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Lazarus in the museum: resurrecting historic specimens through new technology

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ABSTRACT: All scientific and intellectual endeavours advance by building on earlier observations. In organismal biology, we can in fact directly replicate original studies of morphology and anatomy, when the original material is still present and accessible in the permanent care of museums. We refer to the apparently miraculous "Lazarisation" of these historical specimens, when the application of state-of-the-art scientific techniques brings new life to material in natural history collections. Classical anatomical, histological and palaeontological work established our fundamental understanding of the natural world over centuries of meticulous and dedicated research, much of which remains unsurpassed to this day. Many of these original specimens are still available to active researchers through dedicated permanent collections in the care of universities and museums. An explosion of advancing methods in recent decades has opened new avenues of research that can exploit invaluable historical material. We review the application of novel techniques, primarily new imaging methods, to historic and important specimens. The pursuit of ultra-high resolution magnification, three-dimensional digital modelling, non-invasive scanning techniques, and, increasingly, elemental analyses all have enormous implications for the future of morphology. Palaeontology, comparative anatomy, and development in particular make ideal platforms for the exploitation of these new techniques. These methods are revolutionizing our use of museum collections and reinventing their role in modern morphological research, which comes at a time of increasing threat to collections and museum curation funding. Future innovations in imaging and non-invasive analyses will doubtless accelerate the renewed research efforts dedicated to existing specimens. Most importantly, we celebrate the continued contributions to morphology from these invaluable pieces of our scientific heritage.
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KEY WORDS: Museums, natural history collections, imaging techniques, 21st century morphology, tomography.

Лазарь в музее: воскрешение исторических видов при помощи новых технологий

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ABSTRACT: Все научные и интеллектуальные достижения включают в себя, развиваются и базируются на результатах, полученных в ходе исследований предыдущих лет. В организменной биологии мы действительно можем повторить оригинальное исследование морфологии и анатомии, в случае если материал все еще имеется и сохраняется в музее. Мы заявляем о поистине чудотворной «Лазаризации» (воскрешении) исторических видов, когда применение передовых технологий дает новую жизнь материалу исторических природных коллекций. Классические анатомические, гистологические и палеонтологические работы лежат в основе нашего фундаментального понимания мира природы и базируются на столетиях дотошных исследований, многие из которых до сих пор не превзойдены. Многие из этих оригинальных образчиков до сих пор доступны и заботливо сохраняются в коллекциях университетов и музеев. Новые методики, взрывное появление которых наблюдается в последние десятилетия, открывают новые пути исследований и возможность использовать бесценный исторический материал. Мы провели обзор применения новых техник, в первую очередь методов визуализации, в области изучения исторических и важных видов. Погоня за ультравысокими разрешающими способностями, трехмерные реконструкции, неинвазивные методики сканирования и все в большей и большей степени элементарный анализ — все это имеет значительные последствия для будущего морфологии. Палеонтология, сравнительная анатомия, и эмбриология представляют собой идеальную платформу для использования новых техник. Существование этих методов подталкивает нас к использованию музейных коллекций и пересмотру их роли в современных морфологических исследованиях. Будущие инновации в имаджинге и неинвазивном анализе, бесспорно, ускорят появление и обновление исследований на существующих видах. Наиболее важен тот факт, что бесценные кусочки нашего научного наследства продолжают свой вклад в морфологию.

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KEY WORDS: Музеи, естественнонаучные коллекции, имаджинговые техники (методики визуализации), 21 век морфологии, томография.

Introduction

Morphology and anatomy formed the foundation of biological research and taxonomic endeavour, but in the past 20 years there has been increasing concern for the future of morphology in the face of more 'modern' fields, most notably molecular phylogenetic methods (Lee, 2000). In the same time, funding for the curation and use of natural history collections and historic specimens has been repeatedly threatened, and anecdotally it appears that fewer students and early career researchers are pursuing expertise in morphological techniques. Molecular methods, including next generation sequencing, have revolutionised modern biology and are enhancing the pursuit of adaptive evolutionary questions served by comparative morphology. However, these phylogenetic reconstructions alone cannot illuminate evolutionary processes (Chen et al., 2017).

