© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb243016. doi:10.1242/jeb.243016

RESEARCH ARTICLE

How to build a puncture- and breakage-resistant eggshell? Mechanical and structural analyses of avian brood parasites and their hosts

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ABSTRACT

Evolved eggshell strength is greater in several lineages of obligate avian brood parasites (birds that lay their eggs in other species' nests) than in their hosts. Greater strength is typically indirectly implied by eggshell thickness comparisons between parasites and hosts. Nevertheless, there is strong evidence that the eggshell structural organization differentially influences its mechanical properties. Using instrumental puncture tests and SEM/EBSD and XRD techniques, we studied the most relevant eggshell mechanical, textural, ultrastructural and microstructural features between several host species and their parasitic cowbirds (*Molothrus* spp.). These parasitic species display different egg-destructive behaviors, reducing host reproductive fitness, including the more frequently host-egg puncturing *M. rufoaxillaris* and *M. bonariensis*, and the host egg-removing *M. ater*. The results, analyzed using a phylogenetic comparative approach, showed interspecific patterns in the mechanical and structural features. Overall, the eggshells of the two egg-puncturing parasites (but not of *M. ater*) were stronger, stiffer and required greater stress to produce a fracture than the respective hosts' eggs. These features were affected by eggshell microstructure and ultrastructure, related to the increase in the intercrystalline boundary network acting in cooperation with the increase in palisade layer thickness. Both structural features generate more options and greater lengths of intercrystalline paths, increasing the energy consumed in crack or fissure propagation. The reported patterns of all these diverse eggshell features support a new set of interpretations, confirming several hypotheses regarding the impact of the two reproductive strategies (parasitic versus parental) and parasitic egg destruction behaviors (more versus less frequently puncturing).

KEY WORDS: Brood parasitism, Crystallographic texture, EBSD, Eggshell strength, Eggshell thickness, Quasi-static punctures

INTRODUCTION

Among interspecific interactions, obligate brood parasitism is unique in its transfer of full parental care from the parasite to the host. Obligate brood parasitic birds lay their eggs in nests of other bird species, the hosts. The latter provide all parental care to the parasitic eggs, chicks and fledglings (Davies, 2000; Ortega, 1998; Rothstein and Robinson, 1998; Soler, 2017). The fitness of brood parasites depends critically on their ability to deposit their progeny in host nests, whereas the hosts can experience a partial to complete reduction in their reproductive success as a consequence of parasitism (Rothstein and Robinson, 1998). Therefore, obligate avian brood parasitism both provides productive research systems for studying co-evolutionary processes and requires an integrated understanding of the behavioral and morphological adaptations of brood parasites (and their hosts) to the species-specific reproductive strategy of parasites.

The obligate brood parasitic cowbirds (*Molothrus* spp.) usually lay their eggs from elevated positions, thereby damaging some of the host eggs as part of their laying process (Fraga, 2011; López et al., 2018). In turn, parasitic cowbird taxa display varied patterns in the use of their beaks to further reduce the clutch sizes of their selected hosts. Multiple parasitism and parasite-imposed intraspecific competitive damage are especially frequent in nests parasitized by the screaming cowbird (*M. rufoaxillaris*) and shiny cowbird (*M. bonariensis*) in South America (Reboreda et al., 2013). These two species, during the nest visitation and egg-laying events, frequently peck and puncture other eggs already laid in the nest, including other parasites' eggs (Astié and Reboreda, 2006, 2009; De Mársico et al., 2010; Fraga, 1998; Gloag et al., 2014; Hudson, 1874; Massoni and Reboreda, 1999, 2002). The brown-headed cowbird (*M. ater*) in North America typically removes just one host egg when it lays its own, by either grasp or puncture ejection (Sealy, 1992, 1994). Overall, the frequency of egg-puncture behavior by brown-headed cowbirds is much lower than that performed by the other two congeneric cowbirds (Antonson et al., 2020; Hauber, 2003; White et al., 2009). Therefore, species differences in the frequency of parasitic egg-puncturing behaviors could exert differential selection pressures, among cowbirds and hosts, on the eggshell material structural features, which could contribute differentially to their resulting mechanical properties (López et al., 2021).

Obligate brood parasitic bird species are historically known for their greater eggshell thickness beyond what is predicted by allometry, a characteristic across phylogenetically distant parasitic taxa (Icteridae: *Molothrus* spp., Cuculidae: *Cuculus* spp., and Indicatoridae: *Indicator* spp.; Antonov et al., 2012). Evolved eggshell strength is considered to be greater in several brood parasitic lineages than for their hosts, as differential shell mechanical strength is typically indirectly implied by eggshell thickness differences between parasites and hosts (Brooker

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Received 24 June 2021; Accepted 23 July 2021

and Brooker, 1991; Hudson, 1874; Igic et al., 2017; Picman, 1989; Spaw and Rohwer, 1987; Spottiswoode, 2010). Diverse hypotheses have been proposed to explain the benefits of increased parasitic eggshell thickness, both from the perspective of co-evolutionary arms races with the hosts and from intraspecific competition among the parasites themselves (Antonov et al., 2012). For example, the thicker eggshells of parasites eggs may reduce the chances of shell damage during rapid laying from an elevated position (López et al., 2018), prevent damage from the attempts by some of their smaller hosts to pierce-eject parasitic eggs when grasp ejection is impossible because of limited beak gape size (Sealy, 1996; Spaw and Rohwer, 1987; Swynnerton, 1918), and lessen breakage when additional parasite eggs are laid onto them if nests are multiply parasitized (Brooker and Brooker, 1991; Hudson, 1874).

Shell thickness is clearly a factor contributing to egg mechanical strength (Ar et al., 1979; Bain, 1991; Dunn et al., 2008; Romanoff and Romanoff, 1949; Tyler, 1961), and egg shape is probably ranked second among the egg and shell features affecting breaking strength (Anderson et al., 2004; Bain, 1991). The optimally strong avian egg shape should be spherical, because this would provide the highest resistance against external forces (Bain, 1991) while also providing the most effective gas transfer between the embryo and the outside environment (Ar et al., 1979). However, these features on their own are not adequate to explain the diversity of avian egg shapes found in nature (Stoddard et al., 2017), and egg shape, in terms of deviation from a sphere, does not appear to be decisive for resistance to breakage (Panheleux et al., 1999; Nedomova et al., 2009). Nevertheless, there is increasingly strong evidence from a wide range of avian taxa that the ultrastructural and microstructural organization of the eggshell material can further influence its mechanical properties (Carnarius et al., 1996; Igic et al., 2011; Panheleux et al., 1999; Rodríguez-Navarro et al., 2002). Meanwhile, whereas the main ultrastructure characteristics and mineralogical composition of eggshells appear uniform at the species level (Nys et al., 2004), there is detectable variability in shell microstructure and resulting mechanical properties interspecifically (Ahmed et al., 2005; Carnarius et al., 1996; Igic et al., 2011, 2017; Meller et al., 1973; Nys et al., 2010; Panheleux et al., 1999; Petersen and Tyler, 1967; Rodríguez-Navarro et al., 2002; Van Toledo et al., 1982). The structural examination of mainly poultry eggshells has greatly enhanced our understanding of avian shell architecture (Pérez-Huerta and Dauphin, 2016), reinforcing the view that the egg mechanical properties cannot be solely determined by shell thickness.

The avian eggshell is a polycrystalline bio-ceramic combining inorganic compounds (mostly calcite) immersed in an organic matrix (Romanoff and Romanoff, 1949; Gautron et al., 2021). Regarding the shell's ultrastructure, three main mineralized layers can be differentiated (outer prismatic palisade layer, inner palisade or thick spongy layer, and mammillary layer), as well as the gas exchange pore structure and organic membranes (Dennis et al., 1996; Nathusius von Königsborn, 1868; Mikhailov, 1987; Stewart, 1934). Simons (1971) proposed that each of these mineralized layers must differently contribute to an eggshell's performance under load, while the organic membranes do not affect eggshell strength, but are essential during the early formation of the eggshell (Du et al., 2015; Dunn et al., 2011). The palisade layers would be involved in eggshell strength (Van toledo et al., 1982; Carnarius et al., 1996), while the mammillary layer would not contribute to the eggshell mechanical properties (Bain, 1990). The calcite grain (or crystal) morphology, including the size and shape distributions, characteristics of its borders and how they are arranged and oriented define the microstructure and texture (Bunge, 1982).

Regarding avian brood parasite-host systems, the content of inorganic components (Picman, 1989) and the density of the eggshell (Picman and Pribil, 1997) have also been used to indirectly infer differences in the eggshell strength between parasites and hosts. However, there is no evidence for the presence of different relative amounts of organic versus inorganic components C, Ca and Mg on the different eggshell mineralized layers, when these were quantified using the energy-dispersive X-ray spectroscopy technique, among parasitic cuckoos and their hosts/non-hosts (Igic et al., 2011), and southern hemisphere parasitic cowbirds and their hosts (López, 2013). In both systems, magnesium was found in the mammillary layers and its concentration in calcite ($CaCO_3$-trigonal) did not show differences between the parasite and host eggs. In addition, there is also no evidence of differences between the parasitic cowbirds and their hosts in the distribution and density of the rounded vesicles in the inner palisade layer (López, 2013; this study, data not shown). These results support the hypothesis that mineral composition in avian eggshells is a conserved phylogenetic feature (Board and Love, 1980; Nys et al., 2004).

There are no data comparing eggshell mechanical features per se between congeneric cowbird species and many of their main smaller or larger icterid and non-icterid host species. New information on the quantitative interspecific patterns of the parasitic cowbird eggshell mechanical, ultrastructure and microstructure features via a phylogenetic comparative approach is therefore much needed for a full evaluation of alternative hypotheses.

The main aim of this study was to perform an interspecific analysis of a suite of major eggshell mechanical features of both host and parasite eggs in a phylogenetic framework, associating those features with usual egg and shell macroscopic and ultrastructural variables and the new eggshell microstructure and texture characteristics, using scanning electron microscopy (SEM) based on electron back-scattering diffraction (EBSD) and X-ray diffraction techniques, focusing on parasitic cowbirds and several of their larger or smaller and non-icterid or icterid hosts. The mechanical behavior of solid materials can be studied by means of quasi-static puncture tests, obtaining the force–displacement relationships, and the derived eggshell mechanical properties.

