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Marie R. G. Attard, Emma Sherratt, Paul McDonald, Iain Young, Marta Vidal-García, Stephen Wroe

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A new, three-dimensional geometric morphometric approach to assess egg shape variation within and between clutches

Marie R. G. Attard[*1,2], Emma Sherratt[*1,3], Paul McDonald[1], Iain Young[1,4], Marta Vidal-García[3] and Stephen Wroe[1]

[1] Function Evolution and Anatomy Research Laboratory, Zoology Department, School of Environmental and Rural Science, University of New England, Armidale, NSW, 2351, Australia

[2] Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

[3] Department of Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra, ACT, 2601 Australia

[4] School of Life and Environmental Sciences, Faculty of Science, University of Sydney, NSW 2006, Australia

*Co-first Author. These authors contributed equally to this work.

Corresponding Author:
Marie Attard[*1,2]
Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK,
Email address: m.attard@sheffield.ac.uk

Short title: Clutch variation in avian egg shape

Abstract

Avian egg shape can differ markedly within and across families, yet comparative analyses that aim to explain shape variation among avian taxa largely ignore potential biases due to within-species variation. To quantify these levels of variation, we have developed a novel approach using three dimensional geometric morphometrics on micro computed tomography (micro-CT) data to quantify patterns of egg shape variation within and between clutches of four passerine species of different lineages. Major patterns of egg shape variation among bird species were inspected through Principal Components Analysis and estimations of within- and between-species morphological disparity. The results indicate that egg shape differs among species, but with extensive within-species variation so that all four focal species occupy a range of shapes. Within-species variation is attributed to between-clutch differences in egg shape, and within-clutch variation is variable but substantial. These findings suggest that there is appreciable variation in egg shape across clutches and that this variation needs to be accounted for in future work. The approach developed in this study to assess variation in shape is freely accessible and can be applied to any spherical-to-conical shaped object, including eggs of non-avian dinosaurs and reptiles through to other extant taxa such as poultry.

Key words: egg shape; geometric morphometrics, micro-CT, morphospace; passerine; bird eggs; clutch

Introduction

Avian egg shape diversity is a well-known biological phenomenon reflecting taxonomic diversity (Olsen, Cunningham & Donnelly, 1994). Within-clutch variation in egg shape is predicted to be a reflection of parental investment in each offspring, or external factors influencing offspring survival (Briskie & Sealy, 1990). This is because the shape of the avian egg is correlated with egg strength (Gosler, Higham & Reynolds, 2005), gas and heat exchange during incubation (Rokitka & Rahn, 1987; Deeming & Ferguson, 1991; Mao et al., 2007; Šálek & Zárybnická, 2015), use of the brood patch area (Drent, 1975; Barta & Székely, 1997) and detection of brood parasitism (Underwood & Sealy, 2006; Zölei et al., 2012). So far, within-clutch studies have used linear dimensions to characterise egg shape (e.g., length and breadth) (Pearl & Curtis, 1916; Pietiäinen et al., 1986; Fernández & Reboreda, 2008), and relate this to the female's breeding experience (Coulson, 1963) and the position of the egg in the laying sequence (Coulson, 1963; Cutten, 1965; Cucco, Grenna & Malacarne, 2012).

Within the physical constraints imposed by a laying female's reproductive tract (Clark, Ewert & Nelson, 2001), egg shape variation would be expected to be constrained within a species if shape strongly influenced survival of the young. Alternatively, if shape is relatively unimportant for egg hatchability and subsequent offspring survival, then large variation in egg shape within a given species might be expected. For example, selection for pyriform (pointed or pear-shaped) eggs may be desirable for species that lay eggs on ledges or cliffs to reduce rolling (Ingold, 1980; Board, 1982; Tschanz, 1990), whereas spherical eggs, considered to be the most optimal shape for shell resistance to external forces (Bain & Solomon, 1991), may be more common among larger sized species, as they could place greater stress upon eggs during incubation (Bain & Solomon, 1991; Barta & Székely, 1997). The influence of eggshell shape on its mechanical behaviour has been widely studied (Richards & Staley, 1967; Anderson et al., 2004; Nedomova, Severa & Buchar, 2009). Spherical eggs also have the smallest surface area of all three-dimensional (3D) solids of a given volume, hence, reduce calcium investment and should therefore be advantageous to females that may be calcium limited (Gosler, Higham & Reynolds, 2005). Elongated eggs provide greater volume to facilitate higher energy and nutrient storage capacity then more spherical eggs of the same width, and thus, provide the hatchling with greater fitness potential (Rose, Simpson & Manning, 1996). In host-parasite arms race, similarities in the shape of eggs within host species clutches is believed to assist in the detection of brood parasitism (Underwood & Sealy, 2006; Zölei et al., 2012).

Avian egg content (albumen and yolk) volume available for embryo growth (Pearl & Curtis, 1916) and the final size reached by the chick at hatching are physically limited by the size and shape of the egg which remain unchanged throughout incubation (Narushin & Romanov, 2002). Egg size and elongation may be constrained by pelvis size. For example, petrel species that lay the most spherical eggs tend to have a deeper and more caudally spread pelvis than those that lay elongate eggs (Warham, 1968). Poultry eggs distinctly elongated or oval in shape were found to have a lower success of hatching (Kumar & Shingari, 1969; Tsarenko, 1988; Burtov, Goldin & Krivopishin, 1990) as these shapes are considered to obstruct the rotation of the embryo inside the egg at later stages of development (Ragozina, 1961; Rolnik, 1968; Narushin & Romanov, 2002). As such, artificial breeding of hens by the poultry industry has produced rounder, stronger eggs (Anderson et al., 2004; Altuntaş & Şekeroğlu, 2008), enabling the morphology of eggs to serve as an indicator of productivity (Mänd, Nigul & Sein, 1986). Hatchability based on egg shape varies depending on the species, being highest for relatively elongated eggs in the turkey *Meleagris gallopavo* (Erisir & Ozbey, 2005), less elongated eggs in two lines of hens (Narushin, Bogatyr & Romanov, 2016), and more pointed eggs in White leghorns *Gallus gallus domesticus* (Provizen & Lvova, 1982). In contrast, Encabo et al. (2001) observed in two passerine species (Great Tit *Parus major* and Blackbird *Turdus merula*) that egg shape did not have fitness consequences in terms of chick survival, and thus failed to find any evidence for an optimal egg shape.

