studies provide detailed information on how samples were mounted and/or how moisture was retained during scanning.

Soft-tissue scanning and staining techniques

Detailed morphological visualisation and quantification of soft tissues form an integrated part of herpetological studies, particularly those focussing on anatomy and developmental biology, with over 30 studies to date reporting the use of soft-tissue scanning and staining techniques. Soft tissues display very low x-ray absorption compared to skeletal elements and samples should ideally be stained with contrast-enhancing chemicals (Krings et al., 2017b; supplementary fig. S2). Visualisation of low absorbing tissues without staining is possible but requires specific x-ray beam characteristics typically found at synchrotron facilities. Fortunately, a variety of effective staining agents is currently available and their use is well-documented in the literature (Gignac and Kley, 2014; de Souza e Silva, 2015; Gignac et al., 2016; see Keklikoglou et al., 2016a for limitations). While osmium tetraoxide (OsO_4) has long been among the most successfully used contrast-enhancing chemicals (see Willaert et al., 2013 for an example of OsO_4 use in anurans), much recent attention has been devoted to cost-effective, non-toxic staining agents. Iodine staining (I_2E, I_2M or I_2KI, also known as Lugol's solution) is characterised by rapid tissue penetration and excellent tissue contrast (Metscher, 2009; Pauwels et al., 2013; Gignac et al., 2016), and is particularly suitable for larger samples (Pauwels et al., 2013). Phosphotungstic (PTA) and phosphomolybdic (PMA) acid staining likewise produce excellent tissue contrast (Metscher, 2009; Pauwels et al., 2013; Krings et al., 2017; supplementary fig. S2), often superior to iodine staining (Krings et al., 2017b), but penetration rate is considerably slower. Both PTA and PMA are particularly suitable for smaller samples (e.g. tadpoles; Krings et al., 2017b), and/or if structures within tissues are to be revealed (Pauwels et al., 2013; Goyens et al., 2018). Iodine staining provides the best result when freshly fixed material is used (Gignac et al., 2016), whereas PTA/PMA have been advised for Bouin- or formalin-fixed ethanol-preserved specimens (Metscher, 2009). Non-surprisingly, iodine staining and PTA/PMA are the most commonly used stains in herpetological studies that employ x-ray microtomography and staining techniques (57.7% and 26.7%, respectively).

Despite the fact that soft-tissue staining can provide sharp image contrast, staining of specimens might be considered an invasive technique in some cases. Firstly, PTA and PMA are acidic in solution and might result in decalcification, particularly of porous bone structures (Metscher, 2009; Pauwels et al., 2013). Yet, the exact extent to which these staining agents decalcify tissue remains to be tested. Secondly, staining is difficult or even impossible to remove from samples (Faulwetter et al., 2013; Pauwels et al., 2013; Fernández et al., 2014; Keklikoglou et al., 2016a). Hence, non-reversible staining methods might not be suitable for reference (museum) specimens. Iodine staining is more suitable in this regard as it is considered a reversible process (Akkari et al., 2015; Gignac et al., 2016), although any long-term effects have yet to be quantified. Nevertheless, approaches to remove contrast-enhancing

chemicals from tissues are receiving much attention in literature (Schmidbaur et al., 2015; Gignac et al., 2016). Lastly, contrast-enhancing chemicals have a higher osmolarity than biological tissues and might cause additional shrinkage in soft tissues (Vickerton et al., 2013; Buytaert et al., 2014). In a recent study, Hedrick et al. (2018), suggest that fixation and/or preservation agents, besides other factors such as storage duration, temperature and agent concentration, have a stronger effect on tissue shrinkage than the staining itself (here: iodine). In addition, shrinkage also appears to depend on the tissue type, with brains generally speaking experiencing significant levels of shrinkage (Buytaert et al., 2014; Hedrick et al., 2018). The use of water-based contrast enhancement agents might provide a solution to this problem, or alternatively, a gradual dehydration procedure should be implemented (Keklikoglou et al., 2016a).