Specifically, within a dynamic co-evolutionary process and in the context of adaptations to brood parasitic reproduction, we predicted that the eggs of both frequently egg-puncturing parasitic species, the screaming and shiny cowbirds, would have greater shell mechanical strength than eggs of the other, typically non-egg-puncturing parasite, the brown-headed cowbird, and all the hosts (regardless of host size), because increased eggshell strength would prevent successful puncture of the parasitic eggs by conspecific parasites in nests of all host types. In turn, we predicted that the brown-headed cowbird egg would have greater shell mechanical strength than the eggs of its smaller sized hosts to resist puncture ejection by these hosts whose beaks are too small and cannot be used to grasp-eject the parasitic eggs. However, shell strength of this cowbird's eggs may or may not be greater than that of the eggs of their larger hosts and of the egg-puncturing cowbirds, because eggshell mechanical properties would not be directionally selected as its larger hosts typically reject the parasitic eggs through grasp-ejection and conspecific parasitic puncture events are infrequent. According to previous work (see above), we also expected that eggs of the three brood parasitic cowbirds would have shells thicker than expected by allometry, when compared with other related but non-parasitic icterid species, because shell thickness is a characteristic that positively contributes to eggshell mechanical strength. Furthermore, we expected that eggs of the screaming and shiny cowbirds would show eggshell microstructures and microtextures differing from those of both the brown-headed cowbird and host species, because calcite crystal structural shifts could act synergistically with the shell thickness on the resulting mechanical properties.

Considering the egg and shell features commonly used to infer eggshell strength differences between parasites and hosts, and including the new mechanical and microstructural data reported by the current work, we investigated not only evolutionary associations but also evolutionary singularities (Nunn and Zhu, 2014) and performed multimodel-inference analyses, as new methods are needed to integrate the understanding of general phenotypic regressions, fundamental to the comparative approach to testing pattern convergence in parasites, and alternative adaptive biological hypotheses.

MATERIALS AND METHODS

Study areas and egg species

The fieldwork was conducted in Buenos Aires Province, Argentina (37°26′S, 61°53′W), during the southern breeding season (October to January of 2014/15 and 2015/16), and in Ithaca, NY, USA (42°26′N, 76°30′W), during the northern breeding season (May to July of 2015). We collected egg samples (one egg per nest) during the laying period (i.e. unincubated eggs) from the parasite species: screaming cowbird (Icteridae: *Molothrus rufoaxillaris* Cassin 1866; *n*=12, mass 52 g; Reboreda et al., 1996) and shiny cowbird [*Molothrus bonariensis* (Gmelin 1789); *n*=15, 46 g; Reboreda et al., 1996], and their host species: great kiskadee [Tyrannidae: *Pitangus sulphuratus* (Linnaeus 1766); *n*=8, 61 g; Dunning, 1992], house wren (Troglodytidae: *Troglodytes aedon* Vieillot 1809; *n*=12, 15 g; Dunning, 1992), rufous-bellied thrush (Turdidae: *Turdus rufiventris* Vieillot 1818; *n*=11, 80 g; Dunning, 1992), chalk-browed mockingbird [Mimidae: *Mimus saturninus* (Lichtenstein 1823); *n*=10, 75 g; Dunning, 1992; Fig. 1A], red-crested cardinal [Thraupidae: *Paroaria coronata* (Miller 1776); *n*=6, 36 g; Dunning, 1992) and chestnut-capped blackbird [Icteridae: *Chrysomus ruficapillus* (Vieillot 1819); *n*=12, 32 g; Dunning, 1992]. All these host species are frequent hosts of shiny cowbirds; the brown-and-yellow marshbird [Icteridae: *Pseudoleistes virescens* (Vieillot 1819); *n*=12, 78 g; Dunning, 1992] is also a frequent host of shiny cowbirds, but is an occasional host of screaming cowbirds; by contrast, the grayish baywing [Icteridae: *Agelaioides badius* (Vieillot 1819); *n*=14, 40 g; Dunning, 1992] is an occasional host of shiny cowbirds, but is the main host of screaming cowbirds (e.g. Fraga, 1986; Mermoz and Reboreda, 1996). We also collected fresh eggs from the northern parasite the brown-headed cowbird [Icteridae: *Molothrus ater* (Boddaert 1783); *n*=11, adult female body mass: 38 g; Dunning, 1992] and its host species: eastern phoebe [Tyrannidae: *Sayornis phoebe* (Latham 1790); *n*=10, 19 g; Dunning, 1992], Carolina wren [Troglodytidae: *Thryothorus ludovicianus* (Latham 1790); *n*=9, 17 g; Dunning, 1992], American robin (Turdidae: *Turdus migratorius* Linnaeus 1766; *n*=10, 78 g; Dunning, 1992; Fig. 1A), gray catbird [Mimidae: *Dumetella carolinensis* (Linnaeus 1766); *n*=11, 35 g; Dunning, 1992], northern cardinal [Cardinalidae: *Cardinalis cardinalis* (Linnaeus 1758); *n*=10, 43 g; Dunning, 1992] and red-winged blackbird [Icteridae: *Agelaius phoeniceus* (Linnaeus 1766); *n*=11, 42 g, Dunning, 1992] (see Fig. S1 and Table S1). The parasites and their host species coexist in their respective breeding areas. We classified a host species as 'large host' when the mass of its egg was greater than the mass of the respective parasite's egg, and 'small host' when the mass of its egg was less than the mass of the respective parasite's egg (see Fig. S1 and Table S1). Body mass (g) values correspond to the mass of adult females.

Egg mechanical properties

The mechanical properties were evaluated with a Universal Testing Machine (model 3345, Instron Corp., Norwood, MA, USA) on intact eggs (without denting or cracking) at room temperature. The egg puncture tests were performed with a punch probe, which was displaced perpendicular to the bearing surface (Fig. 1B). A cylindrical probe with a 1.8 mm diameter and flat tip was used, and it moved with a constant speed (0.5 mm min^{-1}). Each egg was placed on a flat rigid horizontal surface and the probe made contact in the zone of maximum diameter (equatorial section) of the deforming egg (Fig. 1B). The force versus displacement curves were recorded (Fig. 1C; Fig. S1) for each test and the maximum force (*F*) needed to fracture the egg (breaking strength or egg puncture resistance; Table S1), the egg deformation (*d*) until failure and the mechanical stiffness (*S*) were calculated. The egg's equatorial section represents the weakest and most uniform part of the egg, thus providing the best estimate of the lower limits of egg resistance (Voisey and Hunt, 1974); moreover, the pecking and puncturing behaviors of cowbirds and hosts are focused to attack this part of the egg (fig. 2 in López et al., 2018). To compare eggs of species with different sizes and shape, we calculated the major specific (or intrinsic) macro-mechanical features of a material, such as the shear stress (or shear strength, τ; Eqn 1; Hunt et al., 1977), eggshell elastic modulus (or Young's modulus of the shell, *E*; Eqn 2; Bain, 1990), the eggshell fracture toughness (or resistance to fracture, K_c; Eqn 3; Bain, 1990), and eggshell puncture energy (e_p; Eqn 4; López et al., 2021) of each egg:

$$\tau = F/(\pi \times \delta \times T), \tag{1}$$

$$E = C \times ((S \times R)/T^2), \tag{2}$$

$$K_c = K_{nd} \times (F/T^{3/2}), \tag{3}$$

$$e_p = \int_0^{D/B} (f/a)\, d(d/B), \tag{4}$$

$$C = A \times (0.408 + (3.026 \times T)/R), \tag{5}$$

$$K_{nd} = 0.777 \times (2.388 + (2.9934 \times (6/R))), \tag{6}$$

$$\begin{aligned} A =&(-0.666 + (1.8666 \times \mathrm{SI}) - (0.907 \times \mathrm{SI}^2) \\ &+ (0.153 \times \mathrm{SI}^3))/0.444, \end{aligned} \tag{7}$$

where τ is shear stress (N mm^{-2}); *F* is breaking strength (N); *T* is eggshell thickness (mm); δ is punch diameter (mm); *E* is eggshell elastic modulus (N mm^{-2}); *S* is eggshell stiffness (N mm^{-1}), calculated as the slope of the force–displacement curves; *R* is the radius of curvature, calculated as egg breadth/2 (mm); K_c is eggshell fracture toughness (N mm$^{-3/2}$); e_p is eggshell puncture energy (J), *f* is applied force on the egg (N), *a* is punch area (mm^2), *d* is punch displacement (mm), *B* is egg breadth (mm); and *D* is punch displacement until failure (mm). *C* (compliance), *A* and K_{nd} (non-dimensionalized stress-intensity factor) are shape-dependent parameters calculated by Bain (1990); SI is shape index, calculated as egg length/breadth (mm mm^{-1}).

Breaking strength describes the response of the eggshell as a composite complex structure (Voisey and Hunt, 1974) and it is used as an index of eggshell strength (Bain, 1990). Shear stress is the breaking strength per unit punch contact area (i.e. cross-sectional area of eggshell, with area parallel to the applied force vector), unaffected by egg shape but accounting for shell thickness (Hunt et al., 1977). During puncture test, a tensile crack develops from the