Invertebrates typically receive less public attention, conservation effort, and large-scale funding in proportion to vertebrates and particularly tetrapods. In times of increasing pressure for 'applied' results attached to research funding applications, this problem is worsening as species of unknown economic value are sifted to the bottom of the pile. Little-known, less 'charismatic', and physically smaller taxa are at risk of being reduced to lines in sequence matrices. Yet these are the very organisms we know the least about, that likely perform important ecological roles and upon which basic research efforts should be focussing. Regarding these organisms especially, museum collections represent the largest, or even the only, sources of knowledge.

Museum collections are most closely connected with the fields of taxonomy and systematics. There is a vast number of undescribed species, and there may be around 0.5 million unnamed species already present in museums as unidentified specimen material (Costello et al., 2013). Cryptic or pseudo-cryptic species may be identified through genetic barcoding but this is somewhat unsatisfactory for finding any new additional material of the newly discovered species, either in collections or in the field. Genetics is therefore not a panacea to the identification of new taxa, and furthermore morphology is important in its own right to understand how organisms interact with their environment.

The role of museum collections and historic specimens has also seen a transformation. These vaults of biodiversity have, in a sense, been unlocked for large-scale studies by these new and time-efficient methods, and the accelerating availability of collections data through massive digitisation efforts (Beaman, Cellinese, 2012; Rogers, 2016). Collection materials are now routinely used to identify cryptic species, reconstruct fine resolution, comprehensively sampled phylogenies, and examine biodiversity and distribution data in specimen-based time series (Sikes et al., 2016). An acknowledged barrier to harnessing museum data is that it is not obvious what specimens are available; this is in part solved by opening collections data through digitisation (Sikes et al., 2016; Davies et al., 2017). Another impediment is that museums are not always the habitual first port of call to researchers seeking specimens for their research. The staff within museums are often increasingly focussed on the application of molecular techniques rather than microscopy and morphology (Boxshall, Self, 2010), though again this is changing in the face of increasing efforts of digitally image specimens. Unless a species is very rare, or unless one is on the museum staff, the amount of effort to extract specimens from a museum is perhaps far more than that involved in acquiring new material, which is assuredly collected and preserved according to specific research needs. Combined, these factors can explain the lag in morphological studies of museum materials, but they do not detract from the enormous research potential that is being overlooked.

We consider advances in several fields that are broadly applicable to the study of invertebrate morphology using historical material: electron microscopy, tomography (constructing 3D digital models from 2D image data), pigment analysis, and the use of microscope slide collections. The increasing accessibility of micro-CT scanning, elemental analysis, digital tomography, molecular probes and increasingly power-

ful microscopes has beckoned in a new era of morphology that is transforming the role of collection material once again. Improvements in processing time, reliability, resolution, quantifiability and digitisation are propelling morphology back to the forefront of biological research. Crucially, the use of non-destructive techniques is also on the rise, most notably including X-ray computed tomography and MRI. This opens the way for increased use of museum collections, without any risk to the integrity of specimens. Natural history collections worldwide are estimated to house between one and two billion specimens (Ariño, 2010; Beach et al., 2010), including many precious animals which have not been accessible for invasive study: types, rare or extinct species, samples from inaccessible habitats and fossils, for example. Many of these have been meticulously and expertly described from external examination, and increasingly tissue samples are being taken for molecular analyses, especially in new additions. But where dissections and histological studies are impossible, researchers only have part of the full picture. Conversely, many specimens, including new species, remain undescribed as a result of the time and expertise required simply being unavailable in the ever-intensifying academic environment. The rapid and reliable generation of high-resolution data from one or several of the new generation of morphological techniques is bringing the field back to the forefront of research in museums, both applied to new acquisitions and historic specimens. Here, we review the breadth of these applications and encourage researchers to broaden their uses of natural history collections and perhaps revisit specimens of particular historical importance, armed with this extensive new toolkit of non-destructive techniques.

The 21st Century morphologist's box of tricks

We have broadly divided the wealth of modern morphological techniques into several themes, according to their particular strengths and applications. Some of these approaches have been present in the scientific sphere for several decades, but have only become usable or commonly available to invertebrate zoologists more recently, owing to substantial technological developments or improved accessibility and decreasing costs. The nature of the study subject, of course, is of enormous importance, and parts of this (inexhaustive) list of methods will naturally not be applicable to every reader's area of interest. For example, palaeontological specimens present challenges which overlap with historic biological material in some aspects — the loss of original colouration, for example — but are more distinct in others, such as the penetration of solid remineralised media to even access the specimen itself. For the purpose of this review we focus on approaches that are as inclusive as possible for all areas of invertebrate morphology.

Look harder: high-resolution microscopy and environmental SEM

The pursuit of greater magnification and resolution has been an ongoing field in biological imaging since Hooke's (1665) *Micrographia* was published over 350 years ago. Pioneers of microscopy, including Robert Hooke (1635–1703), Antonie van Leewenhoek (1632–1723) and Santiago Ramón y Cajal (1852–1934), were not only the first to observe and describe microorganisms, living cells and superfine anatomical details, but they were able to study animals in new and astonishing detail.

Distinguishing differences between many taxa relies on using microstructural characters such as the arrangements and numbers of hairs, papillae, pores, details of genital morphology, shell ultrastructure and many more. These have become accessible only since the development of sufficient magnification microscopes, including not only optically improved light microscopes but also electron and laser microscopy. Scanning electron microscopy (SEM) in particular has been a great asset to morphologists since becoming commercially available 50 years ago, allowing high resolution visualisation of

surfaces and structures with reasonably straightforward sample preparation. The necessary drying and coating methods are potentially less destructive and time-consuming than histological preparation and sectioning, and of course SEM is particularly well-suited to materials such as pinned arthropods, shells, and other hard parts, which may well already be dried for preservation. However, for wet specimens these irreversible processes may not be appropriate for their potential future use in research, especially in the case of rare species or soft-bodied taxa that are more likely to undergo dramatic distortion in processing. Since the 1980s and 1990s, though, wet or environmental SEM has become increasingly accessible, allowing the study of precious samples without the need for coating or for dehydration. The specimen chamber can be flooded with water vapour, without the requirement for a vacuum in regular SEM, and this simultaneously removes the need to coat the specimen with a chargeable substance such as gold sputter. As such, ESEM offers an ideal opportunity to study museum specimens without substantial damage (Valdecasas, Camacho, 2005). While more traditional SEM equipment is commonplace in most institutions, this is expensive equipment with a long depreciation that is not frequently replaced or upgraded, meaning that so far ESEM is still not widely available, and the constraints of drying and coating specimens still limit the extent of use of SEM in museums.

The rise of x-ray computed tomography

Digital tomographic imaging includes analytical approaches that are familiar vernacular from medical applications such as CT (computed tomography, generally from serial x-ray images) and MRI (magnetic resonance imaging) scans. Tomography can refer to the digital reconstruction of a 3D model from any 2D slices including histology, discussed below, but it is most familiar from non-destructive scans (Fig. 1). This technology is perhaps the most significant development that will be discussed in the current review. Used for decades to visualise and reconstruct skeletal structures in vertebrates, CT and associated scanning methods are now deployed to image an enormous range of biological subjects in three dimensions (Gignac et al., 2016). These penetrative imaging techniques allow researchers to examine internal structures within intact specimens, without the need for dissection or histological sectioning (Fig. 1A). Specimens do not require drying and can be scanned in ethanol for both CT and synchrotron visualisation (e.g. Wood et al., 2016). Contrast enhancement, resolution and scanning times are improving rapidly, expanding the potential for applications across all areas of invertebrate zoology (Gignac et al., 2016). Synchrotron tomography, or CT using a high-energy light source from a particle accelerator, is available at publicly funded physics research facilities, which increasingly encourage broadening participation from life sciences projects; synchrotron tomography has advantages over "ordinary" micro-CT in that the higher energy light source dramatically increases the speed of obtaining scans. Some of the results obtained to date are breath-taking, and CT is becoming an increasingly common tool for museum researchers across the world. These are 'big data' projects, which generate gigabyte to terabyte scale image datasets and many institutions are investing in their own visualisation laboratories with computer infrastructure that can accommodate the graphics processing needs of these approaches.