Scanning set-up and settings

Microtomography systems – A wide variety of commercially available x-ray microtomography systems that differ considerably in their capabilities and performance exist, ranging from small desktop models to large walk-in scanners. Desktop microtomography systems typically have an x-ray source voltage of 20-130 kV and are suitable for smaller samples up to approximately 200 mm. Examples of such systems used in herpetological research include the Bruker Skyscan and Scanco μCT series, amongst others. In contrast to these desktop models, core facilities at universities, museums or other research institutes typically possess larger microtomography instruments (e.g. du Plessis et al., 2016a), which include microfocus x-ray sources up to 225 kV and allow larger samples to be scanned. Additional advantages of these advanced systems include more flexible scan durations compared to desktop models and a higher x-ray tube voltage which allows larger samples to be imaged with sufficient x-ray penetration. The latter is especially important for fossils embedded in rock (see below). Many manufacturers of this type of system can be found with GE v|tome|x and nanotom, Nikon XTH series, Zeiss Xradia MicroXCT, Bruker Skyscan (e.g. model 2211) and Viscom systems to be most commonly used in herpetological studies. For relatively large samples (up to 60 cm wide and 2 m long), conventional (medical) CT systems may provide comparable imaging quality (du Plessis et al., 2016b). *In vivo* small animal imaging can be performed in similar gantry-style instruments (but see below), which allows scans to be completed faster than the microtomography instruments listed above. However, as mentioned before, the rotating gantry design limits variations of scan parameters and sample sizes. Although *in vivo* microtomography imaging devices are typically used for preclinical studies, models including GE eXplore Locus, Scanco Viva-CT, Bruker SkyScan *in-vivo* Micro-CT, Siemens Inveon MicroCT and MicroCAT are sometimes used in herpetological research.

Tube voltage – While the peak voltage of the x-ray tube of medical CT scanners is optimised around 80-140 kV, microtomography systems can be operated at a range of tube voltages, as mentioned above. Tube voltage is highly dependent on the type of sample, with suggested values of 30-100 kV for biological samples, 60-150 kV for small rock-embedded fossils and 160-240+ kV for large rock-embedded fossils (du Plessis et al., 2017b). These reference values are comparable to those used in herpetological studies: average tube voltage across all studies was 101 $\pm$ 46 kV (range: 30-420 kV; $n = 148$), with biological samples generally exposed to less voltage (92 $\pm$ 40 kV; $n = 107$) than fossil samples excluding those preserved in amber (126 $\pm$ 53 kV, $n = 40$). Lower tube voltages usually result in improved image quality (Broeckhoven et al., 2017c; Shu et al., 2018) and smaller samples generally require lower voltage than larger specimens (du Plessis et al., 2017b). A recent study by Shu et al. (2018), shows that at lower tube voltage

(i.e. 30 kV compared to 70 kV) tadpole vertebrae cannot be distinguished from polypropylene pipette tips, whereas at higher tube voltage (i.e. 90 kV compared to 70 kV), differentiation between vertebrae and preservative fluid (here: ethanol) proved difficult. In biological samples, higher tube voltage is often warranted when discrimination of soft-tissues (i.e. contrast-enhanced specimens) is important (Gignac et al., 2016) or if structures-of-interest are shielded by dense material (e.g. carapaces of testudines, osteoderms; Broeckhoven et al., 2017c). In all cases, it is important to bear in mind that scanning parameters, particularly tube voltage, might need to be adjusted for a specific sample through trial-and-error to minimise noise and artifacts, such as beam hardening (see below). We refer to du Plessis et al. (2017b) for an overview of possible errors and artifacts encountered during scanning with reference to tube voltage.

Tube current – An increase in current increases the number of electrons generated in the x-ray tube, which, in turn, increases the intensity of the beam. The main advantage hereof is that it allows for an improved signal-to-noise ratio. An increase in current, however, increases the x-ray spot size, which may result in blurred images and consequently poor imaging resolution. Nonetheless, the impact of the tube current on image quality appears to be less important than the effect of tube voltage (figs 10B1-C1; Broeckhoven et al., 2017c; Shu et al., 2018). More importantly, x-ray tube increase might be detrimental to the system's hardware, hence it is common practice to limit the current in microtomography systems to 100-200 μA. Indeed, average tube current across the herpetological studies was 175 ± 120 μA (range: 20-1800 μA; $n = 142$). It must be noted that tube current is limited by the maximum power capability of the system (i.e. high power is not possible when using desktop systems).