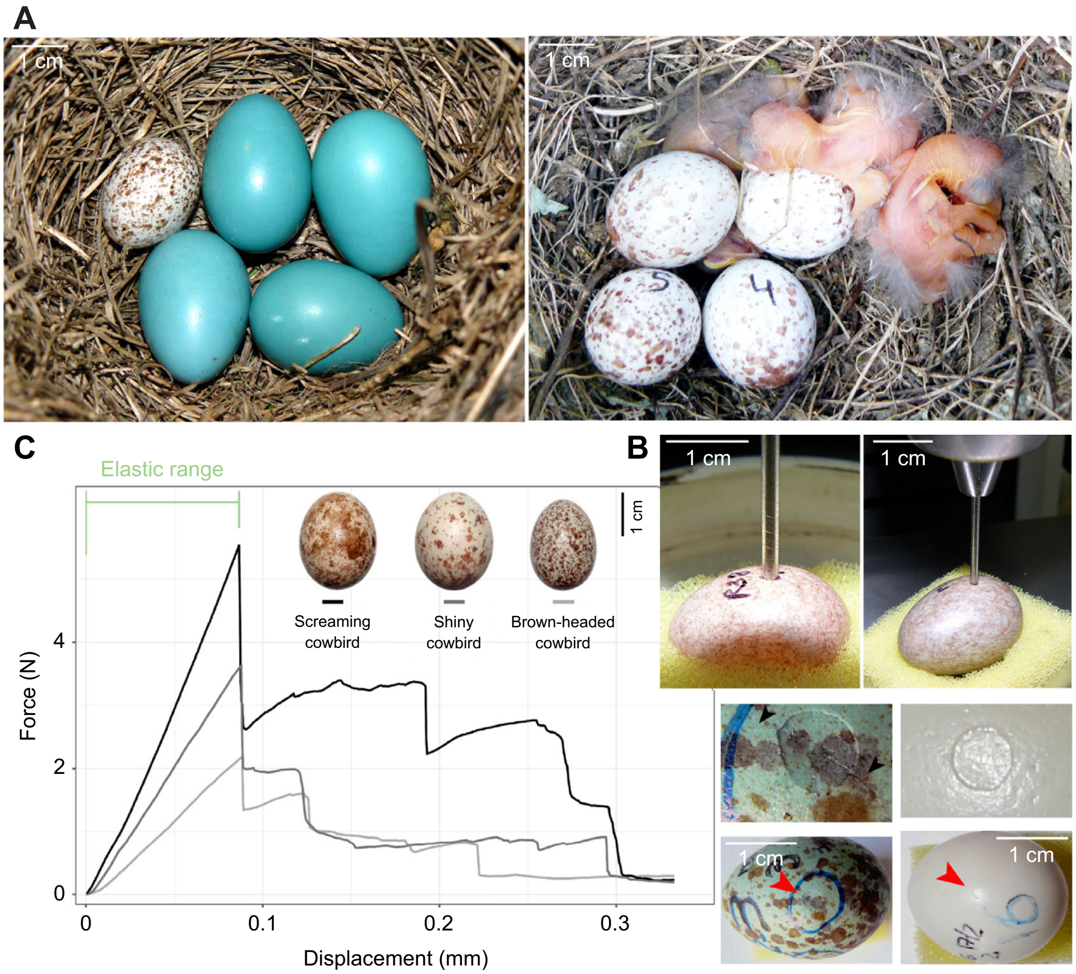

Fig. 1. Study species' eggs and their mechanical properties. (A) Active nests of an American robin (in North America) with 4 of its own eggs, parasitized by 1 brown-headed cowbird egg (left), and a chalk-browed mockingbird (in South America) without its own eggs, parasitized by 7 shiny cowbird eggs and chicks (right). The mockingbird experienced a complete reduction in the size of its clutch (4 eggs) as a consequence of multiple parasitism and intraspecific competitive damage imposed by the southern parasitic cowbirds. (B) Puncture tests on eggs of a small host (house wren; left) and a large host (mockingbird; right). The red arrow shows the macroscopic circular crack on the load site in eggs of a large host (mockingbird; left) and a parasite (shiny cowbird; right); this type of cracking occurs at maximum load (the breaking strength). (C) Force versus displacement curves recorded during puncture tests on eggs of the parasitic screaming, shiny and brown-headed cowbird. Photo credit: A.V.L.

edge of the punch and spreads through the shell as a local tensile rather than shear failure, as is frequently characterized in the literature (Macleod et al., 2006; Stevenson et al., 1979). The punch periphery shears an annulus of the shell material (Fig. 1B); although this seems to be a puncture test, it uses a different principle known as the 'punch and die test' (Bourne, 2002; Stevenson et al., 1979). Shear stress provides a measure of stress causing eggshell fracture (Hunt et al., 1977). The slope of the force versus deformation curve (*S*) provides a measure of the overall stiffness characteristics of an eggshell (Voisey and Hamilton, 1975) and represents a measure of the force per unit of deformation within the elastic range. The elastic modulus describes the contribution made by the eggshell material to the overall stiffness characteristics of the eggshell (Bain, 1990). The ability of a material to resist the propagation of small cracks can be described using the mechanical property known as fracture toughness (Taylor et al., 2016), which is expressed through the parameter K_c (Bain, 1990; Mabe et al., 2003). Clark and Acree (1974) showed, for eggs of the same species, that puncture force increased with punch diameter until the shell fractured as if subjected to compression as the punch became large enough to act as a compression surface. In our case, we used the same type of punch (1.8 mm diameter and flat tip) during our puncture tests on eggs of different species covering a wide range of sizes; therefore, we have standardized the values of force (f) and displacement (d) per unit of contact area of the punch (a) on the egg, and per unit of egg breadth (B) (place of contact with the punch), respectively. The eggshell puncture energy can be determined by integrating the (f/a) versus (d/B) curve from the origin point to the point of egg fracture (elastic linear range; Fig. 1C), and it provides a measure of the amount of energy that eggshell material can absorb during puncture tests within the elastic deformation range (López et al., 2021). The slope and the integral of the (f/a) versus (d/B) curves were calculated using 'deSolve' package in R (v.1.27; Soetaert et al., 2010).

Although puncture force is closely related to compression fracture force (Hunt et al., 1977), we note that the equations provided by Bain (1990) were developed for tests where an egg is compressed between two parallel plates. Under compressive loading, domestic hen (*Gallus gallus*) eggs deform in an essentially linear elastic manner until the maximum load, when one or more macroscopic cracks extend radially from the load point, causing an abrupt load drop and a loss of stiffness, as illustrated in figs 3 and 4 of Macleod et al. (2006). These authors also found that flat-ended punches with diameters of 2 mm and above behaved as infinite flat plates and caused failure by macroscopic cracking. In the current study, we used flat-ended punches with a diameter of 1.8 mm; however, the egg sizes of the host and parasite species are overall 4–14% of the hen egg size, the patterns of the force versus deformation curves and macroscopic cracks (Fig. 1B) are visually equivalent to those shown by Macleod et al. (2006). Therefore, we assume that the equations developed by Bain (1990) are suitable for use in our analyses.

Critically, according to Simons (1971), the organic membranes do not affect eggshell strength. Therefore, we also assume these did not affect our measurements of the eggshell breaking strength, stiffness and estimated mechanical properties as we worked only within the linear elastic range of force versus displacement curves (Fig. 1).

Egg macrostructure measurements

Prior to performing the puncture tests, the egg length (L), maximum breadth (B) and egg mass (M_e) were measured on all individuals using a caliper (Mitutoyo, accuracy ±0.02 mm) and a digital scale (Precisa 200A, accuracy ±0.001 g). The egg volume (V; ml) and egg aspect ratio (SI) were calculated as $V=498\times10^{-6}\times L\times B^2$ and SI=L/B (Picman, 1989; Spaw and Rohwer, 1987). After performing the puncture tests, the eggshells were washed with water and 70% alcohol and dried at room temperature for at least 48 h, and their mass (M_s) was measured and the shell's relative proportion of the total egg mass (hereafter eggshell ratio) was calculated as $100\times M_s/M_e$. The eggshells were kept in a desiccator over silica-gel until further examination.

Eggshell ultrastructure measurements

The ultrastructure of shell cross-sections was analyzed from the images obtained by SEM with a Zeiss Supra 40 microscope with in-lens secondary electron detector at 5 kV. Eggshell samples of the equatorial region were separated and adhered on aluminium stubs by a double conductive carbon tape and metalized with a gold layer (15–20 nm). The total thickness of each shell specimen was measured as the cross-sectional distance from its outermost surface to the point where the basal mammillary cones get inserted into the organic membranes. The thickness of the mammillary layer was also assessed, this being the distance from the basal cones to the point at which the palisade columns first fuse. The outer palisade (including cuticle) and inner palisade layers were differentiated by visual comparison of rounded vesicle density and topographic roughness (Dennis et al., 1996). We also calculated the layer thickness relative to the eggshell total thickness (%) [hereafter mammillary layer (ML) ratio and palisade layer (PL) ratio].

Eggshell microstructure and microtexture measurements

Pieces of shell from the equatorial region of the eggs embedded in conductive resin and polished with diamond paste of 9, 6, 3 and 1 µm and colloidal silica (0.05 µm). A final polishing step in a vibratory polisher was included to improve the pattern quality. The polished surface was analyzed in low vacuum mode using a field emission gun FEI Quanta 200 scanning microscope equipped with an EDAX TSL-OIM EBSD. The orientation of each scanned pixel is determined by indexing Kikuchi patterns (Adams et al., 1993), and the whole crystalline map can be reconstructed by the proper processing of data. Orientation data obtained by systematically scanning (Adams et al., 1993) the polished eggshell surface enable the spatial distribution of grain boundary networks to be mapped and, correspondingly, the spatial arrangement of crystalline orientations (microtexture) to be displayed. From the images, crystal morphology was characterized by estimating the following variables: grain aspect ratio (as a grain shape index), grain area (as a grain size index), low-angle boundaries, high-angle boundaries and total-angle boundaries, with these last three parameters used here to estimate the complexity of grain boundary networks (Bate et al., 2005). The grain aspect ratio was defined by fitting an ellipse to each grain and calculating the ratio between the lengths of the minor and major axes. By fixing a maximum misorientation of 30 pixels and a minimum of 3 pixels, we could determine continuous regions that constitute individual grains, and measure grain (crystal) sizes. The grain boundary networks could be separated by setting values of the misorientation angle ω, i.e. angular misorientation in the angle–axes orientation scheme, as follows. Low-angle boundaries (LAB, 3 deg<ω<15 deg), which are usually low-energy boundaries, and in contrast to high-angle boundaries (HAB, 15 deg<ω<110 deg), which are high-energy boundaries (Gottstein, 2004). Total-angle boundaries (TAB) are both low- and high-energy boundaries. We tailored the LAB, HAB and TAB densities, dividing the lengths of these boundaries by the scanned area [LAB, HAB and TAB density ($1\ \mu m^{-1}$), respectively]. To compare grain size and boundary lengths between egg species with different boundary densities and shell thicknesses, we defined the dimensionless variables grain size ratio as the division of the grain area by the palisade layer area, and relative LAB, HAB and TAB as the respective multiplication of LAB, HAB and TAB density by the thickness of the palisade layer. That way, they become independent of the scanned area and egg thickness.