Contrast enhancement through staining often produces the best results for soft tissue differentiation, with iodine, Lugol's (iodine potassium iodide) solution, osmium tetroxide and PTA being some of the most commonly employed (Gignac et al., 2016). The application of such stains to historic specimens is obviously at the discretion of the responsible curator or user; iodine staining is reversible, to an extent, but most stains are still considered destructive as they can limit future uses of the specimen (Gignac et al., 2016). This has somewhat constrained the use of rare or type specimens for scanning. Phase contrast enhancement has the potential to provide sufficient contrast to differ-

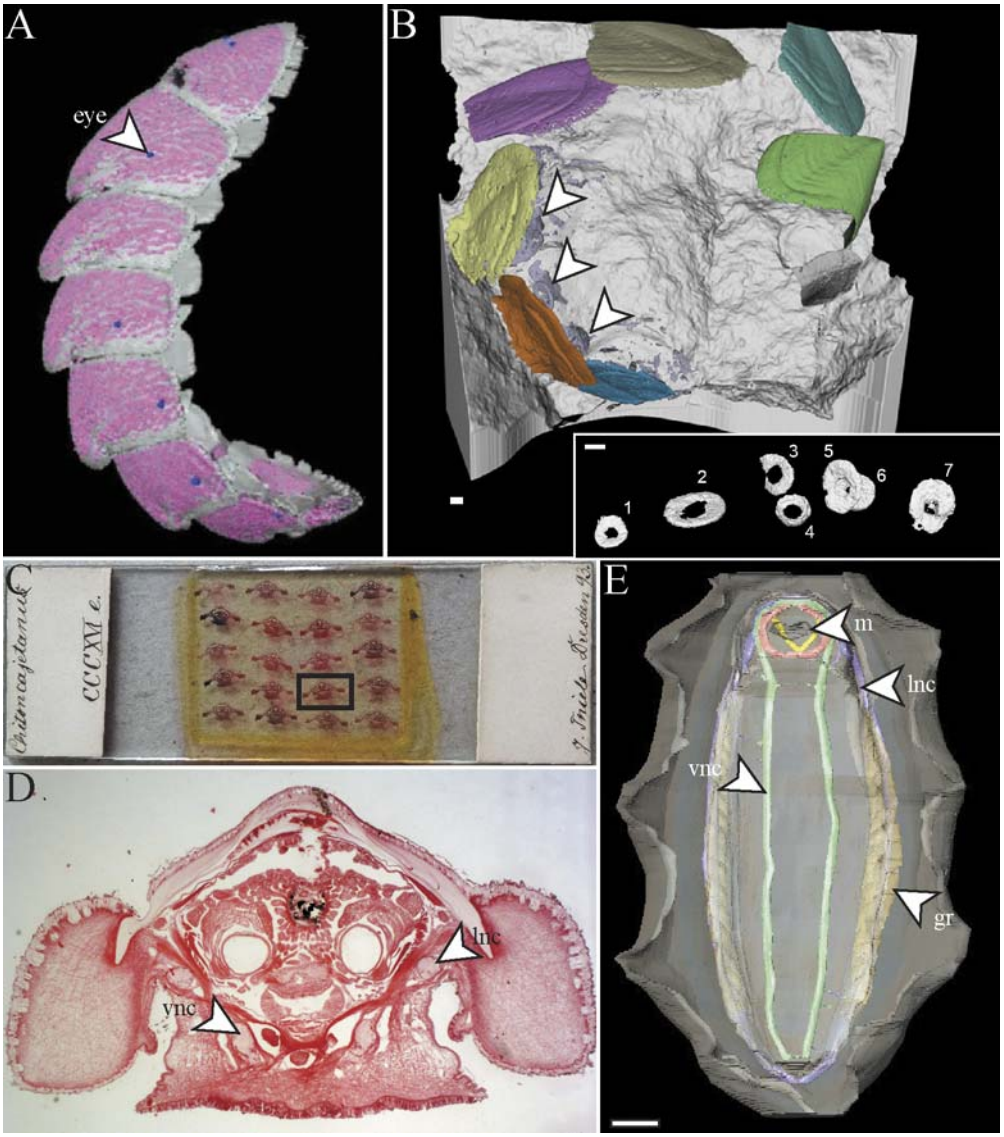

Fig. 1. Tomography in the study of museum specimens. A — digital reconstruction from synchrotron X-ray radiation scan of *Tonicella lebruni*, with differential densities used to identify structures within the valves: internal nerve volume (pink) and the modified aesthetes or shell eyes (blue). Sigwart and Parkinson, unpublished data. B — digital reconstruction from X-ray microtomography of the Ordovician fossil chiton *Helminthochiton thraivensis* (NHMUK PI TG 47258), showing crinoid ossicles positioned within the gut that are invisible in the physical fossil, embedded in the matrix (arrowheads, inset 1–7). Scale bars — 1 mm. Adapted from Donovan et al., 2010. C, D — serial histological sections of *Lepidopleurus cajentanus* produced by Johannes Thiele in 1893 (Museum für Naturkunde, Berlin). E — tomographic model of *Callochiton septemvalvis* reconstructed from digital images of Thiele's original slides, showing little distortion and identifiable organs. Abbreviations: gr — gill row; m — mouth; lnc — lateral nerve cord; vnc — ventral nerve cord. Scale bar — 500 μm. Sumner-Rooney, Sigwart, unpublished data.

entiate between tissue types without the application of chemical stains (e.g. Elfarnawanay et al., 2016). In fossils, which obviously cannot be chemically stained, the resolution of CT scanning depends on the differential density of mineralised parts. Tomography has revealed insights into developmental processes (Donoghue et al., 2015; Yin et al., 2016), the architecture of organ systems (Pohl et al., 2010; Eriksson et al., 2012), lifestyles and ecological interactions (Rahman et al., 2015), and even the cellular structure of eyes (Schoenemann, 2013). The pursuit of high-resolution visualisation methods in wet, unstained specimens will be vitally important to the future use of museum specimens, and that goal is now within reach, having been successfully demonstrated in vertebrate samples.

X-ray tomography provides ability to visualise structures within a specimen, but *ipso facto* also specimens that are embedded in a medium that obscures the view of external examiners. A specimen of a living species may be embedded in wax or resin, but most typically, of course, this includes fossils: amber inclusions and rock-embedded remains that may be wholly or partially hidden from view (Fig. 1B). While the practical difficulties of imaging an animal in rock are easy to imagine, amber inclusions also present considerable obstacles. Amber may be dark or opaque, discoloured over time, contain substantial distortions which impede a clear view of the specimen, or be covered by a naturally-occurring white emulsion (Penney, 2007, 2016). In both cases, digital palaeontology provides unparalleled opportunities for researchers to image external and often internal structures without the impediments of the surrounding media (Sutton et al., 2014). The use of conventional and synchrotron radiation CT has already led to the description and redescription of new species (Dunlop et al., 2011; Henderickx et al., 2012) and even fossilised evidence of behaviour (Lak et al., 2008).

It is important to highlight that there are some remaining issues to consider with the use of computed tomography, which is not the solution to all future morphological studies. Most importantly, the data processing is often labour- and computationally intensive, taking far more expertise and effort than novices sometimes appreciate (Ruthensteiner, 2008). Tomography can be confounded by specimen movement during scanning, inappropriate orientation of the specimen or scanning window, or the optical capabilities of the specific equipment. Resolution using these techniques even in the best facilities is not yet in line with that provided by transmission electron microscopy at a subcellular level, but it is now possible to visualise individual cells and cell layers in a wide range of systems. Resolution decreases with increasing penetration over relatively short spatial scales – so the internal structures of a specimen are potentially not as clear as the more distal aspects. The extent of the trade-off between scanning specimens and potential resultant DNA damage is yet to be resolved (Faulwetter et al., 2013; Gignac et al., 2016). This is also true for the application of iodine-based stains, which may also impair future molecular analyses.

Rebuilding slide collections: we have the technology

”Tomography” refers to any 3D object constructed from two-dimensional slices, not solely the process of MRI or computed X-ray tomography. The same software technology can be used to reconstruct models from other image stack data, including histological serial sections. The microtome has been a key tool for morphologists for more than 150 years, and by the end of the 19th century biologists could reliably cut serial sections at around 10 microns thick (Chandler, Robinson, 2009). Advances made over the last 20 years in software development primarily for use with medical imaging techniques, can be applied to classical histological slides. This approach has been widely used in the 3D reconstruction of internal organs especially for microscopic invertebrates that are too small for conventional dissection, yet perhaps too large to be transparent. Even for moderately large specimens, rendering the organ systems *in situ* in 3D provides new insights that are not

possible from the "flattening" that comes from opening the body cavity to visualise internal structures (e.g. Sumner-Rooney et al., 2015). To achieve high quality sections, generally the material for this technique is fixed in an anatomical fixative such as glutaraldehyde, and embedded in a hard plastic resin to minimise impact distortion during cutting (Ruthensteiner, 2008). However, there are also 150 years of microscope slides, including complete serial sections, held in natural history museums.

Provided the user knows, or can estimate, section thickness and intervals, allows the reconstruction of tomographic 3D models from any material, including slide mounted sections more than a century old. To date, we are not aware of any published studies that have taken this approach. The approach to using such material would follow the steps normally used for tomography of fresh serial sections — capturing digital images of the individual sections in series, digital contrast enhancement, and identification of organ or tissue structures using appropriate tomography software. As discussed above, histological sections do still have some advantages over CT scanning, and slide collections in good condition offer an unappreciated resource for modern digital morphology. Historical slides could provide some efficiency benefit in procuring, preparing, and sectioning specimens. Slide-mounted sections that are stained, sealed with a permanent coverslip, and stored in dark and dry conditions, usually show very minimal fading or yellowing compared to modern material. Masters of the field such as Ludvig Plate (1862–1937), Johannes Thiele (1860–1935), Charles M. Yonge (1899–1986), and others produced stunningly high quality histological sections without modern equipment, and they typically processed multiple species with good taxonomic coverage (Fig. 1C–E; Sumner-Rooney, Sigwart, unpublished data). For larger scale interests, such as whole organ systems, or comparative studies, these represent an excellent potential source of data, if the researcher invests time in examining existing slide collections, and it is a testament to the skill of our invertebrate morphology forebears that their material still offers invaluable insight to modern biological questions.

Colour from the past

Colour is an important aspect of communication and reproductive biology in many species, most notably in animals with clear visual behaviour such as some arthropods and cephalopod molluscs. Understanding colouration patterns or pigment identity can provide a fresh perspective on the ecology, physiology, behaviour and evolutionary history of an animal. Recreating colour in the fossil record has been a challenge for palaeontologists for centuries, and damage to the appearance of extant collection specimens can occur over time despite dedicated and careful curation (Fig. 2).

Identification of pigments in specimens of living species can resolve more accurate images of the animals, but more importantly also facilitates new conclusions about their diets, predator-prey interactions, reproductive behaviours and physiology. In fact, even where pigment is absent, indicators of colour may still be preserved: proposed biomarkers for certain pigments, such as pyrolysates indicating degraded melanins, have been found in some preserved specimens, but caveats remain concerning the ambiguity of some of these, particularly trace metals (Vinther, 2015).

After decades of research into pigmentation and colour, powerful imaging, spectroscopy and chemical analyses now allow us to recapture the original colours of living and fossil species. One of the most notable examples of this, from beyond the invertebrate realm, of course, is the identification of melanosomes in fossil tetrapods (Zhang et al., 2010). However, many invertebrate fossils also show preservation of colour and patterning visible to the naked eye (see Vinther, 2015: figs. 1D–H), and UV illumination and fluorescence microscopy, for example, can reveal preserved colouration patterns (note: not the original colours themselves) in gastropod shells (Caze et al., 2015). The actual extraction and chemical analysis of pig-

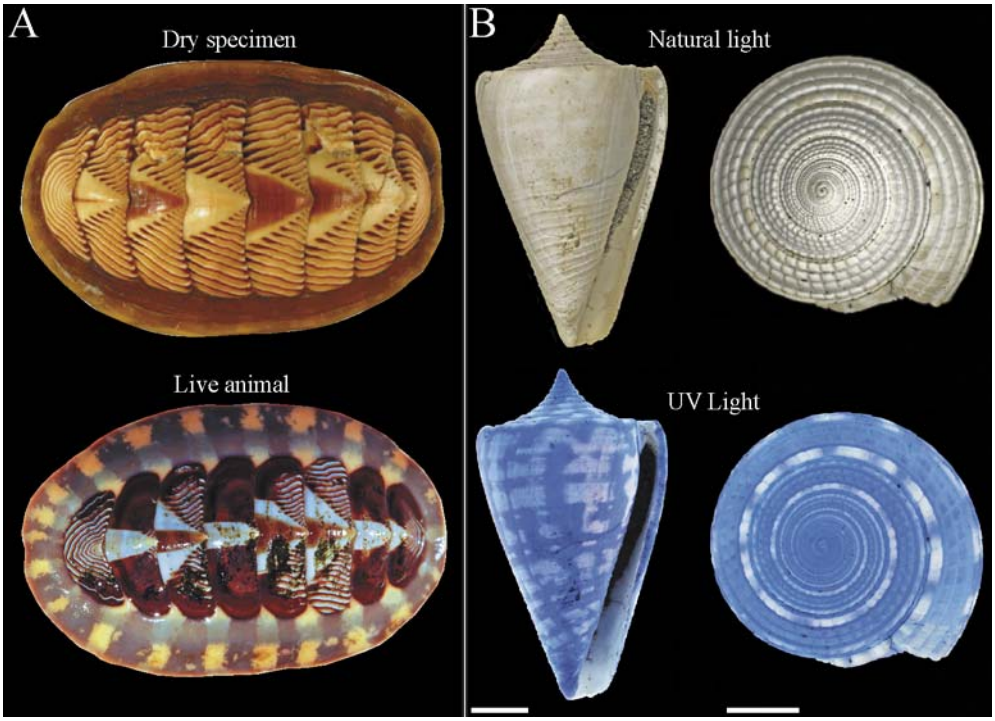

Fig. 2. Loss of colour is extremely common in both fixed and fossilised museum specimens. A — dried (above) and live (below) specimens of the chiton *Tonicella lineata*. Although some of the colouration pattern persists in dried museum specimens, note that there is differential fading in the various pigments. Upper image, museum specimen NMR 53350, courtesy of the Natural History Museum of Rotterdam. B — colouration patterns in fossils are often invisible to the naked eye, as in the gastropods *Conus* sp. (left, NHMUK PI TG 26776, height 29.2 mm) and *Architectonica* aff. *nobilis* (NHMUK PI TG 26777, Diameter 21.3 mm). Scale bars — 5 mm. Images from Williams, 2016, courtesy of Suzanne Williams, Biological Reviews, and Wiley and Sons.

ments from fossil specimens has demonstrated that pigments can be identified after millions of years and included in comparative analyses, using spectroscopy, chromatography, elemental analysis and chemical assays (Wolkenstein et al., 2008; Glass et al., 2012), and these same methods can be effectively deployed to invetigate colour in living taxa drawn from collections (Williams et al., 2016). High-resolution microscopy, particularly (E)SEM, now allows the identification of architectural markers of iridescence and structural colour in fossilised and aged specimens, particularly arthropods. Characteristic multi-layered structures, concaved surfaces and ribbed scales are all reliable indicators, and digital modelling allows researchers to use these details to reconstruct the original colours and recapture the true appearance of extinct animals (McNamara et al., 2012).

Back to the future: exploiting the age of historic specimens

An inherent asset of historic collections is their age. Having access to two or more centuries' worth of fauna allows researchers to incorporate a temporal aspect to their studies. The most obvious application for this is, of course, global changes such as climate, ocean acidification and urbanisation. In the past this approach has been used to monitor changing species distributions in response to biotic and abiotic fac-

tors, to establish species climate indicators, and examine straightforward changes such as body size (e.g. Bartomeus et al., 2013). But it can also be used to explore more specific impacts of global change on the organisms in question. Growth markers, shell composition and thickness, reproductive measures such as gonad size and composition could be compared from animals taken from the same geographic regions along a time series without sacrificing the specimens, thanks to computed tomography and elemental analyses. Such studies could give us the best indicators yet of the physiological impacts of global change, which are currently the subject of enormous research attention and effort. However, a common problem for many physiologists is the inherent disparity between long-term environmental changes and those possible in a simulated laboratory environment in a period of just days, weeks or months (Wernberg et al., 2012). The inclusion of actual historic data in these experiments, or the comparison of historic and artificially reproduced climate change trends, could play a crucial part in validating, or indeed refuting, the efficacy of these studies for the future.

Conclusions: the future of 'Lazarisation'

Even in the digital age, historical museum material continues to find new life. The development of non-invasive techniques is becoming more and more important, and technology is moving forward at a rapid pace. Here, we hope to have given the reader a whistle-stop tour of some of the many modern uses of historic biological specimens in invertebrate morphology. Additional tools are used in specific fields, such as in palaeontology, where elemental analyses can now complement taphonomy to identify different tissue types in cases of exceptional preservation (e.g. Ma et al., 2014). As all new methods develop, it is common to apply them first to freshly purpose-collected material. Of course, the application of pioneering methods is frequently most effective using high-quality tissue that has been collected and preserved under known conditions. Now that many of these modern techniques have been tested and documented (references herein), we encourage morphologists to turn to the enormous wealth of material available in natural history collections. These collections have a key role to play in modern comparative morphology, and offer the fruits of centuries of dedicated collecting around the world. The constant improvement of resolution, scanning time and sample conditions in computed tomography will be among the most important of these developments, especially where staining and sample disruption can be minimised, but CT and similar methods cannot answer all our questions, nor is it always necessary to answer those it can. The production of digital specimen libraries and 'cybertypes' through growing initiatives such as MorphoBank and DigiMorph and iDigBio is commendable, as is the increasing inclusion of 3D data alongside publications (e.g. Sumner-Rooney et al., 2015; Chen et al., 2017; Davies et al., 2017). These will doubtless be a key part of globalising museum collections and morphological data, but we hope that this will include slide collections, SEM data and high-quality macro photography as well as X-ray image stacks and volumes.

Museum collections remain under threat, and funding for collections research is in a highly vulnerable position across the world. The continued efforts of systematics and taxonomic researchers and funding initiatives such as in-house and inter-institutional programmes at museums will help cement the place of historic specimens in the future of morphology, and we hope that this summary of some of the many possible avenues of research will lead readers back into the collections. Finally, and most importantly, rather than diminishing the achievements of our predecessors, we celebrate the incredible work that has been done without this technology, and the care and dedication shown by research and curatorial staff in the preservation of these specimens.

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