Filtration – Pre-filtering removes low-energy photons and offers correction for beam hardening which arises when polychromatic x-ray beams are absorbed differently by the sample and which results in reduced image quality and presence of artefacts (du Plessis et al., 2017b). We found that 15.1% of herpetological studies reported whether filtration was used or not. Of these 58.8% reported the use of 0.5 mm aluminium (Al, range: 0.25-1.5 mm) and 0.1 mm copper (Cu) filters. As evident from figs 10D1-D3, using a thin filter (e.g. 0.1 Cu; fig. 10D1) does not affect image quality, but quality decreases significantly when thicker filters are being used (figs 10D2-D3). Generally speaking, filtration is not required for small or low-density samples (but see below) but should only be used in case excessive beam intensity is available (i.e. to provide similar intensity at higher x-ray tube current).

Spatial resolution – X-ray microtomography systems are predominantly defined by their superior imaging performance which is characterised by a high spatial resolution compared to conventional CT systems. Spatial resolution describes the ability of the system to resolve details in an image and is frequently presented as the dimensions of a three-dimensional pixel or voxel. Spatial resolution was reported by half of the herpetological studies (i.e. 51.4%) and ranges from 0.67 to 250 μm (mean: 29.7 ± 28.1 μm, $n = 141$). Despite being frequently reported, voxel size and actual image resolution (which also depends on contrast) are different concepts (du Plessis et al., 2017b). While references are mostly made to voxel size, detailed information on the scanner set-up is required to ascertain whether image resolution is effectively related to the voxel size. In practice, the best possible voxel size depends on both the size of the sample, its distance from the x-ray source (figs 10A1-A2) and detector, as well as the size of the x-ray detector, and can be easily calculated if this information is available (see du Plessis et al., 2017b). One notable exception is x-ray microscopy, whereby the voxel size is less limited by sample size and distance from the source, making it possible to scan smaller areas within a larger sample at high resolution, compared to typical geometrical magnification