In addition, we performed texture analysis on eggshell samples using the Schultz X-ray diffraction reflection technique (Schulz, 1949). We resorted to this technique because the rather large crystal sizes detected by EBSD suggested that EBSD measurements may present poor statistics for texture determination. Texture is also a dimensionless variable (Bunge, 1982), although it is a density distribution in a three-dimensional space of angular orientations. Eggshell texture measurements and crystallographic characterization were performed by standard diffractograms in a Panalytical X-pert Pro MPD diffractometer equipped with Cu Kα radiation, multifilamentary X-ray concentrator (X-ray 'lens'), parallel plate collimator and Xe detector. In this case, because of the 100–200 µm penetration of the X-rays, the eggshell samples did not need special or specific preparation. Several pole figures were measured and, after orientation distribution function (ODF) calculation, re-calculated to check result consistency. Inverse pole figures (IPFs) and pole figures (PFs) can be calculated from ODFs. Furthermore, the degree of preferred crystalline orientation was analyzed through the alignment of the <0001> directions with the radial direction, either by plotting PF or IPF or by calculating Kearns factors. Kearns factors are a numerical quantity representative of the preferred crystalline orientation (Kearns, 1965), allowing for even further reduction of the complexity of the texture variable and taking advantage of the high dependence of any physical variable on the <c> axis crystal direction. Kearns factor values vary between 1 (all the crystals are aligned pointing with their <c> axis perpendicular to the shell) and 0 (none). They are integral polar values taking in account the fiber-like intensity of <0001> crystal direction with respect to the radial egg direction. They constitute an additional dimensionless variable for representing texture effects in a compact way.

Statistical analyses

Statistical analyses were conducted using R software v.3.6.3 (http://www.R-project.org/). To correct for non-independence because of common ancestry of species (Grafen, 1989), the data were analyzed via phylogenetic generalized linear mixed-effects models (PGLMMs) with Gaussian error, using the Markov chain Monte Carlo (MCMC) approach by Bayesian statistics, via the *MCMCglmm* function from the 'MCMCglmm' package (v.2.29; Hadfield, 2010). A set of 1000 topologies was obtained from the Bayesian posterior distribution provided in www.birdtree.org (Jetz et al., 2012). A unique phylogenetic consensus tree with branch lengths expressing divergence time was generated (Fig. S1) and both block of topologies and consensus tree were used as a framework for performing the Bayesian analyses.

Phylogenetic comparative analyses

To determine whether consistent differences exist between egg groups, we used information on the historical (not-independent) relationships of lineages (phylogenies) to test evolutionary hypotheses. Accordingly, we fitted Bayesian PGLMMs to compare each egg and shell feature (response variable; see below) on the egg groups given by the interaction between egg class (levels: large hosts, small hosts and parasites) and egg pecking (i.e. if the eggs inside the host's nest are frequently pecked and damaged by parasites; levels: yes and no) as fixed effect variables. We included the phylogeny (given by phylogenetic variance–covariance matrix) and the species (as species-specific effect) as the random effect variables. Each response variable was defined as eggshell breaking strength, eggshell stiffness, shear stress, eggshell elastic modulus, eggshell fracture toughness, eggshell puncture energy, eggshell ratio, PL ratio, ML ratio, Kearns factor, grain size ratio, relative LAB, relative HAB and relative TAB, and grain aspect ratio. PGLMMs were run for 5 million iterations with a burn-in of 10,000 and a thinning interval of 500. This generated 10,000 samples from each chain from which parameters were estimated. Before fitting the models, we explored the normality and homogeneity of variance. The violations to these assumptions were addressed by the log-transformation of the variables. For the PGLMM diagnosis, we analyzed the Markov chain to check reliability of the posterior approximation through Trace-plot and Autocorrelation-plot (Hadfield, 2010). We also applied the Heidelberg stationary test with the R package 'coda', a diagnostic test of convergence that uses the Cramér–von Mises statistic to test the null hypothesis that the sampled values come from a stationary distribution (Plummer et al., 2006). The Bayesian contrasts (hereafter BC) were performed using the *tidyMCMC* function from the 'broom' and 'broom.mixed' packages in R (http://github.com/tidyverse/broom, https://github.com/bbolker/broom.mixed). Fitted models and contrasts were considered statistically significant when the 95% credible interval (CrI) did not span 0 or MCMC $P\leq0.05$ (Hadfield, 2010). In the cases where there was evidence of a significant interaction on the response variable, we could re-define the six levels (egg groups) as eggs from: (1) shiny and screaming cowbird's large hosts (or southern large hosts' eggs), (2) brown-headed cowbird's large hosts (or northern large hosts' eggs), (3) shiny and screaming cowbird's small hosts (or southern small hosts' eggs), (4) brown-headed cowbird's small hosts (or northern small hosts' eggs), (5) shiny and screaming cowbirds (or southern parasite eggs) and (6) brown-headed cowbird (or northern parasite eggs).

Phylogenetic evolutionary association models

We assessed the associations between eggshell feature pairs over evolutionary time (i.e. evolutionary association) by a new alternative statistical methodology, to show that the parasitic cowbirds deviate from the previously established allometric equation (i.e. eggshell thickness versus egg size; Mermoz and Ornelas, 2004; Spaw and Rohwer, 1987) and to confirm those indirectly inferred deviations (i.e. eggshell puncture resistance versus egg mass, and eggshell puncture resistance versus palisade layer thickness). We fitted Bayesian PGLMMs using the phylogeny (phylogenetic variance–covariance matrix) and the species (species-specific effect) as the random effect variables. The egg size variables (i.e. volume, mass) are positively correlated with eggshell thickness and eggshell puncture resistance (e.g. Ar et al., 1979; Thompson et al., 1981), so the egg volume and mass are confounding variables that must be controlled in statistical tests. The palisade layers are indeed a more direct measure to determine when describing the egg mechanical strength (Carnarius et al., 1996). The models were fitted using predictor variables with multiple measurements for each egg species, allowing the 'between-species' and 'within-species' slopes to be obtained using the technique of within-group centering (Davis et al., 1961; Van de Pol and Wright, 2009); this separates the fixed effect variables into two components: one containing the species-level mean of the variable (the specific mean) and the other containing the within-species variability (Hadfield, 2010; Van de Pol and Wright, 2009). The models were run for 5 million iterations with a burn-in of 300 and a thinning interval of 100, generating 50,000 samples from each chain from which parameters were estimated. For model diagnoses, see above. We estimated R^2, an indicator of goodness of fit in a Bayesian context, as the amount of variance explained by each model, using an alternative formulation of R^2 into a frequentist context, proposed by A. Gelman, B. Goodrich, J. Gabry and I. Ali (http://www.stat.columbia.edu/~gelman/research/unpublished/).

We calculated the posterior probability of the phylogenetic signal of models, across the entire posterior distribution of model variances, using Lynch's phylogenetic heritability (h^2) equation, equivalent to Pagel's λ in generalized least-squares (GLS) models of phylogenetic signal inference (Garamszegi, 2014; Hadfield and Nakagawa., 2010; Lynch, 1991). The phylogenetic signal is defined as the tendency for related species to resemble each other more than they resemble species drawn at random from the tree, and it can range from zero (no phylogenetic signal, equivalent to a 'star' phylogeny) to one (consistent with Brownian motion) (Blomberg and Garland, 2002). The phylogenetic signal is strongly related to the number of species. The statistical power for detecting phylogenetic signal increases dramatically with sample size, with a good value of 0.8 (range 0–1) reached at approximately 17–20 species (Blomberg et al., 2003).

Evolutionary singularities: how do cowbird species differ from their hosts?

To integrate the understanding of phenotypic evolutionary associations (see previous section) and pattern convergence in the parasites, we investigated the presence of evolutionary singularities under the phylogenetic prediction approach (Garland and Ives, 2000; Nunn, 2011; Organ et al., 2011), using the 'BayesModelS' code in R (Garamszegi, 2014). We focused on a case in which the evolutionary singularity occurs on a terminal branch and thus is autapomorphic (Nunn and Zhu, 2014). 'BayesModelS' generates distributions of predicted trait values for the 'target species' (i.e. the species in which the hypothesized exceptional character occurs) based on phylogenetically controlled analyses of trait co-variation with predictor variables, across a block of topologies (Miller et al., 2019; Nunn and Zhu, 2014). This method investigates change along a single branch of an evolutionary tree, and it can be applied to any branch on a phylogeny, typically focusing on an *a priori* hypothesis for ‘exceptional evolution’ along particular branches (Nunn and Zhu, 2014). ‘BayesModelS’ uses a MCMC to fit parameters of a PGLS model and assumes a Brownian motion model of evolutionary change. The PGLS models are used to generate trait value predictions for the ‘target species’, and the uncertainty in phylogenetic structure can be accounted for by sampling from a set of trees (Freckleton et al., 2002). ‘BayesModelS’ accounts for phylogenetic non-independence of residual trait values by incorporating branch-scaling factors when fitting PGLS models. For specific details about this procedure, see Garamszegi (2014) and Miller et al. (2019). We were interested in how some quantitative (continuously varying) traits (i.e. target trait) differ between the target species (each of the three species of cowbirds) and the rest of the species, on a set of 1000 phylogenetic topologies. It is important to note that we focused on the situation where we had an *a priori* expectation of a singularity in some trait(s) in a particular lineage, rather than searching *post hoc* for exceptional differences predicting a single trait’s value for all species in a clade in a one-by-one procedure through all the species (Nunn and Zhu, 2014). The phylogenetic prediction approach involves three steps: (1) building of phylogenetic regression models to describe how the independent variables predict response variables (target traits) – the target species were not included in the analysis to avoid biasing the predictions, (2) using the regression PGLS model results to predict values of the target traits in the target species, based on measured values of the predictor variables for the target species and its phylogenetic position relative to the other species in the dataset, and (3) evaluating whether the target traits differ from expectations in the target species – the prediction intervals on the predicted value were calculated, again incorporating phylogeny. We used ‘BayesModelS’ code on models setting the burn-in to 100 iterations, a thinning interval of 100 iterations thereafter for 200,000 iterations, resulting in 2000 samples for the posterior distribution of all parameters and the prediction for cowbirds. The response (target trait) and predictor variables were (a) eggshell puncture resistance versus egg mass, (b) eggshell thickness versus egg mass, and (c) eggshell puncture resistance versus palisade layer thickness. In the multimodel-inference of our comparative analyses (see next section), variables related to the complexity and density of the intercrystalline boundary network were selected as predictors of the eggshell mechanical features. Therefore, we also investigated the presence of evolutionary singularities by quantifying the density of the (d) LAB, (e) HAB and (f) TAB, using the thickness of the palisade layer as predictor variable. The cowbirds were identified as ‘missing’ in the analysis through the argument in ‘BayesModelS’. The cowbirds species were excluded from the first step of estimating parameters in the model. Thus, predictions that come from the model are not biased by extreme values in the target species. All cowbird species were identified as ‘singularity’ when their observed trait values fell outside the 95% CrI. We also defined a cowbird species as a ‘positive outlier’ when their observed value fell inside the 95% CrI but above the majority of predictions, and a ‘negative outlier’ when the opposite was true.

Multimodel-inference in comparative analyses

As eggshell mechanical features cannot be inferred by simple measurements of shell thickness (see above), we performed multimodel-inference analyses to find the most plausible combinations of predictors (egg and shell macrostructural, ultrastructural, microstructural and textural characteristics) that best explain interspecific variation in each response variable (eggshell mechanical features). We solved a model selection problem using the intra- and inter-specific dataset under two approaches of inference from multiple biological hypotheses to describe the variation in the degree of how well they fit the dataset at hand. We first solved a model selection problem assuming the absence of phylogenetic effects, fitted through GLS in order to build a set of multiple-regression linear models with Akaike information criterion corrected for small sample sizes (AICc), which selects the optimum models based on maximum likelihood (Akaike, 1998). We generated candidate models with all additive and multiplicative combinations of the covariant predictors and we ran all models using the GLS functions available in ‘nlme’ in combination with ‘ape’ packages (Paradis and Schliep, 2018; https://CRAN.R-project.org/package=nlme) and the ‘AICcmodavg’ functions for multimodel-inference (Burnham and Anderson, 2002; https://CRAN.R-project.org/package=AICcmodavg). We used the predictors with multiple measurements per species (within-species variance), and complemented the model selection analyses using the forward and backward stepwise selection process via the *regsubsets* function in R package ‘leaps’ (https://CRAN.R-project.org/package=leaps). We estimated the test mean square error (test-MSE) through the validation set and K-cross-validation (Kohavi, 1995) for choosing between models with different numbers of predictors, identifying the best models with the lowest validation test error. The continuous covariant predictors were centered prior to the analysis and we investigated pairwise correlations between all predictors to avoid the problem of multicollinearity, and we also used variance inflation factors (VIF; values of VIF <2.5; Zuur et al., 2010) on the selected models using the *vif* function (‘vcar’ package; Fox and Weisberg, 2019) and *vif.MCMCglmm* function (‘MCMCglmm’ package; Hadfield, 2010). The predictor variables of eggshell puncture resistance (or breaking strength) and eggshell stiffness included the egg aspect ratio (as an egg shape index), egg mass (an egg size index), eggshell palisade layer thickness (an eggshell ultrastructure variable), LAB density, HAB density and TAB density (as measures of the complexity of grain boundaries), grain area (a grain size index), grain aspect ratio (a grain shape index), and Kearns factor (an eggshell microtexture variable). The predictor variables of eggshell fracture toughness and eggshell puncture energy included the egg aspect ratio, eggshell ratio, PL ratio, grain size ratio, grain aspect ratio, Kearns factors, relative LAB, relative HAB and relative TAB. Finally, all the candidate models previously selected were fitted as Bayesian PGLMMs using the *MCMCglmm* function (‘MCMCglmm’ package in R; Hadfield, 2010), adding the phylogeny and the species (species-specific effect) as random effect variables. The PGLMM fits were evaluated using deviance information criterion (DIC) and R^2 (http://www.stat.columbia.edu/~gelman/research/unpublished/). For model running parameters and diagnosis, see above.

Ethical note

Research in Argentina was permitted by Organismo Provincial para el Desarrollo Sostenible, Argentina (permit number 71/16-O.P.D.S.) and Ministerio de Agroindustria – Dirección de Flora y Fauna – Buenos Aires Provincia, Argentina (permit number 40/16), and complies with the current laws of Argentina. Research in the USA was permitted by a Hunter College IACUC protocol (MH 2/13-01), a New York State Fish and Wildlife License (78) and US Federal Permits (23681 and GATE-00270).

RESULTS

Phylogenetic comparative analyses

Eggshell mechanical features

Eggs of the two more frequently egg-puncturing parasitic species (screaming and shiny cowbirds) were overall 2.2 and 3.2 times stronger and stiffer than those of their large and small hosts, respectively (BC 95% CrI did not include zero), and overall 2.2 times stronger and stiffer than those of the egg-removing parasite (brown-headed cowbird) (BC 95% CrI [1.39, 3.50] and [11.8, 43.6], respectively). As predicted, the brown-headed cowbird eggs were not statistically stronger and stiffer than their large hosts' eggs (BC 95% CrI [−0.29, 1.36] and [−21.7, 18.4], respectively; Fig. S1) but were 2.2 times stronger and stiffer than their small hosts' eggs (BC 95% CrI [0.05, 1.82] and [−6.05, 19.20], respectively). As for the stress causing shell fracture, the egg-puncturing parasites' eggs showed shear stress values that were overall 1.7 times greater than those of their own large and small hosts (BC 95% CrI [1.16, 3.94] and [2.311, 4.88], respectively) and the egg-removing parasite eggs (BC 95% CrI [1.19, 3.40]). Brown-headed cowbird eggs showed shear stress values that were overall 1.4 times greater those of their small hosts' eggs (BC 95% CrI [0.24, 1.50]), and did not show differences from those of their large hosts' eggs (BC 95% CrI [−0.02, 1.69]). As for the mechanical properties allowing a more direct comparison of the eggshell stiffness characteristics, the more frequently egg-puncturing parasites' eggs showed elastic modulus values that were overall 1.4 times higher than those of all the hosts' and egg-removing parasites' eggs (BC 95% CrI did not include zero). Eggs of the brown-headed cowbird and its large and small hosts did not show statistical differences in their elastic modulus values (BC 95% CrI [−645, 6258] and [−687, 6637], respectively). As for the ability of the shell material to resist the spread of small cracks (K_c), the more frequently egg-puncturing parasites' eggs were overall 1.5 times tougher than eggs of the egg-removing parasite and hosts (BC 95% CrI did not include zero). The brown-headed cowbird eggs were not statistically tougher than their small hosts' eggs (BC 95% CrI [−48.1, 38.9]) but were 1.2 times tougher than their large hosts' eggs (BC 95% CrI [−3.39, 82.0]). Finally, regarding the ability of the eggshell material to absorb energy during elastic deformation, we found no evidence of an interaction between the factors (egg class and pecking) on eggshell puncture energy; therefore, the main effects were analyzed. Those parasitic and host eggs frequently punctured by the parasites showed puncture energy values that were overall 1.7 times higher than eggs of the parasite and hosts that are rarely punctured (i.e. egg-removing parasite's own and its hosts' eggs) (BC 95% CrI [0.87, 1.65]). Overall, small and large hosts' eggs were similar in their puncture energy values (BC 95% CrI [−1.56, 0.71]), whereas the parasites' eggs showed puncture energy values that were 2.4 times higher than those of the small and large hosts' eggs (BC 95% CrI [1.47, 4.48] and [1.17, 3.94], respectively) (Fig. 2; Table S2).

Eggshell ultrastructure characteristics

The usual shell ultrastructure of the cross-section of avian eggs was identified by us in all species of parasitic cowbirds and hosts under study (Figs S2). We could easily recognize and measure the thickness of the three mineralized layers on the SEM micrographs of complete eggshell cross-sections. We found no evidence of an interaction between the predictors egg class and egg pecking on the eggshell ratio (shell mass relative to total egg mass; Table S2). However, there was evidence of an eggshell ratio versus egg class effect, with the result that the large and small hosts did not show evidence of variation in their eggshell proportion values (overall 6.5%; BC 95% CrI [−1.70, 0.75]). All parasite eggs had higher proportions of eggshell mass (overall 9.3%) than both host groups (small and large, BC 95% CrI [0.96, 3.92] and [1.25, 4.47], respectively). Regarding the thickness of the mineralized layers relative to the total eggshell thickness, evidence of statistical variation in PL ratio and ML ratio was not found between the egg-removing parasite and host groups (overall PL ratio=69% and ML ratio=31%; BC 95% CrI overlapped with zero). However, the egg-puncturing parasites' eggshells showed a greater PL ratio (76%) that the egg-removing parasite eggshells (BC 95% CrI [0.13,10.20]) and all host group eggshells (BC 95% CrI did not include zero). Consequently, egg-puncturing parasites' eggshells showed smaller ML ratio (24%) than those of the egg-removing parasite (BC 95% CrI [−9.58, −1.09]), and the host groups (BC 95% CrI did not include zero) (Fig. 3; Table S2).

Eggshell microstructure and texture characteristics

As expected for avian eggs, diffractograms from parasite and host eggshells showed the predominance of calcium carbonate ($CaCO_3$) in calcite form. Textures were measured according to the peak positions of each diffractogram. We found no evidence of appreciable variation in Kearns factors among all egg groups. Kearns factors covered a range of 0.38 to 0.72, with a mean of 0.54 ±0.03. From EBSD data, the textures were obtained by three methods (IPF maps, IPF sections, PF). These methods indicated that eggshell texture was characterized by a single fiber component with the <0001> direction along the radial direction of the eggshells. The EBSD IPF orientation maps clearly showed columnar grains (palisade layers) in eggshells of all species except screaming and shiny cowbirds. Screaming and shiny cowbirds' eggshells also had columnar grains, but frequently showed grains that had lateral deviations and very irregular boundaries, characterizing a more complex and dense grain boundary network (Figs 4–7).

Concerning the calcite grains that make up the eggshell palisade layers, there was no evidence of variation in the grain size values between eggshells of the brown-headed cowbird and host groups (BC 95% CrI included zero). In contrast, grain size in eggshells of the more frequently egg-puncturing parasites was overall between 2.0 and 2.6 times smaller than in eggshells of the third parasite and all host groups (BC 95% CrI did not include zero). As for the complexity of the grain boundary network, quantified through the low-, high- and total-angle boundary lines included in the palisade layers (i.e. relative LAB, relative HAB and relative TAB, respectively), we found no evidence of an interaction between the factors (egg class and egg pecking) on the relative LAB; however, the analysis of the main effects indicated that the parasites' eggshells showed a LAB line length (relative LAB) that was overall 2.0 times greater than those of the large and small hosts' eggshells (BC 95% CrI did not include zero). The screaming and shiny cowbirds' eggshells showed HAB line lengths (relative HAB) that were on average 2.9, 1.8 and 1.9 times greater than the eggshells of the small and large host groups (BC 95% CrI did not include zero) and brown-headed cowbird (BC 95% CrI [1.63, 4.74]), respectively. There was no evidence of variation in relative HAB in the brown-headed cowbird, the large host groups and the small hosts whose eggs are frequently punctured (BC 95% CrI included zero). Brown-headed cowbird eggshells showed HAB line lengths (relative HAB) that were on average 1.9 times greater than those of the eggshells of their own small hosts (BC 95% CrI [0.17, 2.84]). Similarly, the egg-puncturing cowbirds' eggshells showed TAB line lengths (relative TAB) that were on average 3.1, 2.0 and 1.9 times greater than those of the eggshells of the small and large host groups (BC 95% CrI did not include zero) and the brown-headed cowbird (BC 95% CrI [3.67, 8.64]), respectively. In turn, there was no statistical evidence of variation in the relative TAB for this parasite, the two large host classes, and the small host class whose eggs are frequently punctured (BC 95% CrI included zero). The egg-removing parasite eggshells showed a TAB line length (relative TAB) that was

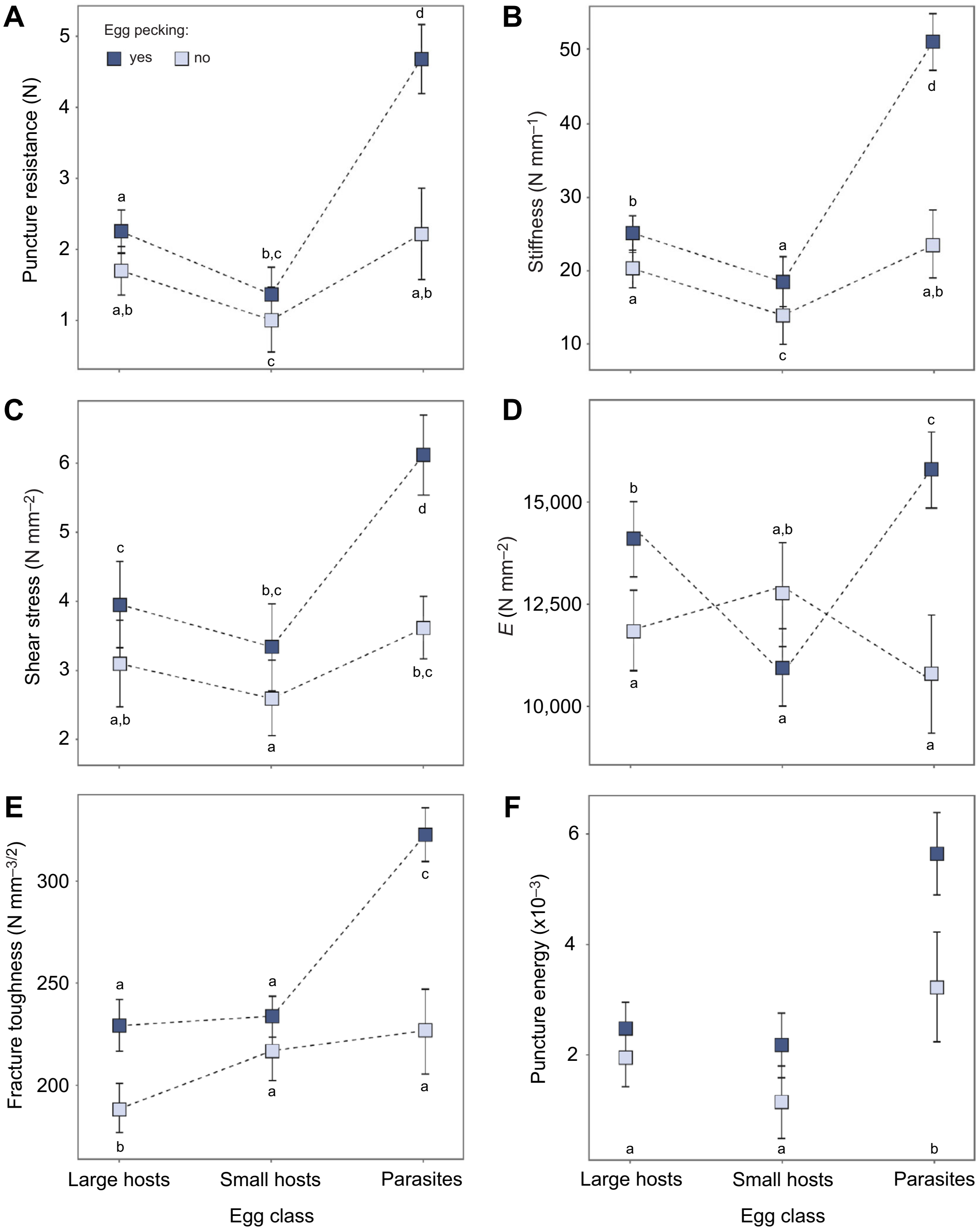

Fig. 2. Interaction plots of the change in mechanical properties for each egg class. (A) Puncture resistance, (B) stiffness, (C) shear stress, (D) Young's modulus (*E*), (E) fracture toughness and (F) puncture energy. Measurements were taken from eggs that are frequently and rarely punctured by cowbirds during parasitism events (egg pecking). The values and bars indicate the means of posterior densities and associated 95% credible interval. Lowercase letters indicate significant differences between the means of groups ($P<0.05$). There were significant differences between the levels of egg pecking (yes, no) in F.

on average 2.1 times greater than that of the eggshells of their own small hosts (BC 95% CrI [0.45, 5.34]). Finally, we did not find evidence of variation in the grain aspect ratio between egg groups. Overall value grain aspect ratio was 0.32±0.02, covering a range from 0.21 to 0.48 (Fig. 3; Table S2).

Phylogenetic evolutionary association and evolutionary singularities

As has been shown in previous work and as predicted here, parasitic cowbirds deviated positively from the typical allometric equation of non-parasitic species. Eggshell thickness of the parasites' eggs was 1.3–1.6 times greater than that predicted by the regression when egg mass was the independent variable, being above the predicted upper 95% CrI (Fig. 8A, Table 1). However, we combined our understanding of phenotypic evolutionary associations and species-specific pattern convergence in parasites, and investigated the presence of evolutionary singularities. Under phylogenetic prediction, the more frequently egg-puncturing parasites (screaming and shiny cowbirds) were identified as positive outliers, as their eggshell thickness mean values fell above the vast majority of the predicted posterior distributions (Fig. 8B, Table 2). Around 5% of predictions were found to be above their respective mean observed

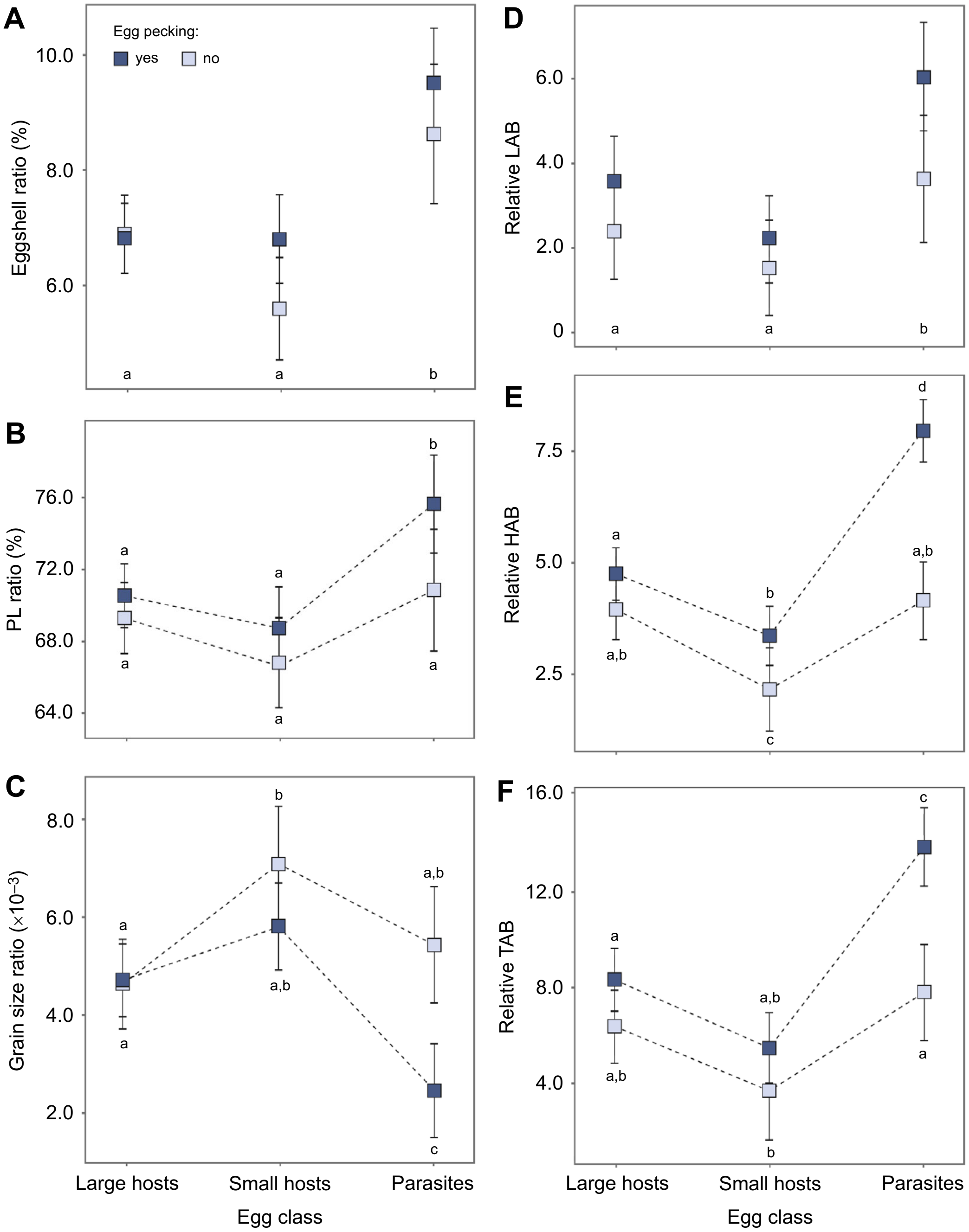

Fig. 3. Interaction plots of the change in eggshell ultrastructure and microstructure features for each egg class. (A) Eggshell ratio (shell mass relative to total egg mass), (B) palisade layer (PL) ratio, (C) grain size ratio, (D) low-angle boundary (relative LAB), (E) high-angle boundary (relative HAB) and (F) total-angle boundary (relative TAB). Measurements were taken from eggs that are frequently and rarely punctured by cowbirds during parasitism events (egg pecking). See Fig. 2 for descriptions. There were no significant differences between the levels of egg pecking (yes, no) in A and D.

values, whereas the egg-removing parasite (brown-headed cowbird) was not identified as an outlier. Its observed mean value was included within the predicted 95% CrI, and more than 20% of predictions fell above their observed mean value (Fig. 8B, Table 2).

In addition, the parasitic cowbirds also deviated positively from those allometric equations that typically had been indirectly inferred (see Introduction). Specifically, eggshell puncture resistance in the parasitic cowbirds was 1.6–3.3 times greater than that predicted by the regression using egg mass as the independent variable, each being above the upper 95% CrI (Fig. 8C, Table 1). Under the phylogenetic prediction approach, the egg-puncturing parasites were deemed as evolutionary singularities, as the mean values exceeded the predicted upper 95% CrI, with more than 97% of predictions falling below their mean values (Table 2). By contrast, although the mean value for the egg-removing parasite was greater than the mean value of the predicted posterior distribution, the observed mean value was included within the predicted 95% CrI; only 26% of predictions fell above the mean observed value, indicating this parasite was not an outlier (Fig. 8D, Table 2).

Finally, based on the pattern that the palisade layers would be involved in eggshell strength (see Introduction), the observed eggshell puncture resistance for cowbirds was included within the

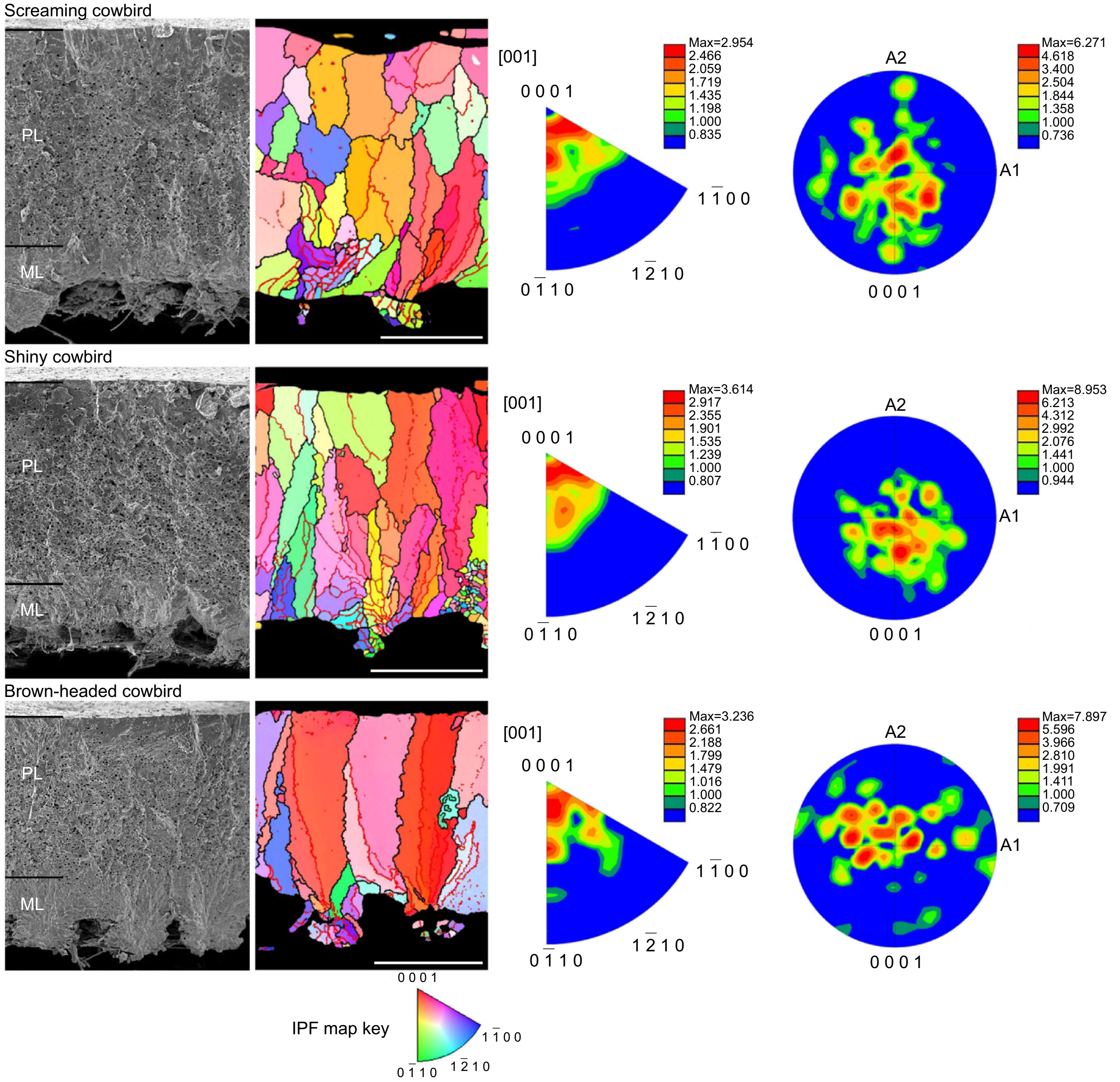

Fig. 4. Cross-sectional profile of transverse eggshell cuts from the icterid parasitic cowbird species. Profiles were obtained by (left to right): scanning electron microscopy (SEM), inverse pole figure (IPF) maps, pole figures (PFs) and IPFs. The orientation information was rotated so the colors in each map indicate the crystal direction aligned with the radial direction. The points in the PFs and IPFs are ordered by size to allow easy visualization of the orientation of the bigger grains. The low (3 deg<ω<15 deg) and high (15 deg<ω<110 deg) angle boundaries are shown as red and black lines between grains, respectively. White scale bars in the IPF maps indicate an approximate reference length of 50 µm. PL, palisade layer; ML, mammillary layer.

95% CrI, when the independent variable was the thickness of the two palisade layers (Fig. 8E, Table 1). However, the screaming and shiny cowbirds were still identified as positive outliers, whereas the brown-headed cowbird was again not an outlier. For example, the observed screaming cowbird eggshell puncture resistance value was 130% greater than the mean of the predicted posterior distribution. This parasite's position was the only one strongly supported as an evolutionary singularity, as its observed value exceeded by 50% the predicted upper 95% CrI, with more than 99.9% of predictions falling below the observed mean value. Meanwhile, the observed shiny cowbird eggshell puncture resistance was 86% greater than the mean of the predicted posterior distribution, with more than 93% of predictions falling below the observed mean value. Finally, the brown-headed cowbird eggshell puncture resistance was 10% lower than the mean of the predicted posterior distribution, with more than 52% of predictions falling above the observed mean value (Fig. 8F, Table 2). These last novel parasitic species-specific patterns appear to indicate that other egg and shell structural characteristics contribute differentially to the shell breaking strength in the eggs of the screaming and shiny cowbirds.

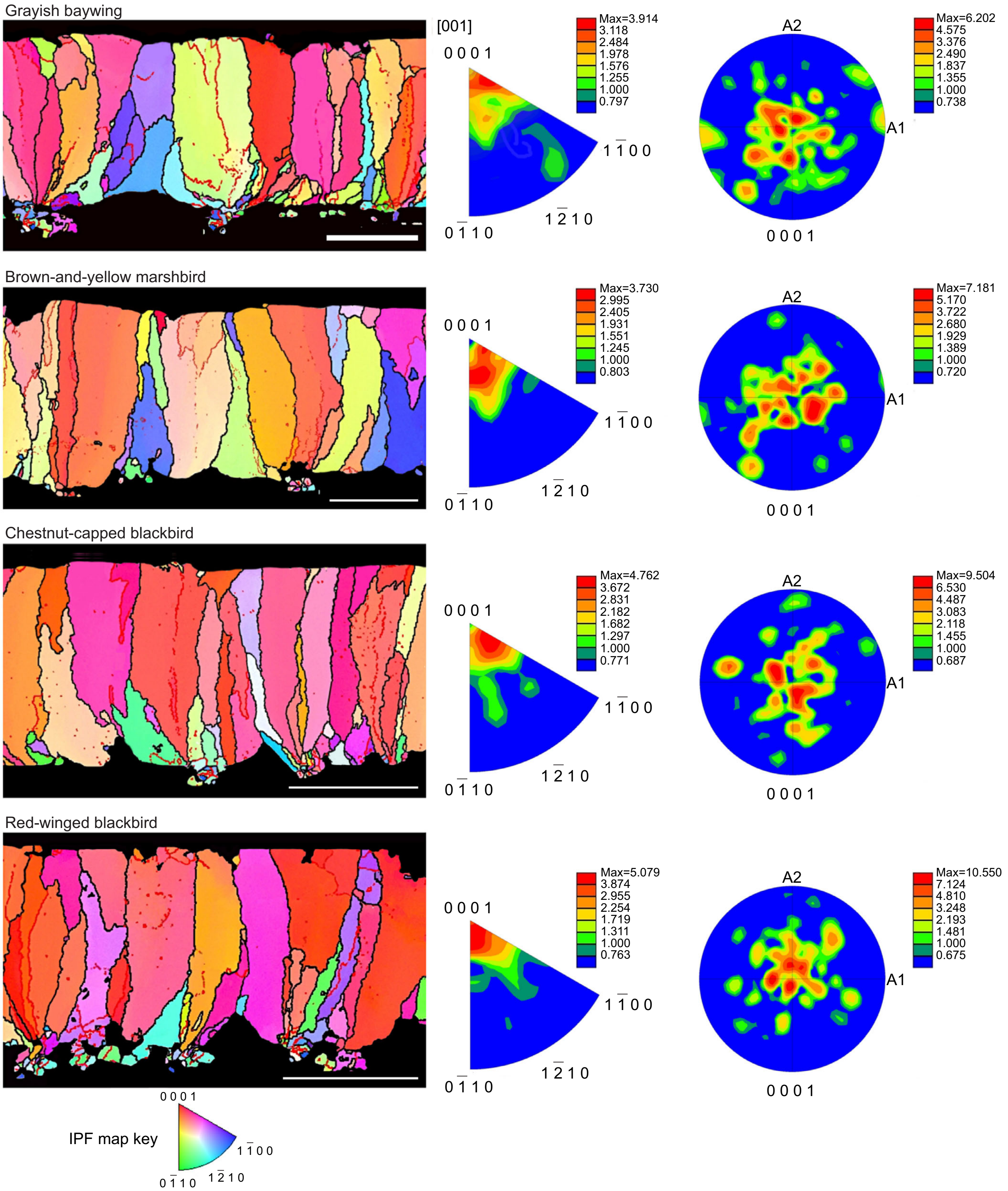

Fig. 5. Cross-sectional profile of transverse eggshell cuts from icterid host species. From left to right: IPF maps, PFs and IPFs; for details, see Fig. 4.

We also investigated the presence of evolutionary singularities by quantifying the intercrystalline boundary densities using the palisade layer thickness as the prediction variable. Screaming and shiny cowbirds were deemed as evolutionary singularities, as the TAB density mean values exceeded the predicted upper 95% CrI, with more than 95% of predictions falling below the mean observed values. Furthermore, these two egg-puncturing parasites, screaming and shiny cowbirds, were considered as positive

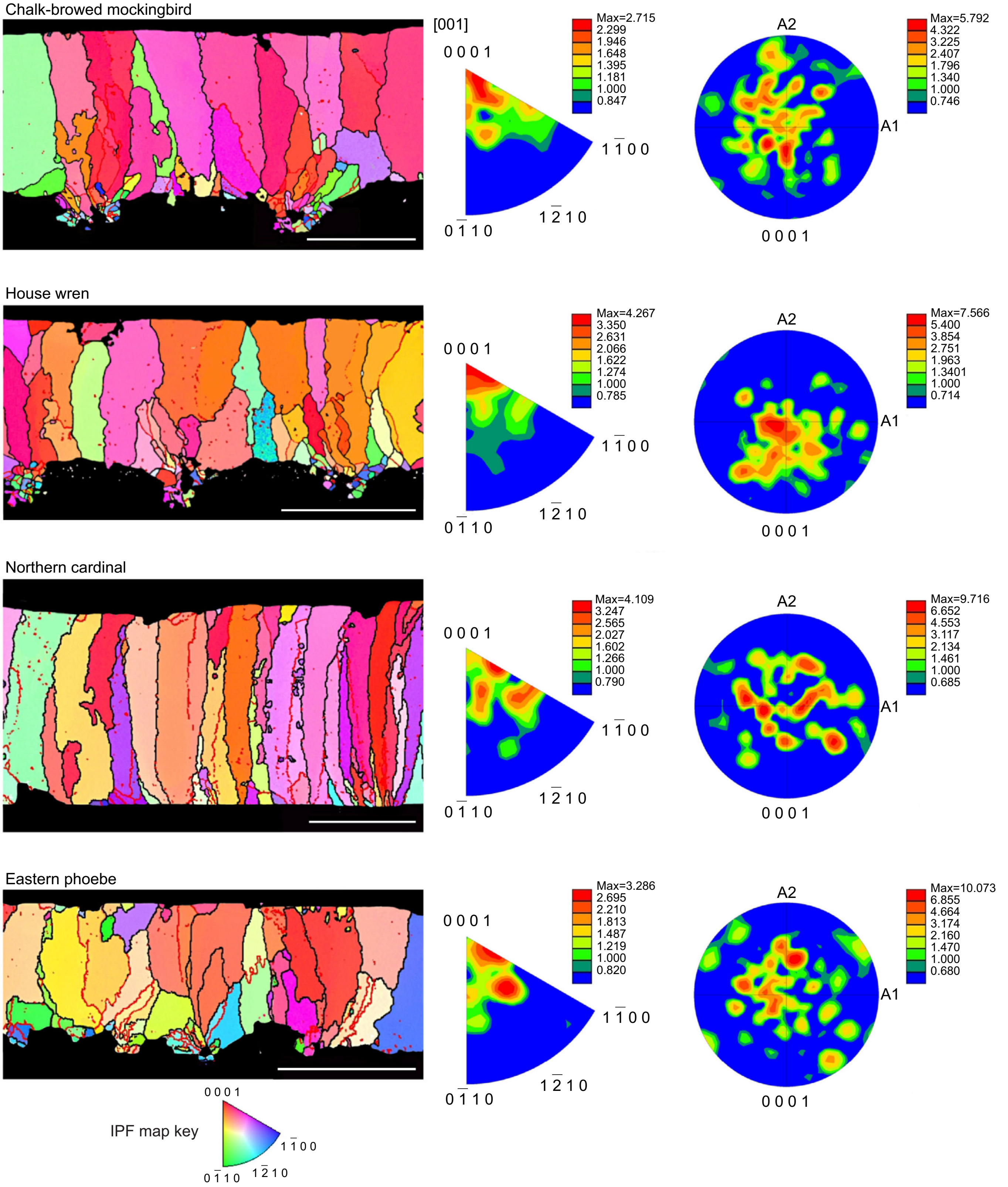

Fig. 6. Cross-sectional profile of eggshell cuts from non-icterid host species. From left to right: IPF maps, PFs and IPFs; for details, see Fig. 4.

outliers for LAB and HAB density, respectively (Fig. 7). By contrast, the LAB, HAB and TAB density observed mean values for the egg-removing parasite were included within the predicted 95% CrI, and more than 50% of predictions fell above each observed mean value, indicating this parasite was not an outlier (Fig. 7).

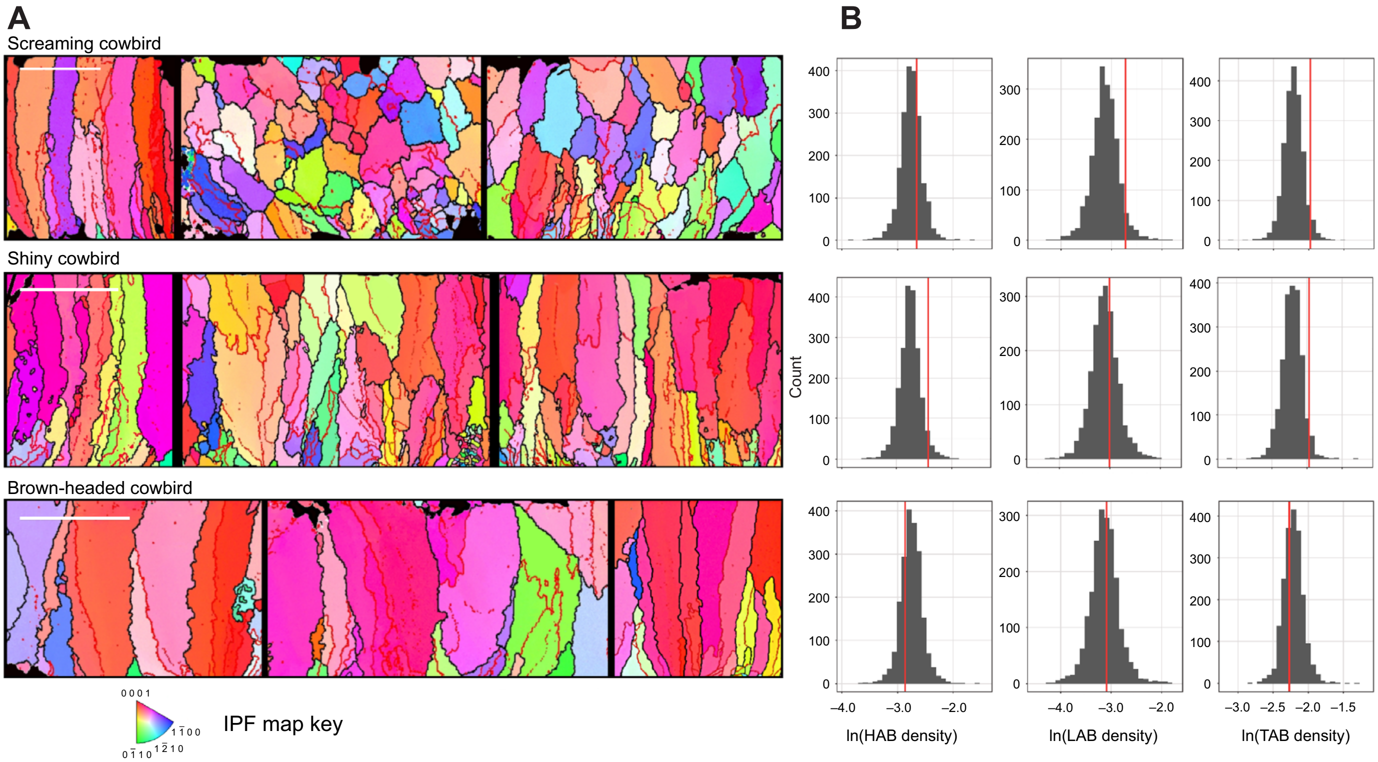

Fig. 7. Cross-sectional profile and boundary density of parasitic cowbirds. (A) Cross-sectional profile by IPF maps of transverse eggshell cuts (palisade layer) from parasitic cowbirds. For details, see Fig. 4. (B) Posterior distributions of predicted LAB, HAB and TAB density (μm^{-1}) for cowbird species using palisade layer thickness as a predictor variable. Vertical red lines indicate the observed values.

Multimodel inference in comparative analyses

Through multimodel-inference analyses, the parsimonious GLS and PGLMM models that best explained the interspecific variation in eggshell breaking strength and stiffness included the interaction between an ultrastructural variable (palisade layer thickness) and microstructural variables related to the complexity of the intercrystalline boundary networks. