The Hidden Secrets of Dinosaur Eggs

Revealed Using Analytical Scanning Electron Microscopy

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In this article we take a new look at the structure of fossilised eggshells, using an advanced scanning electron microscopy technique: electron backscatter diffraction (EBSD). The resulting maps, highlighting the crystallographic structure of the shells with sub-micrometre resolution, are both beautiful and essential for the rigorous characterisation of the shells. We demonstrate how this new application of a technique predominantly used in materials science can assist palaeontologists with the complex task of assigning dinosaur eggs to specific taxa.
Introduction
Dinosaurs capture people's imagination. In any natural history museum, a dinosaur exhibition will invariably draw in the largest crowds as adults and children alike try to envisage a world full of these majestic and occasionally terrifying animals. When we think of palaeontologists researching dinosaurs, we tend to imagine a team of scientists uncovering large, fossilised bones in some remote, dusty desert, or tracing a line of huge footsteps across an ancient layer of mudstone. However, there is another, fascinating biomineralised system left behind by the dinosaurs: fossilised eggs.

Dinosaur nesting sites are being discovered all across the world, complete with an amazing array of different sized and shaped eggs. There is no official record of the number of dinosaur eggs that have been found, but there are at least several hundred known sites and probably many times that number. The eggs are unquestionably and sadly collectable items, often selling for inordinately large sums in dedicated auctions, but are they of any scientific value to palaeontologists? The answer is an emphatic yes: the eggshells are biomineralised skeletal systems like the bones in our bodies and, therefore, supply important taxonomic and functional data. Increasingly it is the application of advanced micro-analytical techniques that is driving research in this field. Non-destructive techniques such as X-ray micro computed tomography (micro-CT) are able to reveal in extraordinary detail the contents of fossilised eggs, allowing confident identification of the dinosaur type based on the nature of the embryonic remains (e.g. Grellet-Tinner et al., 2011). This approach is sure to find many further applications in this field in the coming years, in the same way as it has done recently in other branches of palaeontology (e.g. Sutton, 2008).

Unfortunately, finding embryonic remains in fossilised eggs is extremely rare: cracking of the eggs during the burial process results in the embryos being destroyed by parasitic or bacterial activity, by the passage of fluids through the egg or simply because the embryos were not yet formed at the time of death. Regardless, very few embryos survive the fossilisation process. This is not the case for the eggshells themselves. Dinosaur eggshells, like their modern day avian descendants, were made of the mineral calcite, a trigonal variant of calcium carbonate (CaCO₃). Calcite is a stable mineral, and survives almost any fossilisation process completely intact; therefore, the eggshell that encases a fossilised dinosaur egg retains the exact structure of the original eggshell. And eggshell structures vary significantly between different types of dinosaurs, much as they do in the dinosaurs of our time—the birds. The eggshell of the boiled egg on your Sunday breakfast table has a completely different structure to that of an emu egg laid way out in the Australian outback. Therefore, studying the structures of fossilised dinosaur eggshells allows palaeontologists to distinguish different groups of dinosaurs based solely on the eggshell remains found at nesting sites, and advanced classification schemes have been devised to do just that (Grellet-Tinner, 2006; Grellet-Tinner et al., 2006). No longer are they reliant on uncovering fossilised skeletal remains, and no longer do they rely on finding eggs containing well-preserved embryonic remains. Even so, finding embryonic remains is a major bonus as it enables accurate benchmarking of a specific eggshell structure to the relevant dinosaur taxon (Grellet-Tinner et al., 2011).

In this study we show how we can use a modern electron microscopy technique, namely electron backscatter diffraction (EBSD), to aid dinosaur identification based on eggshell structures. We show how EBSD can be applied with great success to a range of different eggshells, and give an insight into the wealth of detail that the technique can uncover in this fascinating research field.

Eggshell structures
Scientists have been looking at eggshell structures for many decades, often utilising a range of analytical techniques, from light microscopy to advanced scanning electron microscopy or X-ray diffraction (e.g. Young, 1950; Nys et al., 1999; Al-Bahry et al., 2009). Transmitted light microscopy, following the preparation of thin sections from the eggshells, remains the most common approach to studying eggshell structures, and is the basis for most attempts at classifying eggshells based on their structure. In general, the structure of any reptilian
or even avian and non-avian dinosaur eggshell can be summarised by the schematic illustration in figure 1. For dinosaurs and their descendants, the inner part of the eggshell is typically characterised by radiating crystals of calcite from discrete nucleation points; this part is sometimes referred to as the cone layer or the mammillary layer, and in some cases may be the only structure in the eggshell, extending all the way to the outer surface (in which case the shell is said to have a spherulitic structure). Commonly, the mammillary layer is followed by a less clearly structured layer that may either have a prismatic crystal appearance (then known as the prismatic layer), or have a more indistinct, spongy texture in which case it is termed the squamatic layer. Avian eggshells have an additional layer on the outside of the shell, referred to simply as the external layer. These structural layers are now defined as layers 1, 2, and 3 from the inner to the outer surface. The boundaries between these layers may be sharp or gradual, and it is often difficult to determine how many layers exist in a single eggshell, and where exactly one layer changes to another.

Other structures in the eggshells also aid taxonomic identification, such as the shape and spacing of pores (allowing the all-important exchange of gases between the embryo and the atmosphere) and the morphology of the outer surface of the eggshell. For example, figure 2 shows the contrasting surface morphologies of a fossilised sauropod eggshell and a modern day emu eggshell.

**Analytical techniques**

In this research we used a scanning electron microscope (SEM) based analytical technique, EBSD, to characterise the microstructures of a range of fossilised and extant eggshells. EBSD is a common technique in the materials sciences and, to a lesser extent, in the geological sciences (Prior et al., 1999; Schwartz et al., 2009). The electron beam is focused onto a point on the surface of a well-polished, highly tilted sample and, where the conditions for Bragg diffraction are met, individual lattice planes in the target crystalline material project bands (known as Kikuchi bands) onto a large detector, positioned close to the sample. The array of bands forms an electron backscatter diffraction pattern (EBSP) and this can be rapidly processed by commercially available software to identify the phase in question (from a list of candidate phases) and the crystalline orientation of the crystal lattice at that point. The process of indexing the EBSP is fully automated and extremely fast, providing an accuracy of better than 0.5° at speeds often in excess of 100 analyses per second. This high speed and high level of automation allows the user to define a grid of analysis points on the sample surface that, once analysed, can be reconstructed into an orientation map – a map of the sample showing the orientation of the crystal lattice across the surface. In many ways an orientation map may resemble a light photomicrograph of the same region, but the advantage of EBSD is that it fully quantifies the microstructure, characterising the grain structure, the level of deformation, the nature of the individual grain boundaries and the degree and nature of any preferred orientation of the crystals (sometimes referred to as the crystallographic preferred orientation – CPO – or texture).

EBSD has been used on a number of occasions to study shell structures, including studies of marine shells (e.g. Macdonald et al., 2010; Goetz et al., 2009), bird eggshells (Dalbeck and Cusack, 2006) and even fossilised structures (e.g. Lee et al., 2007). However, there has been little or no application of EBSD to the study of dinosaur eggshells until the last year or so (e.g. Grellet-Tinner et al., 2011). For this research, we have been fortunate to have access to a diverse range of fossilised eggshells as well as to have the advanced analytical facilities at the Australian Centre for Microscopy & Microanalysis (ACMM) at the University of Sydney, Australia. The ACMM is equipped with a wide range of electron microscopes, including 2 field emission gun SEMs equipped with EBSD systems, plus the comprehensive sample preparation facilities necessary to prepare polished cross sections of small pieces of eggshell.

Preparing the eggshells for EBSD analysis involved mounting them in epoxy blocks, careful grinding and polishing down to a final stage using colloidal silica suspension, followed by coating with a thin (approximately 2 nm) layer of gold to assist in charge removal in the SEM. The samples were mounted on a pre-tilted holder (tilted to 70° from horizontal) and then were analysed using a commercially available EBSD system at speeds ranging from 15 to 50 analyses per second.
Overnight, automated EBSD analyses with spacing between analysis points ranging from 0.25 to 2 μm generated orientation maps with between 1 and 3 million individual measurements. The quality of these data sets was generally excellent, enabling the complete reconstruction of the microstructure of each eggshell. Many of the resulting orientation maps were visually stunning, as can be seen in some of the images in this article, but there remained a clear scientific objective for the analyses: to develop a rigorous and systematic technique to characterise the microstructures of fossilised eggshells and, where possible, to use the results to assist in the assignment of the egg to a particular taxon. The results have been exciting and, occasionally, quite unexpected.

Results
A selection of different eggshells have been analysed at the ACMM, and these can be broadly divided into 2 different subgroups, that we will discuss in greater detail. These are:
1. Fossilised non-avian dinosaur eggshells;
2. Fossilised avian dinosaur eggshells and their modern day descendants.

In each case we have analysed a number of different samples, of which a selection are presented here. We have chosen to show only a few of the EBSD orientation maps that best summarise the structures within each eggshell: more detailed microstructural analyses, including the CPOs and the boundary misorientation distributions, will be published elsewhere.

Non-Avian Dinosaur Eggshells
The majority of the dinosaur eggshells that we have studied come from sauropods and, more specifically, the titanosaurs. These are the huge, plant-eating dinosaurs that roamed many parts of the Earth in the Jurassic to Cretaceous periods and included some of the heaviest of all dinosaurs.
including Argentinosaurus. A number of nesting sites have been discovered, including several in Europe, particularly in Romania, and a well documented site, Auca Mahuevo, in Argentina. The Auca Mahuevo site includes a number of embryonic remains, allowing confident assignment of the eggs to a particular titanosaur taxon (Chiappe et al., 1998; Grellet-Tinner et al., 2004).

The titanosaur eggshells are characterised by a spherulitic microstructure in cross section, with discrete eggshell units consisting of nucleation sites on the inner eggshell surface and large, fanning calcite crystals radiating out to the exterior surface. This is clearly shown in the EBSD orientation map in figure 3. The calcite crystals have a trigonal symmetry, indicating that the arrangement of atoms in the crystal lattice can be approximated to a hexagonal cylinder. In the eggshells, the long axis of the cylinder (known as the c-axis) preferentially aligns perpendicular to the surface of the shell. The colouring scheme used in figure 3 highlights this orientation: the blue colour indicates regions that have a perfect alignment of the calcite c-axis with the radial direction, whereas a red colour indicates a c-axis orientation 45° from the radial direction.

It is clear from figure 3 that this titanosaur eggshell has only a single layer: even a closer look at low angle boundaries within the shell (marked by the light grey lines) does not indicate any layering. These low angle boundaries represent places in which the orientation of the crystal lattice changes from pixel to pixel by a small angle, typically between 2° and 10°, whereas high angle boundaries (often referred to as grain boundaries and marked by the black lines), indicate orientation changes of more than 10°. Low angle boundaries are unlikely to be seen clearly in any light micrograph and they therefore can provide new information about the structure of the shell.

Most of our information about the eggshell structures is derived from studies of cross sections through shells. However, if we take a look at the plan view of this titanosaur eggshell, sectioned about 0.75 mm from the exterior surface, we can see the shape and distribution of the individual shell units with great clarity (figure 4). This orientation map uses the same colour scheme as in figure 3 and we can see the expected change from the ideal c-axis orientation in the centre of the shell units (blue colours) to oblique orientations at their edges (yellow to red colours). However, we can also see that there exists significant heterogeneity between the structures of individual shell units: some of the units are almost perfectly circular in cross section, whereas other units appear to contain many smaller sub-units, separated by high angle boundaries. This is especially noticeable in the large, dominantly blue area in the lower left corner of the map. In addition, some of the inter-unit pores are more clearly visible in this section than in the standard cross sections. Clearly, analysing the structures in this plan view provides a useful complement to the cross section view.

The titanosaur egg shown in figures 3 and 4 was found in Romania. Another dinosaur eggshell is shown in figure 5: although this sample was found in France, it clearly shows many similar traits to the Romanian example. Indeed, it is inferred to have also come from a titanosaur, and the similar radiating eggshell units, the arrangement of low and high angle boundaries and the nodular outer surface visible in the orientation map all reinforce this belief.

However, not all titanosaur eggshells display this same structure. The EBSD orientation map shown in figure 6 is known to have come from a titanosaur eggshell collected from a site in Mongolia as the egg itself contained embryonic remains that have been identified as lithostrotian (nemegtosaurid) titanosaur remains (Grellet-Tinner et al., 2011). Although figure 6 uses a different colour scheme (based on the crystallographic direction that is parallel to the eggshell’s normal direction, as shown in the inset), we can see that the structure is significantly different. The clear eggshell units visible in the two European examples (figures 3 and 5) are not present, although some differences may be due to localised diagenetic alteration (e.g. in the lower left corner of the map) and to erosion of the outer surface of the eggshell. However, a closer look at the distribution of low angle boundaries in this sample (shown by the grey lines in figure 6) also shows something else unexpected: a significant increase in the number of low angle boundaries in the outermost third of the shell. Compare this
with figures 3 and 5, in which there is no noticeable difference between the inner and outer parts of the shell with regards to low angle boundary population. An advantage of the EBSD technique is that we can process the data in many different ways. Figure 7 shows the distribution of strain within this cross section using an algorithm developed for mapping strain in shot-peened aluminium samples: brighter regions (greens, yellows and reds) mark areas that have greater variations in the crystal lattice orientation (i.e. more low angle boundaries). It is clear that the outer part of the sample has significantly more “strain” than the inner part, yet we do not see the same results on the previous examples.

This is fascinating new information: it is not layering in the strict sense, but these subtle variations have been seen on a few other samples that we have analysed, including from eggs with titanosaur embryonic remains. Perhaps we need to reassess the classification of the earlier European examples as titanosaurs? Certainly a more in-depth analysis of these structures and associated classification schemes is essential, and this is research that we hope to publish in the near future.

**Avian Dinosaur Eggshells**

We have also had the opportunity to look at a number of fossilised eggshells that are believed to have come from avian dinosaurs (in a number of cases these remains are found in rocks from the Cenozoic, after the extinction of the non-avian dinosaurs). The structures of these shells show greater diversity than the Mesozoic dinosaur eggshells, and in some cases can be directly compared to modern day descendants.

The EBSD orientation map shown in figure 8 is taken from a fossilised egg collected in Spain. This egg is not from an extinct avian group, but from a direct ancestor of a modern species of bird. There are many differences to the large, spherulitic structures seen in the titanosaur eggshells, and the shell structure can be split into 3 distinct layers, much as in the schematic diagram in figure 1. Yet how does this compare with a present day bird eggshell? Following a desperate hunt around a number of museums in both Australia and the US, we finally managed to get our hands on a few small fragments of a flamingo egg, and the resulting EBSD orientation map (figure 9) does indeed show many similarities to the fossilised sample. There is the same relatively narrow mammilla at the base, indistinctly altering into a thick middle layer with elongate grains and extremely irregular grain boundaries, with a thin exterior layer characterised by straight grain boundaries and a marked increase in low angle boundary frequency.
If we take a look at the structures in another present day eggshell, this time from an emu (figure 10), then we can see a contrasting structure to the flamingo egg. There may be the same broad 3-layer structure as for the flamingo, but a pronounced exterior layer exists, with a radial grain structure and a clear, discrete boundary to the inside layer. This exterior layer gives the emu eggshell the broad, gentle nodular morphology on the outer surface that is visible in figure 2. However, it can become a little more complex when we take a look at yet another present day eggshell – this time a hen egg taken off the shelves of a local supermarket. The EBSD orientation map in figure 11 is remarkably similar to that of the flamingo egg – the same slightly indistinct mammilla, the same long grains in a central layer with clear, irregular boundaries and a similar, narrow outer layer with straight boundaries. The outermost layer lacks the significant increase in low angle boundaries seen in both the fossilised avian egg and the modern day flamingo egg, but the differences are relatively subtle. Clearly EBSD analyses cannot totally replace the expert judgement of both field and hand specimen studies just yet.

Conclusions
We have shown in this article how a new application of an SEM-based diffraction technique, namely EBSD, can provide a whole new insight into the structure and classification of eggshells, both from extant species and, more interestingly, from fossilised species. Not only are the EBSD orientation maps beautiful, but they also contain a wealth of information about the eggshell structure. Although this article only touches the surface in terms of the detailed information contained within each EBSD dataset, it hopefully demonstrates clearly how the technique can uncover significant differences and similarities between eggshell samples. The next step is to use the data to improve the existing schemes of eggshell classification, and to apply EBSD to a wide range of fossilised eggshells to assist in taxonomic assignment. The future is certainly not going to be dull!

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References


Grellet-Tinner, G., Chiappe, L.M., Bottjer, D., Norell, M. (2006). Paleobiology of dinosaur eggs and nesting...


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Patrick has been working in electron microscopy for over 17 years. Following a degree in geology at Oxford University, he completed his PhD at the University of Liverpool, focusing on the use of electron diffraction and imaging techniques to understand the microstructures of deformed rocks. A short spell as a postdoctoral researcher at Utrecht University in the Netherlands was followed by a decade in commercial electron microscopy. During this time he worked as an applications specialist in SEM based microanalysis, with a particular emphasis on electron backscatter diffraction. In 2010 he returned to academia, taking up a position at the Australian Centre for Microscopy & Microanalysis at the University of Sydney, where he currently holds the position of SEM manager. Patrick spends a large amount of his time helping users from across the complete spectrum of science to get the best out of the fantastic equipment available to them, as well as assisting and collaborating with visiting researchers such as Gerald.

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Gerald completed his PhD at the University of Southern California in 2005 focusing on the palaeontology and palaeobiology (namely) of the dinosaur lineage that led to modern birds. He became intrigued by the palaeobiology of extinct and modern dinosaurs and their nesting paleoenvironments. His research led to a few important discoveries and over 25 publications in professional journals, including Nature. The latest discovery describes the association of geothermal environments as preferred nesting sites for the giant sauropod dinosaurs. However, to understand the world of these giants, he extensively uses the minuscule features of their egg and eggshell microstructures for taxonomic identifications and functional morphology. He specialised in the use of SEM, BESEM, and CL, and now applies new microcharacterisations (EBSD, micro and nano-CT) in collaboration with the Sydney ACMM researchers to further elucidate the palaeobiology of extinct oviparous vertebrates, which in turn helps understanding the ecology and preservation of their modern counterparts. He is currently a member of the Argentinean CONICET scientific institution but also aims to ground his research in Australia.