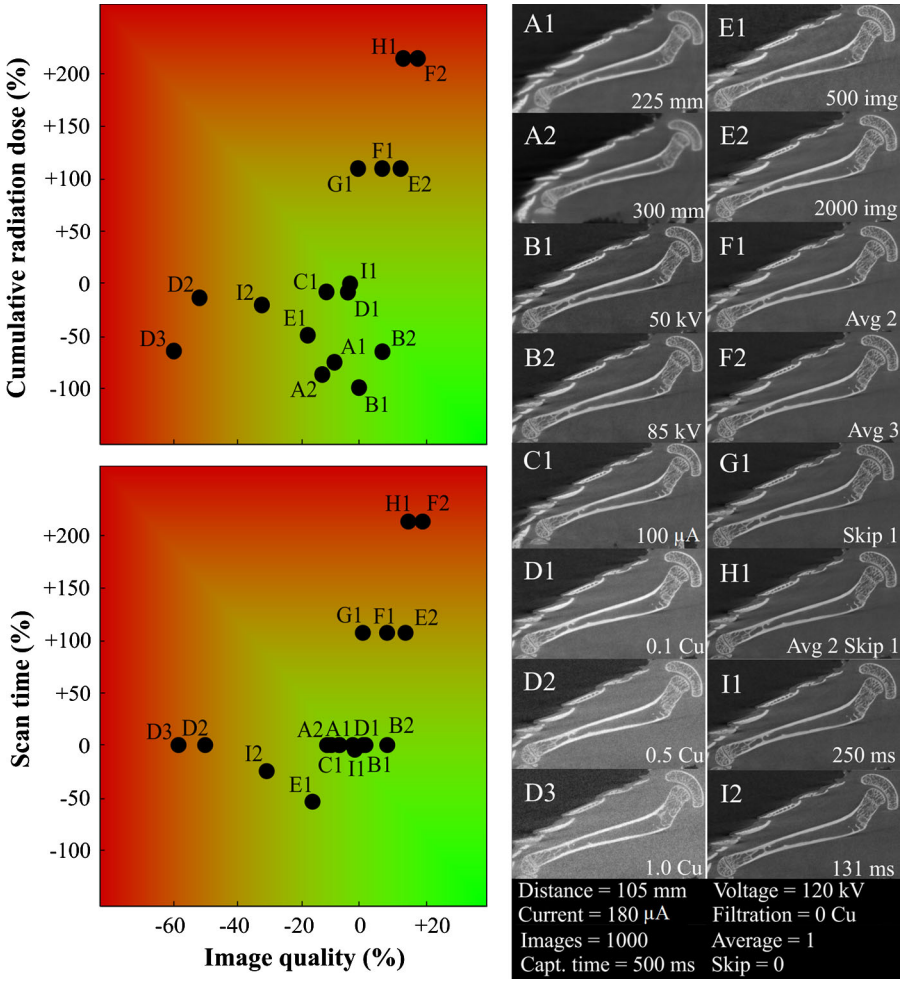

Figure 10. Scatterplots depicting the effects of altering scanner parameter settings on total radiation dose, total scan time and image quality. Each black circle represents an increase or decrease in one specific parameter value (indicated in bottom right corner of the slice views) relative to the reference settings (indicated in the black box). The images are slice views through the hind leg of *Ouroborus cataphractus* and show the femur as well as the osteoderms in the skin. Modified from Broeckhoven et al. (2017c) with permission.

systems. The most important factor to consider is the partial volume effect in which the feature of interest nears the voxel size, leading to erroneous determinations of dimensions.

Scan time – Total scan time depends on a variety of factors including, but not limited to, number of images, image averaging, image acquisition or exposure time and rotation steps (degree). Given that each system has its own unique combination of settings, total scan time can vary considerably among studies. Many setups use a continuous scanning method (i.e. continuous rotation and image acquisition without steps), which, for instance, does not allow image averaging. Total scan time is rarely explicitly reported by herpetological studies (9.4% and 17.6% of the cases, respectively), but is generally around 39 $\pm$ 29 minutes (range: 10-120 minutes; $n = 27$). In exceptional circumstances, for instance when x-ray microscopy is used, a significantly longer scan time (up to 20 h) might be required due to its different geometry and detection system.

Total scan time has important implications for imaging quality, often defined by the signal-to-noise ratio (SNR; e.g. Broeckhoven et al.

2017c) or contrast-to-noise ratio (CNR; e.g. Goyens et al., 2018). Image quality, up to a certain point, increases with scan time (Shu et al., 2018), number of images (figs 10E1-E2) and exposure time (figs 10 I1-I2). Furthermore, in some systems the sample is rotated in a stepwise fashion during which multiple images can be acquired and averaged per step, or the first image(s) at each new step position can be skipped. These averaging and skipping methods might reduce noise and contribute greatly to improved image quality (figs 10 F1-H1). Especially for samples that might experience vibrational movement during rotation, such as the adhesive setae on the feet of geckos (Green et al., 2018), using averaging and skipping methods or switching to a set-up that uses continuous instead of step-wise scanning, is warranted (du Plessis et al., 2017b).

Nevertheless, despite improved image quality, prolonged scan times might (1) induce shrinking effects when samples are scanned outside of preservative fluid or significantly increase radiation dosage in case of live animals (see above); (2) incur significant financial costs, especially if an external scanning facility with fixed rates has to be used by the researcher and (3) increase data size, especially in case of a higher number of images, which may adversely affect data analysis, storage and sharing. Hence, a trade-off must be made between image quality and total scan time, based on the above-mentioned reasons.