Given that eggs must contain all required nutrients for offspring through to hatching, insufficient space to store nutrients within an egg may thus lead to a reduced growth rate, final size and ultimately lower survival rate for offspring, unless measures are put in place post hatching to compensate for any early deficits in growth. Females breeding in privileged circumstances might benefit from reductions in egg investment if the future fitness payoffs from doing so exceed the current fitness payoffs from increasing egg investment (Metcalfe & Monaghan, 2001). For example, fairy-wren mothers lay smaller sized eggs when assisted by helpers as a ‘catch up’ growth strategy, whereby the initial nutritional setback of developing inside a small egg is compensated for by accelerating the growth of young post hatching from additional food provisions supplied by helpers (Russell et al., 2007). The life history of egg laying species may be closely linked to breeding and nutritional conditions to enhance the growth rate, final size and survival of offspring.

It is also important that within- and between-clutch variation in egg characteristics are considered for large comparative studies across many species (Garamszegi & Møller, 2010). Comparative analyses that aim to explain variation in phenotype among taxa require that within-species variation is lower than variation between species or taxa (Felsenstein, 1985). Low repeatability in species-specific characteristics occurs where there is large within-species variation due to phenotypic plasticity, population differences or measurement errors (Ives, Midford & Garland, 2007), which can lead to misinformed conclusions. The magnitude of bias depends on within-species variation and sample sizes.

The aim of this study is to investigate within- and between-clutch variation of avian egg shell shape. Natural variation in egg shape can be quantified using different methods, with some more comprehensive in their evaluation of shape differences than others (Havlíček et al., 2008). To be able to quantify subtle differences in the curvature of egg shells, we present a new method to quantitatively analyse the shape of eggs in three-dimensions. Microcomputed tomography (micro-CT) data were used to generate 3D models and a 3D geometric morphometrics toolkit was used to quantify egg shape in four Australian bird species (four clutches per sp.): the grey shrike-thrush *Colluricincla harmonica*, red-browed finch *Neochmia temporalis*, spiny-cheeked honeyeater *Acanthagenys rufogularis* and superb fairy-wren *Malurus cyaneus* (Figure 1A). With these data, we investigate whether there is greater shape variation within a clutch than between clutches for a given species, and ask if egg shape can be used to distinguish between bird species~~?~~ According to general assumptions in evolutionary comparative approaches across species or taxa, intraspecific variation in egg shape should be lower than interspecific variation (e.g., Felsenstein, 1985). If natural selection is acting on egg shape, we ~~would~~ expect that differences in egg shell shape ~~will~~ reflect adaptations ~~that will optimise~~ incubation and/or hatchability (Hoyt, 1976).

Method

Specimens

Egg shape was compared for taxa from four distinct families: the Maluridae (fairy-wrens), Meliphagidae (honeyeaters), Pachycephalidae (whistlers and thickheads) and the Estrildiae (wax bills, grass finches, munias and allies) respectively (Table 1). All species except the grey shrike-thrush are endemic to Australia. These taxa cover a range of body sizes (10-68g), diets, clutch size (2-5 eggs per clutch), life histories (pair breeders versus cooperative breeders), evolutionary origins (Australian Old Endemics versus more recent Eurasian colonists) (Table 1). Within each species, four clutches from different collection locations were preferentially selected ~~for this study~~ to ensure that different clutches were ~~could~~ not ~~have been~~ laid by the same breeding female (Figure 1B). All eggs are held at the Australian National Wildlife Collection, Canberra. Collection date, location and clutch size were available for all eggs included in this study (Table 2). Only clutches collected after dichlorodiphenyltrichloroethane (DDT) was banned in 1987 were included to minimise the risk of reported pesticide-induced changes in avian egg shell characteristics (Fry 1995), although, given their dietary preferences, none of these taxa are likely to have been affected by this process in any case. All eggs from each clutch were included in this study, with the exception of one of the seven eggs from clutch 10376, which was too damaged to reliably reconstruct digitally.

Micro-CT Scanning

The intact egg shells (N=55) were scanned using a compact desktop micro-CT scanner (SkyScan 1174, Bruker micro-CT, Kontich, Belgium) at the Australian National Wildlife Collection, Canberra. The following scanning parameters were used: 50 kV source voltage, 80 µA source current, 33.28 to 33.45 µm pixel size, 360° rotational angle, 0.8° rotational step, 2.3 s exposure time, 40% sharpening, 2 frame averaging, random movement and flat field correction turned on and a 0.5 mm aluminium filter. The isotropic voxel size used for each scan was specimen specific, and was based on the resolution required for the length of the egg to fill 90% of the field of view.

The acquired shadow projections (16-bit TIFF format) were further reconstructed into two-dimensional (2D) slices of the structure of each sample using the NRecon software interface (v.1.6.9, Brueker micro-CT, Kontich, Belgium). The reconstruction parameters used were: smoothing (5%), ring artefact correction (20%), beam hardening correction (100%) and setting of contrast limits between 0 and 1.4. A 1.5ml eppendorf tube containing MilliQ water was included in all scans. Micro-CT Hounsfield units (HUs) were calibrated using known water CT density (HU = 0) during 2D slice reconstruction. The cross-section slices were stored in DICOM format and are available on the Figshare repository (https://figshare.com/s/3af9f0cf5346e9b881f6).

3D Volume Reconstruction

Digital segmentation and solid meshing of micro-CT scan data was performed with medical imaging software, Mimics (v. 16.0) and 3-matic (v. 8.0) using protocols adapted from Attard, Parr et al. (2014). In brief, thresholding and segmentation of egg micro-CT data was conducted in mimics to create a 3D object of the egg shell. The 3D object was imported in 3-matic where the geometry was wrapped, reduced and smoothed, while still preserving egg shape and size without data loss. All eggs had been blown cleanly through a hole. To fill in the hole, the surface around the hole of each egg was selected and filled using the freeform tool in 3-matic. The egg shell was separated into two surfaces; one of the interior egg shell surface and one of the exterior egg shell surface. A surface mesh of the exterior egg shell surface consisting of approximately 10,000 triangles was produced for each specimen and exported as a .PLY file for shape analysis, and are available on Figshare (https://figshare.com/s/3af9f0cf5346e9b881f6).