In vivo scanning and radiosensitivity

In vivo scanning is frequently used in the biomedical field to investigate the anatomy of live animals and gain insight into disease status or progression (reviewed in Ritman, 2004; Schambach et al., 2010). The main advantage of *in vivo* x-ray microtomography is that repeated measurements can be taken at different time points without the need to sacrifice study subjects (e.g. tooth cycling in alligators; Wu et al., 2013; Widelitz et al., 2017). The latter advantage makes the technique particularly suitable for ecological and evolutionary studies that rely on large sample sizes, include protected or rare species, or both (e.g. osteoderms in armadillo lizards; Broeckhoven et al., 2018b). The high demand for three-dimensional imaging of live experimental animals for preclinical research has resulted in the availability of multiple purpose-built *in vivo* scanners with a rotating gantry design (i.e. similar to medical CT-scanners) to facilitate the use of inhalation equipment needed for the administration of anaesthesia (Schambach et al., 2010). Nevertheless, Broeckhoven et al. (2017c) recently developed a protocol to scan reptiles and amphibians, *in vivo*, using more commercially available scanners with rotating sample design. By benefitting from their poikilothermic nature, reptiles and amphibians can be immobilised by cooling, thereby avoiding risks associated with the administration of anaesthesia (Broeckhoven et al., 2017c). However, *in vivo* scanning requires several deviations from the aforementioned guidelines. The foremost concern is reducing exposure to ionising radiation which can be accomplished by (1) making use of metal filtration to filter out the low-energy photons, (2) decreasing x-ray tube voltage, (3) keeping scan duration to the minimum and (4) limiting proximity of the sample to the x-ray source (i.e. lower radiation exposure at the expense of spatial resolution). An overview of the effect of adjusting scanner settings on total radiation exposure and image quality is illustrated in fig. 10. We refer to Broeckhoven et al. (2017c) for guidelines on radiation dose calculation, as well as radiosensitivity (LD_{50}) in reptiles and amphibians. *In vivo* x-ray microtomography provides great advantages over traditional scanning methods (Holdsworth and Thornton, 2002) if the above-mentioned concerns are considered. Yet, its use in herpetological research is still at an early stage and future applications (e.g. use of radiocontrast agents) should be further explored.

Radiation exposure risk is not only limited to live organisms, but concerns have also been

made with regards to its detrimental effects at cellular level. Paredes et al. (2012) found no significance difference between DNA strand length of tissues exposed and unexposed x-ray radiation. Similarly, Faulwetter et al. (2013) show no effect of x-ray radiation on sequence fragments of 16S rRNA even when repeatedly exposed. Other studies show a clear relationship between increasing x-ray dose and ancient DNA damage, yet no negative effects appear to take place when the radiation dose is kept below 200 Gy (Immel et al., 2016). This limit is significantly higher than the radiation dose typically accumulated during a conventional microtomography scan (e.g. Broeckhoven et al., 2017c report values of only 0.003-0.017 Gy min^{-1}).

Challenges and present considerations

The vast amount of possibilities with regards to sample preparation, scanner set-up and scanning parameters, as indicated in the aforementioned sections, make the presence of confounding factors unavoidable, which, in turn, could increase measurement error and have a significant effect on the interpretation of results. From our review of herpetological literature, we found that detailed methodological information is lacking in most studies.

Firstly, the preservation method and preparation of samples for x-ray microtomography (e.g. staining) might cause shrinkage and therefore have an important effect on morphological measurements (Vervust et al., 2009; Buytaert et al., 2014). The degree of shrinkage not only depends on tissue type, but also on preservative agent, drying method prior to scanning and staining method (Buytaert et al., 2014; Sombke et al., 2015). Although fresh or previously frozen material is more suitable in this regard (Vickerton et al., 2013), most specimens in herpetological collections have been subjected to fixation and preservation methods. Hence, future studies should provide sufficient details on sample preparation and/or take any potential effect of variation in preparation method into consideration.

Secondly, another potential source of error might arise from variation in spatial resolution. Scanning with low resolution relative to the actual structure size might cause an overestimation of the object due to partial-volume effects (Bouxsein et al., 2010) and this effect might be more prominent in smaller specimens. Supplementary fig. S3 shows the effect of spatial resolution (i.e. 35 μm versus 100 μm) on measurement errors in osteoderm volume based on data from Broeckhoven et al. (2017c). While no noticeable differences in slopes are present, the intercepts of the regression lines differ significantly. Here, we suggest that comparison should be limited to scans obtained at similar spatial resolution or, alternatively, that spatial resolution should be included as a random factor in analyses, especially when a large number of samples is used.

Lastly, we emphasize that few herpetological studies that make use of x-ray microtomography report to have extracted data from more than a single individual per species. The median ratio of specimens per species was 1 (range: 1-211 specimens/species). Limited sample size even appears to be the general trend, especially in ecological studies that make use of x-ray microtomography (Gutiérrez et al., 2018). Low sample sizes preclude powerful statistical analyses, especially those conducted in a comparative context, and limit interpretations if variation in the trait of interest has an intra- or interspecific basis. While in some cases, limited sample sizes are due to the rarity of samples (e.g. fossil specimens or para- and holotypes), in many cases, more material would have been available, but it is not included. Despite the effort needed to process multiple samples, researchers could easily generate large amounts of data (i.e. large sample sizes) within a short time span by carefully selecting scanner parameters (see above). This might be more meaningful for future herpetological studies than investing time/effort in attempting to maximise the image quality of a

single scan or improve the visual appeal of microtomography images.

In addition, making data available to other researchers can facilitate the process of obtaining a larger sample size, provided that differences in scanning parameters among data files are considered. A large number of microtomography scans can already be accessed through online repositories including the Digital Morphology Library (http://www.digimorph.org), MorphoBank (https://morphobank.org) and MorphoSource (http://morphosource.org) amongst others. We refer to Lebrun and Orliac (2017) for an overview of virtual repositories that provide access to 3D datasets of biological specimens.

Conclusions and future directions

The ability to generate three-dimensional models at high resolution in a relatively non-invasive, cost-effective way, render x-ray microtomography a particularly useful technique for (palaeo)biological studies. X-ray microtomography has been utilised in herpetological research over the last two decades and its use has grown exponentially in the last few years (fig. 3). Microtomography instruments are becoming more accessible to researchers, with recent advancements in hardware manufacturing (e.g. helical scanning trajectories, multi-energy x-ray detectors) and software (e.g. reconstruction, data analysis and visualisation programs), facilitating data collection and the quality thereof. Despite the various methodological hurdles mentioned in this review which have to be overcome in the near future, x-ray microtomography has the potential to become the golden standard for morphological studies in herpetology. Novel insights have already been gained into the development of anatomical structures (e.g. Wu et al., 2013), systematic placement of fossil taxa (e.g. Xing et al., 2018a, b), as well as the evolution of morphological traits (e.g. Yi and Norell, 2015; Broeckhoven et al., 2016, 2018c; Da Silva et al., 2018), among others. Although still in their early stages of experimentation and implementation, *in vivo* and soft-tissue scanning, or a combination of these methods, will certainly become of great value to herpetologists, or developmental biologists in general. Furthermore, the ongoing trend to increase spatial resolution combined with advancements in staining techniques could provide an alternative to the time-consuming manual process of histologic examination.

The ever-growing number of digital specimens that can be easily accessed through online repositories (e.g. Keklikoglou et al., 2016b; Davies et al., 2017; Lebrun and Orliac, 2017) already allow researchers to rapidly compile or supplement comparative datasets. Additionally, x-ray microtomography could boost the development of these virtual specimen collections by including virtual type material, so-called "cybertypes" (Faulwetter et al., 2013). Making cybertypes of the holotype of newly discovered or previously described species available to researchers, will certainly revolutionise our current approach to taxonomy. In light of this, soft-tissue staining techniques could be used for additional type material to provide complementary morphological information and to make the accuracy of the cybertypes closer to the physical type material.

In conclusion, x-ray microtomography is improving at a rapid rate and increases in computing power and imaging resolution will continue to broaden its application in herpetological research, leading to exciting discoveries in an emerging research field. On a final note, it is important to mention that data obtained through x-ray microtomography extend well beyond (palaeo)biological research. The use of virtual models and/or three-dimensional prints, for instance, is well on its way to transform current teaching systems and educational exhibits (e.g. du Plessis et al., 2015; Porro and Richards, 2017; supplementary fig. S4).

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