Quantifying Egg Shape

Historically a range of methods have been applied to quantify egg shape; in morphometrics the term ‘form’ refers to size plus shape, and ‘shape’ is defined as “all the geometric information that remains when location, scale and rotational effects are filtered out from an object” (Kendall, 1977). Numerous different indices have been used in the past to evaluate deviation of a true shape from a model object based on linear dimensions (e.g., index of pear-shapedness, plumpness, conicity, equality and roundness) as summarised by Havlíček et al. (2008) and Narushin (2001). Egg shape is often studied by computing ratios from linear distance measurements. The index of egg elongation, calculated as the ratio of egg length (L) to breadth (B, i.e., maximum diameter), was one of the earliest and widely used approaches used to quantify egg form (Pearl & Surface, 1914; Asmundson, 1931; Marble, 1943; Shultz, 1953; Kern & Cowie, 1996; Harun et al., 2001; Hesna Sahin et al., 2009), and continues to be applied in ecological research (Deeming & Ruta, 2014) and to assess eggshell quality in poultry industry (Nedomova, Severa & Buchar, 2009). The relationship between egg length and breadth varies with egg size in the Great Tit (Van Noordwijk et al., 1981), and was used to infer differences in egg shape and volume with egg size. The index for egg asymmetry is calculated based on the ratio between the distance from the lower vertex of the egg to the point where the polar axis intersects the equatorial axis (D) to egg length (L) (Deeming & Ruta, 2014). High within-clutch repeatability in particular characteristics (egg length, width and elongation index) has been suggested in several bird species (Hendricks, 1991; Petersen, 1992; Mónus & Barta, 2005).

We chose to not use these historical methods because the limitations of ratios from linear measurements are apparent: two eggs having the same elongation index does not necessarily imply the same shape, because the same ratio may be obtained if the widest point is at the middle of the egg, or towards the base. Consequently, multiple indices are often used in combination to obtain a better description of egg shape, however, these variables may be correlated with one another, leading to difficulties in identifying the extent of shape variation between samples and selecting which indices best characterise egg shape for a given species (Narushin, 2001). This variation in curvature is an important component of eggshell shape. Eggshell curvatures were superficially created in the past based on direct measurements and mathematical equations to approximate the profile of an egg (Hutt, 1938; Bonnet & Mongin, 1965; Besch, Sluka & Smith, 1968; Carter, 1968), though this methodology is often tedious to measure and it is difficult to determine how closely the calculated curvature mimics the true profile of the egg (Carter, 1970). Other methods have been developed in attempt to capture the precise geometry of such contours. Curve-fitting equations have been used to estimate egg shape through 2D contour profiles (Nedomova, Severa & Buchar, 2009), with some requiring separate equations for each species (Preston, 1953, 1968; Smart, 1991). Another study was able to fit an egg silhouette of any species as long as the egg was oval (Baker and Brawn 2002).

Our approach builds upon more recent developments that have utilised approaches to precisely measure the 2D contours of the eggshells from digital photographs (Elliptic Fourier analyses and semilandmark-based geometric morphometric methods) (Johnson, Leyhe & Werner, 2001; Havlíček et al., 2008; Bravo & Marugán-Lobón, 2013; Murray et al., 2013; Deeming & Ruta, 2014). Each method takes a different approach to capturing the egg outline and turning the contours into 2D shape variables (see Iwata & Ukai, 2002 and; Murray et al., 2013 for details of each method), however the methods perform similarly in capturing shape variation of biological structures (Loy et al., 2000; Sheets et al., 2006). Sometimes only one half of the egg is examined owing to the radial symmetry of the eggshell, and therefore have a plane of symmetry that is considered to remain the same with rotation along the egg's long axis (Deeming & Ruta, 2014). However, assuming complete radial symmetry can be misleading, as it ignores subtle differences in curvature around the entire eggshell. We suggest that the two-dimensional approach is prone to error associated with aligning a highly-rounded object exactly parallel to the imaging plane, and thus a three-dimensional (3D) approach is preferable. To date there have been no 3D analyses of egg shape.

In the present study we characterised egg shape using three-dimensional semilandmark-based geometric morphometrics (Bookstein, 1991; Mitteroecker & Gunz, 2009; Adams, Rohlf & Slice, 2013). On the 3D surface models we placed 206 landmarks to cover the contours of the egg using a digitising routine written in R v.3.2.2 (R Core Team, 2015) modified from those in the *geomorph* library (Adams & Otárola-Castillo, 2013; Adams, Collyer & Sherratt, 2016). Firstly, the egg model was centred and rotated by its principal axes so that the x-axis lay along the longest dimension of the egg. The top and bottom of the egg were defined by dividing the egg into two halves and calculating the volumes (convex hull volume) of each; the top of the egg is defined as the smaller half. Two landmarks were placed at the poles by taking the minimum and maximum values along the x-axis when y is zero. Four landmarks were placed on the y- and z-axes by the same means, resulting in 6 landmarks that together demark the height, width and depth, and are positionally homologous on every egg (Figure 2, red points). Then, 200 equally-spaced semilandmarks were fitted to the first shell surface (Figure 2, black points) following the algorithm outlined in Gunz et al. (2005) and Mitteroecker and Gunz (2009), producing a template that could be fitted to every other specimen by thin-plate spline (TPS) warping (Bookstein, 1989), using the 6 polar landmarks to orient the template. This method assures that positional homology is maintained between every semilandmark and works well with curved 3D structures with few homologous landmarks (e.g., Serb et al., 2011).

The landmark data were aligned using a generalised Procrustes superimposition (Rohlf & Slice, 1990); all 206 semilandmarks were permitted to slide in either direction on two planes tangential to the surface in order to minimise bending energy between specimens (Gunz, Mitteroecker & Bookstein, 2005). The resulting Procrustes shape coordinates were ordinated using a Principal Components Analysis (PCA), allowing visualisation of the variation in shape across all 55 eggs. The shape changes described by the main PC axes were visualised using the surface warp approach (Drake & Klingenberg, 2010; Klingenberg, 2013, 2015; Sherratt et al., 2014); an egg model derived from micro-CT reconstruction was warped to the mean shape using the TPS method, and then this reference egg was warped to the shapes representing the minimum and maximum values of PCs 1 and 2. For 3D objects this is one of the most visually accessible ways to view the shape variation described by each PC axis.

To test for differences in egg shape between species and between clutches of each species, we used the Procrustes ANOVA (D-ANOVA; Goodall, 1991). This distance-based ANOVA uses Procrustes distances among specimens rather than explained covariance matrices among variables, but is statistically equivalent to a regular analysis of variance and is beneficial for high-dimensional datasets since only the number of individuals is important in the model. We evaluated a nested model of egg shape ~ species / clutches. Significance testing was achieved through permutation using a residual randomisation permutation procedure involving 1000 permutations (Collyer, Sekora & Adams, 2015).

To quantify the amount of egg shape variation within-species, as well as within-clutches of each species, we measured the dispersion of all observations around the mean shape for the group. For shape data, this is the Procrustes Variance, which is the mean squared Procrustes distance of each specimen from the average shape, and can be calculated as the sum of the diagonal elements of the covariance matrix of that group (e.g., Zelditch, Swiderski & Sheets, 2012). To test for statistical differences in disparity between clutches, we calculated absolute differences in Procrustes variances between clutches and used these as test statistics in a permutation procedure, where the Procrustes variances residuals are randomised among groups. For each species, 1000 permutations were performed.

All analyses were performed in R v.3.2.2 (R Core Team, 2015) using the geomorph library v.3.0 (Adams & Otárola-Castillo, 2013; Adams, Collyer & Sherratt, 2016). Digitising routines are provided in the Figshare Repository (https://figshare.com/s/45fee8f96179b19d7988).

Results

Our data indicate a broad range of egg shapes for all species studied (Figure 3). The first two PCs accounted for 82.5% of the total egg shape variation across species, and therefore together they provide a reasonable approximation of the total variation. The remaining PCs each contributed less than 5% of the total variation and are not discussed further. PC1 (73.5%) is associated with an elongation of the egg; negative PC1 scores correspond with shorter, squatter eggs, while positive PC1 scores correspond with taller, more slender eggs. Eggs from all species were spread along the PC1 axis, revealing high variability in egg elongation within each species. PC2 (10.7%) is associated with tapering in egg shape, with negative PC2 scores being associated with eggs that were conical (i.e. cone-shaped) and positive PC2 scores associated with eggs that were more oval in shape, with egg breadth only increasing slightly at the bottom pole from low to high values of this axis.

The four species do not have significantly different egg shapes when the clutches per species are considered, however the clutches within-species are significantly different (Table 3). Figure ~~2~~ 3 3 shows clearly that there is substantial within-species variation in egg shape for three of the four species: within-species disparity (Procrustes variance) is high for three of the four species, excluding the superb fair-wren (Figure 4A). The high disparity in egg shape observed within-species is due to between-clutch shape differences as well as within-clutch disparity (Figure ~~2, 3~~B).

Clutch disparity appears quite variable within each species (Figure 4A), however the Procrustes variances of clutches in all but two pairwise comparisons were not significantly different from each other within each species (Figure 4B, Table 4). Overall mean clutch disparity was not significantly different among species (ANOVA, $F_{3,12} = 0.936$, $P = 0.453$). Together these results indicate that within-species egg shape variation is due to between-clutch differences as well as within-clutch differences in egg shape.

Discussion

Variation in egg shape is thought to be brought about due to a combination of genetic (Marble, 1943; Shultz, 1953; van Tijen & Kuit, 1970; Anderson et al., 2004) and adaptive (Grant, 1982) differences. In the latter case, various possible natural selective pressures have been proposed for birds to develop particular traits in egg features to improve chances of offspring survival. Surprisingly, we find no evidence of selective pressure in the form of a constraint on egg shape for the species investigated. Overlap in shape independent of size across species suggests that either there is little constraint or selection on shape across these diverse taxa, or that shared selective influences have led to convergence. A larger study incorporating taxa that exhibit a broader range of nesting behaviours on different substrates will be required to investigate these issues in more detail and better understand the factors influencing egg shape.

The variety of egg shapes extrapolated in this study concurred with that previously described for each species in the literature. The only shape described for superb fairy-wren eggs is an elongated oval, with markings commonly concentrated at the broader end (North 1901-14; Schodde, 1982; Campbell, 1990), implying that their eggs are only slightly rounder at one end. In contrast, at least several different shapes have been used to describe the eggs of all other species included in this study (Marchant and Higgins 1990). The eggs of all grey shrike-thrush subspecies have been described as either oval, rounded oval, stout oval to thick oval, or elongate-oval, with some being slightly pointed at each end (Marchant and Higgins 1990). PCA results show that all grey shrike-thrush eggs incorporated in the present study were at least slightly pointed at one pole, with some clutches being clearly more elongated than others. Spiny-cheeked honeyeaters have been previously reported to have had oval or elongate-oval eggs (North 1910-14; Campbell, 1990), with spots and blotches usually concentrated at the broader end (North 1910-14; Serventy & Whittell, 1962; Campbell, 1990). Only one clutch of eggs from this species included here (E06324) was very elongated along PC1, with all other eggs being only slightly elongated. This may possibly be attributed to geographic location; E06325 was from New South Wales, while the other clutches used from this species were from South Australia (Figure 1B). Similarly, the grey shrike-thrush clutch collected from South Australia (E14518) grouped together with low PC1 values (Figure 3, red square symbol), signifying less elongated eggs compared to the three clutches collected from New South Wales for this species (Figure 1B). All clutches within a given species are from the same subspecies, with the exception of the superb fairy-wren, which had one clutch from subspecies *leggei* (E14555), and the others from *cyanochlamys*. Our PCA results for eggs of the red-browed finch encompass a wide range of eggshell shapes (Figure 3), with this species producing the largest clutch size of the species included in this study (Table 1). Egg shapes of the Pied flycatcher *Ficedula hypoleuca* are high variability within populations, ranging from pointed to ellipsoidal to nearly spherical, and is proposed to be linked to clutch size (Kern & Cowie, 1996).

Surprisingly, a large range of egg shapes was found within each species expressed as between clutch. Whether variation in egg shape between clutches for these species is due to differences inherent in individual breeding females, or the location and year of collection remains unknown. Further information and quantification of impacts of hatch order and geographic location are required to better understand the processes involved in producing particular shaped eggs**.** We believe that the present study provides a method that can achieve this, allowing the quantification of fine-scale differences in egg shape not discernible using previously published approaches. This approach can be applied to any spherical to conical shaped object, including eggs from various taxa; from birds, and extant reptiles to non-avian dinosaurs and pterosaurs.

Our methods may also be of relevance to poultry science, enabling the inference of egg quality based on egg geometry parameters (Narushin & Romanov, 2002). The physical structure and chemical composition of an eggshell is commonly used as an indicator of egg quality and stability during storage and can have significant and direct effects on prices when eggs are graded (Narushin & Romanov, 2002). As egg quality traits are associated with hatchability and are moderately heritable, it is important that birds producing eggs with desirable traits are retained for future breeding (Ragozina, 1961; Rose, Simpson & Manning, 1996). Physical factors generally appreciated in eggs are shell appearance and strength, egg size, weight and the size and appearance of the albumen and yolk (Murray et al., 2013). Such information can also be used to provide tangible advice to farmers on the appropriate requirements for good quality eggs. Previous studies have used traditional linear measurements to evaluate egg geometry parameters to infer egg quality of domesticated chickens. Micro-CT may provide a viable option to provide precise measurements of egg volume and size, and eggshell thickness and shape. Egg quality traits are greatly influenced by breeding and environmental factors (Clark, Ewert & Nelson, 2001; Johnson, Leyhe & Werner, 2001). As eggs are a primary source of animal protein globally, with levels of egg consumption rising particularly in developing countries (Kern & Cowie, 1996), it is important that new scientific approaches are adopted to evaluate egg quality and production.

Data accessibility

Micro-CT scans and surface meshes of all specimens included in this study (in DICOM and PLY format, respectively) and the R scripts to perform the analyses are available on Figshare (see https://figshare.com/s/3af9f0cf5346e9b881f6 and https://figshare.com/s/45fee8f96179b19d7988).

Author contributions

Marie R. G. Attard conceived and designed the experiments, performed the experiments, analysed the data, wrote the manuscript, prepared figures and/or tables, reviewed drafts of the manuscript. Emma Sherratt conceived and designed the experiments, performed the experiments, analysed the data, wrote the manuscript, prepared figures and/or tables, reviewed drafts of the manuscript. Paul McDonald conceived and designed the experiments, wrote the manuscript, reviewed drafts of the manuscript. Iain Young conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the manuscript. Marta Vidal-García performed the experiments, prepared figures and/or tables, reviewed drafts of the manuscript. Stephen Wroe conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the manuscript.

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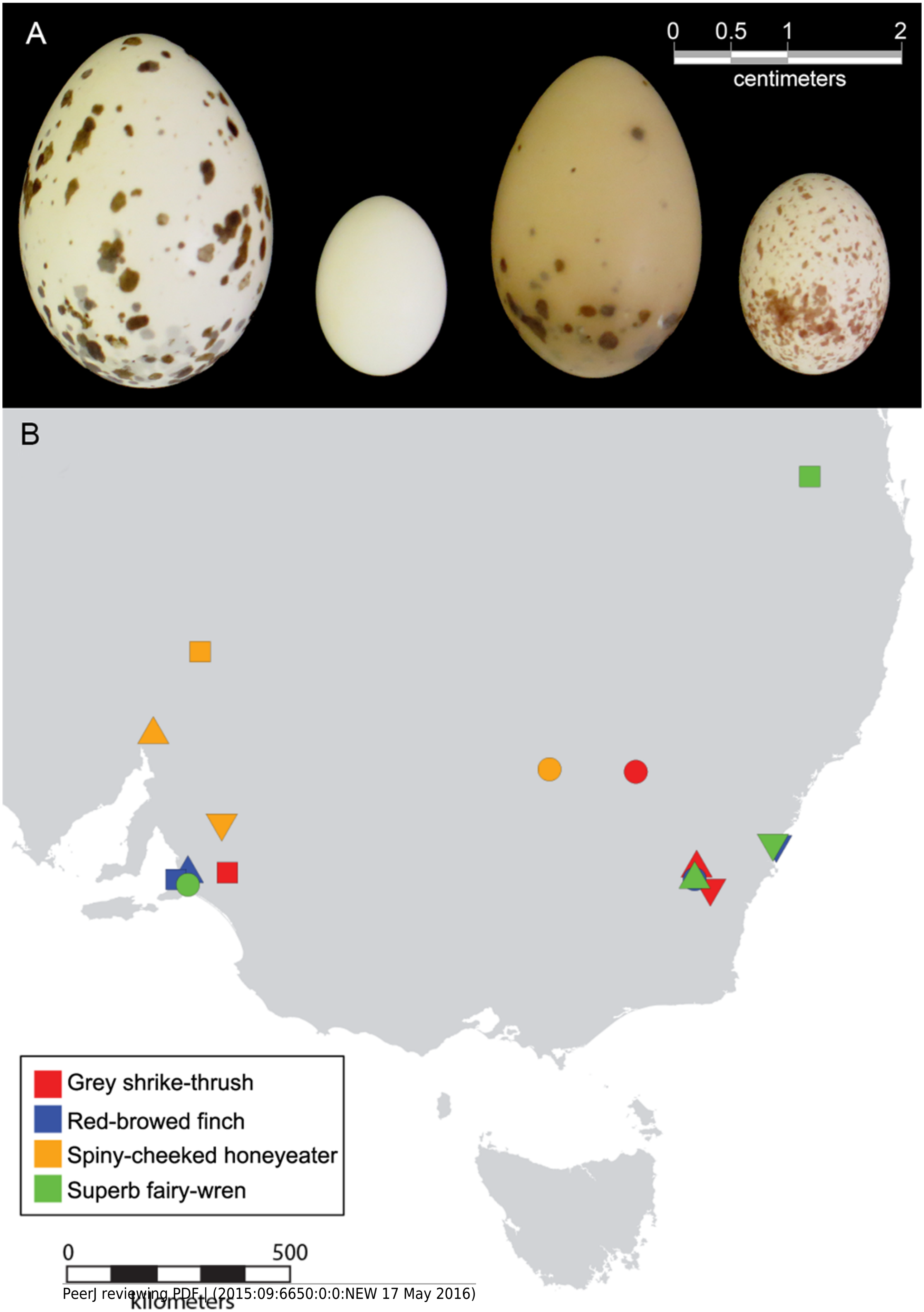
1

Photograph of eggs and geographical range of clutches included in this study.

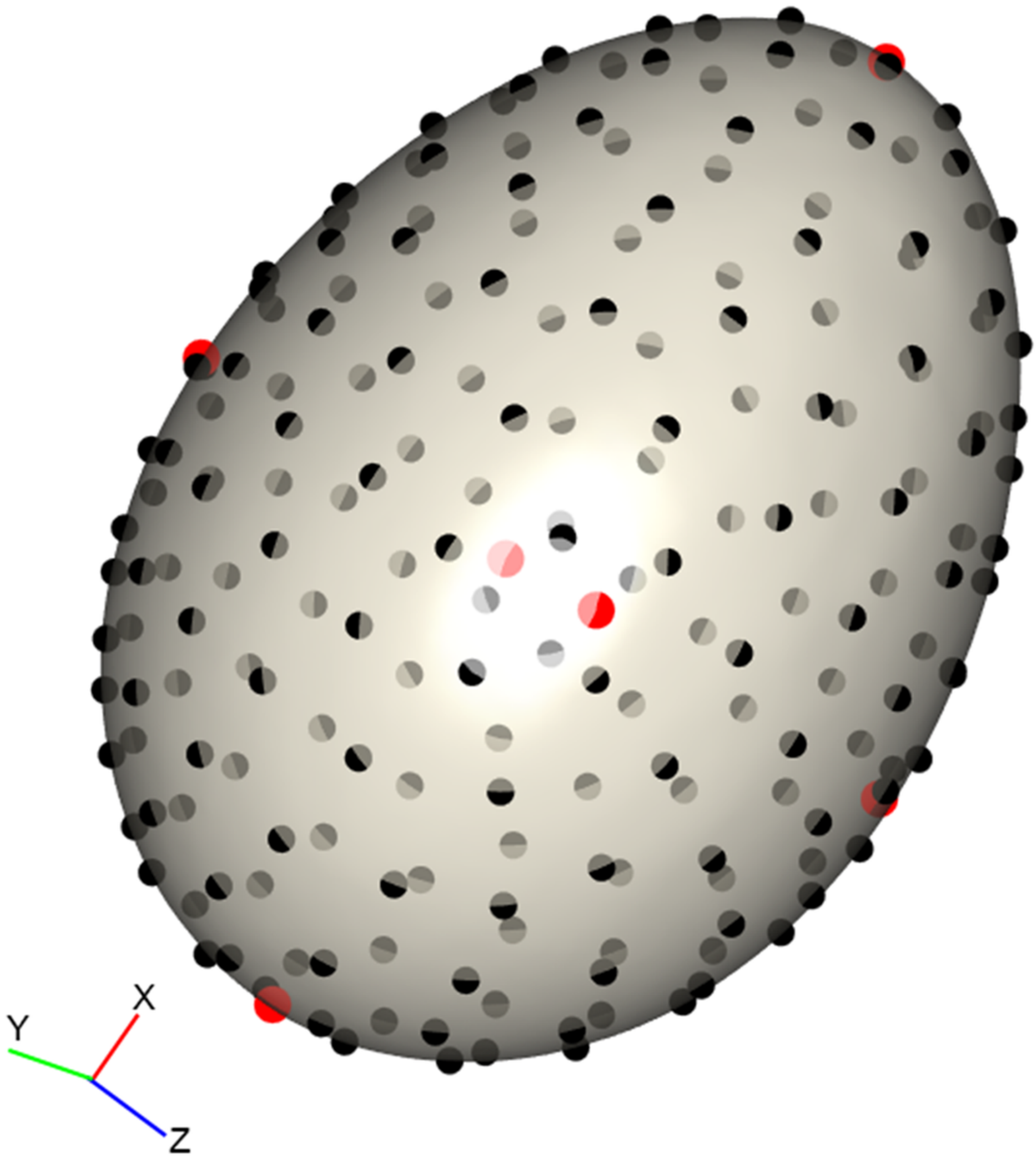
(A) Photographs of eggs from the four species of bird included in this study; from left to right, grey-shrike thrush, red-browed finch, spiny-cheeked honeyeater and superb fairy-wren. (B) Geographical range of bird clutches. See Table 2 for symbol shape used to represent each clutch.


A
0
0.5
1
2
centimeters
B
🟥 Grey shrike-thrush
🟦 Red-browed finch
🟧 Spiny-cheeked honeyeater
🟩 Superb fairy-wren
0
500
kilometers

2

Example of the digitisation of landmarks on a virtual egg.

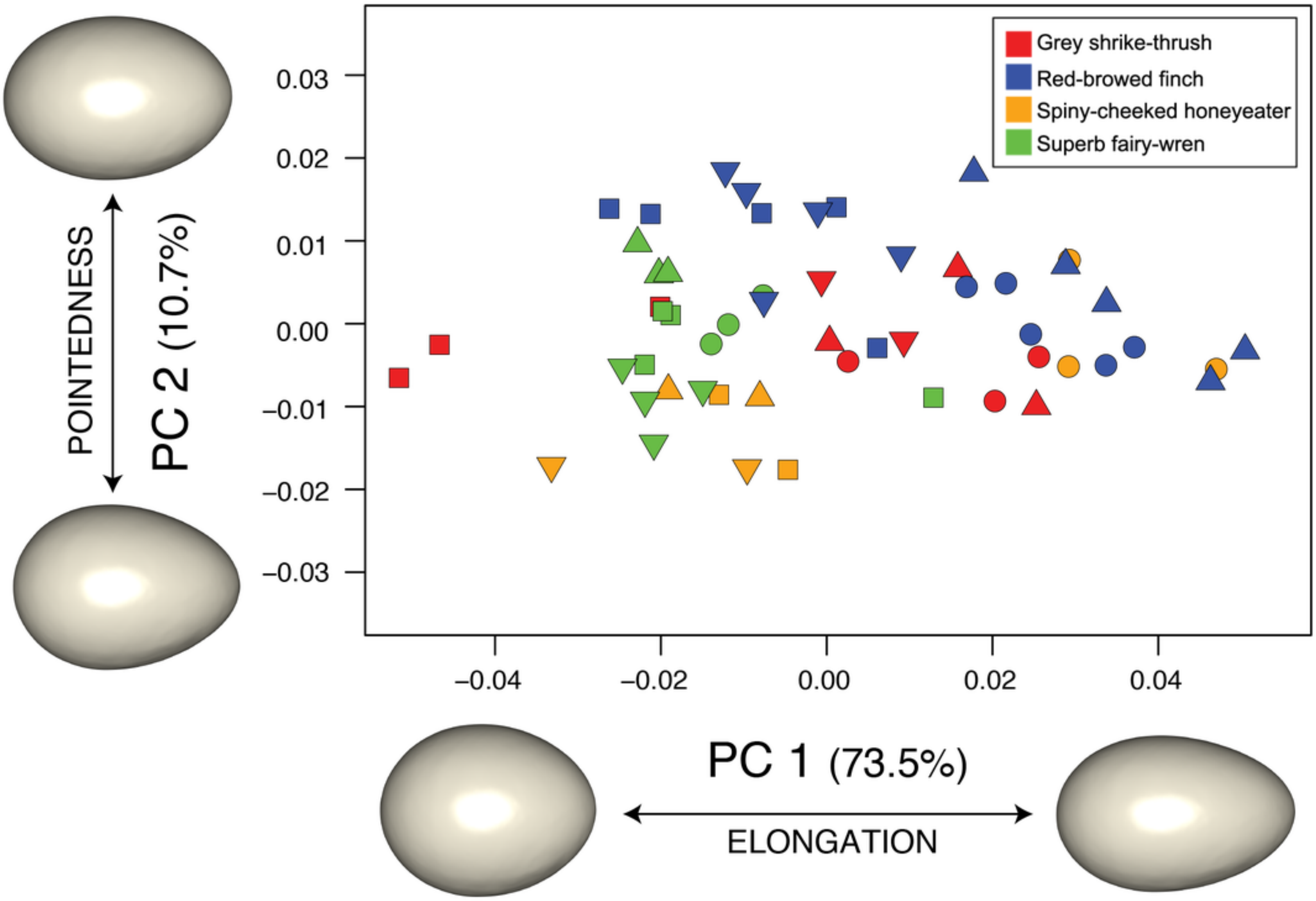
The position of six homologous landmarks (sharp pole, blunt pole and four equidistant points around equator) are shown in red, and the template of semilandmarks are shown in black. The surface mesh of the egg was reconstructed from micro-CT data.


Y
X
Z

3

Morphospace defined by the two first principal components (PC’s) of shape variance among four bird species.

The percentage of total variance described by each axis is shown in parentheses. Shapes associated with the extreme ends of each PC axis are shown as warped surface models (see text for details). Each symbol in shape space represents a single egg, and the origin point corresponds to the mean shape. Proximity of each symbol indicates similarity in shape. Symbol colour represents species and shape represents clutch (Table 2).



4

Bird egg shape disparity within species and clutches.

Disparity, measured as Procrustes variance, of egg shape within (A) species and (B) clutches. Colours correspond to species as in Figure 1. The y-axes of both graphs are plotted to the same scale. Pairwise comparisons of clutch disparity are presented in Table 4.

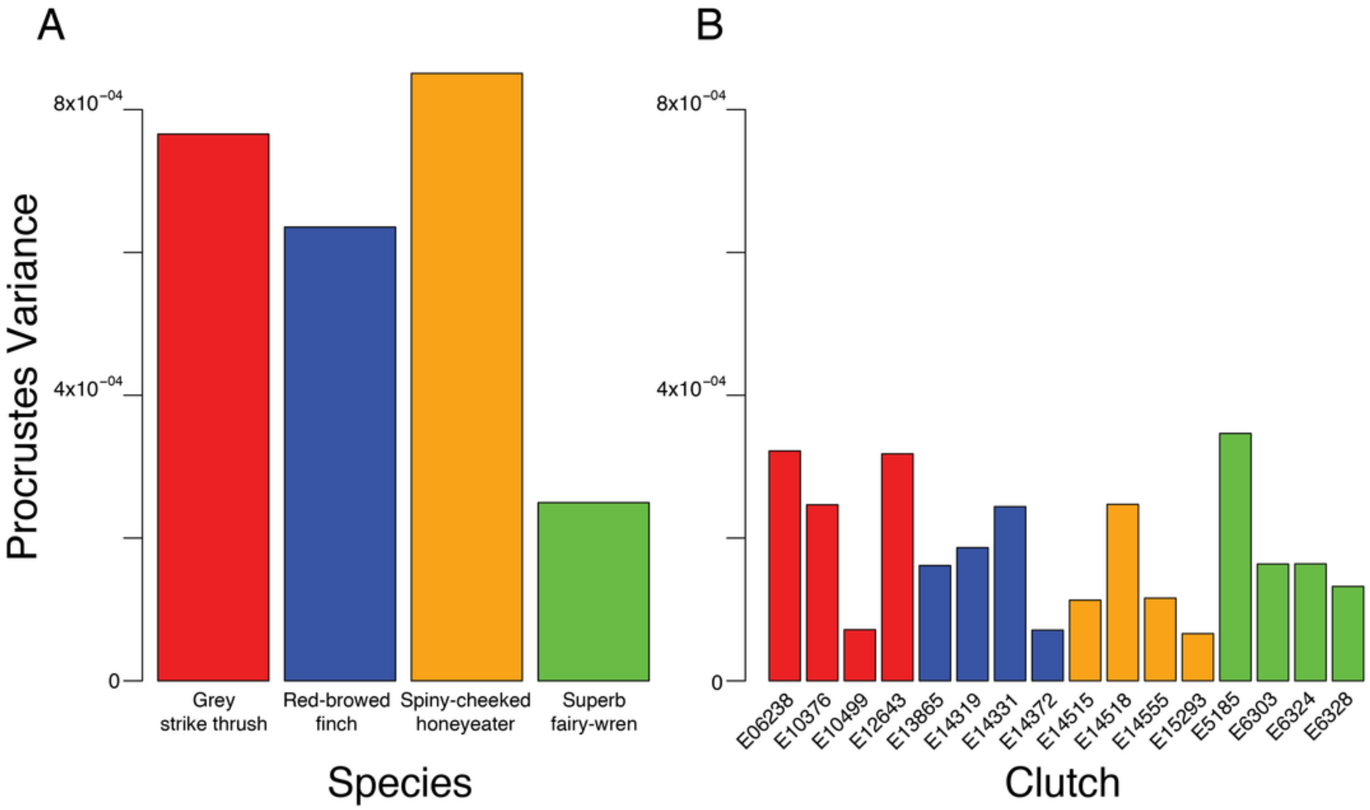


Table 1(on next page)

Body size, life history and evolutionary origin of four species of bird included in this study.

Incubation period is from completion to of clutch to hatching. Sources are Higgins, Peter & Steele, 2001; Higgins & Peter, 2002; Higgins, Peter & Cowling, 2006.

Common name	Family	Order	Clade	Population description	Body length range (mm)	Body mass average (mm)	Primary diet	Clutch size average	Clutch size range	Nest material	Nest shape	Nest site	Incubation	Incubation period (days)	Young development
Grey Shrike-thrush	Pachycephalidae	Passeriformes	Whistlers, Allies	Australian	22-27	67.5	Vertebrates, invertebrates	3	1-4	Strips of bark	Cup	Hollow	Biparental	16.9	Altricial
Red-browed Finch	Estrildidae	Passeriformes	Waxbills, Allies	Endemic	10-13	9.5	Small seeds and grasses	5	2-8	Dry or green grass	Dome or bottle-shaped with tunnel or spout entrance.	In fork among dense foilage	Biparental	14.6	Altricial
Spiny-cheeked Honeyeater	Meliphagidae	Passeriformes	Honeyeaters	Endemic	22-27	44.7	Fruit, nectar, insects	2.2	1-3	Grass, spider web	Cup	Live tree or shrub	Unknown	14.5	Altricial
Superb Fairy-wren	Maluridae	Passeriformes	Australasian Wrens	Endemic	11-14	10.6	Invertebrates	3.2	1-5	Grass, strips of bark, twigs, moss, fern fronds, spider web/egg-sacs	Dome with side entrance	Live plant	Female	14	Altricial

1

Table 2(on next page)

Museum accession and collection information on each clutch included in the study.

Common name	Catalogue Number	Clutch size	Latitude	Longitude	Date collected	Symbol
Grey shrike-thrush	E06328	3	-33.01	147.92	15/09/1999	circle
Grey shrike-thrush	E14331	3	-34.96	149.17	14/01/2004	triangle
Grey shrike-thrush	E14518	3	-35.11	139.54	06/09/1991	square
Grey shrike-thrush	E15293	2	-35.42	149.45	01/10/2006	inverted triangle
Red-browed finch	E06238	5	-35.1	138.73	27/10/1987	triangle
Red-browed finch	E10376	6	-35.28	138.57	08/11/1988	square
Red-browed finch	E14319	5	-34.57	150.77	11/12/2003	inverted triangle
Red-browed finch	E14515	5	-35.22	149.13	03/01/2005	circle
Spiny-cheeked honeyeater	E05185	2	-30.56	138.98	17/09/1987	square
Spiny-cheeked honeyeater	E06303	2	-34.1	139.43	27/08/1992	inverted triangle
Spiny-cheeked honeyeater	E06324	3	-32.97	146.15	14/09/1999	circle
Spiny-cheeked honeyeater	E14372	2	-32.19	138.02	12/09/1988	triangle
Superb fairy-wren	E10499	3	-35.22	149.13	15/10/2002	triangle
Superb fairy-wren	E12643	4	-26.97	151.5	15/09/2002	square
Superb fairy-wren	E13865	4	-34.55	150.73	31/10/1998	inverted triangle
Superb fairy-wren	E14555	3	-35.34	138.69	16/11/1992	circle

1

Table 3(on next page)

Nested D-ANOVA evaluating variation in shape between species and between clutches within each species.

P-values based on 1000 random residual permutations.

	Df	SS	MS	R^2	F	Z	Pr(>F)
species	3	0.00857	0.00285676	0.20656	1.5446	0.88833	0.32967
species:clutch	12	0.022194	0.0018495	0.53493	6.7251	2.92343	0.000999
Residuals	39	0.010726	0.00027501				
Total	54	0.04149					

Table 4(on next page)

Pairwise comparisons of Procrustes variance between clutches.

Values in the lower triangle are the observed pairwise absolute differences (distances) among clutch Procrustes variances. Upper triangle values are P-values associated with pairwise differences (1000 permutations). P-values in bold are significant at the 5% level.

Grey shrike-thrush (*Colluricincla harmonica*)

	E14331	E14518	E15293	E6328
E14331	-	0.9790	0.0989	0.2867
E14518	3.20E-06	-	0.0969	0.2812
E15293	1.78E-04	1.81E-04	-	0.5744
E6328	1.12E-04	1.15E-04	6.61E-05	-

Red-browed finch (*Neochmia temporalis*)

	E06238	E10376	E14319	E14515
E06238	-	0.4735	0.1658	**0.0320**
E10376	7.53E-05	-	0.5614	0.1588
E14319	1.35E-04	6.01E-05	-	0.4476
E14515	2.09E-04	1.34E-04	7.35E-05	-

Spiny-cheeked honeyeater (*Acanthagenys rufogularis*)

	E14372	E5185	E6303	E6324
E14372	-	**0.0025**	0.3861	0.3412
E5185	2.75E-04	-	0.0924	0.0554
E6303	9.26E-05	1.83E-04	-	0.9980
E6324	9.28E-05	1.83E-04	2.02E-07	-

Superb fairy-wren (*Malurus cyaneus*)

	E10499	E12643	E13865	E14555
E10499	-	**0.0290**	0.4985	0.6404
E12643	2.46E-04	-	0.2318	0.1239
E13865	8.99E-05	1.56E-04	-	0.6553
E14555	4.43E-05	2.02E-04	4.56E-05	-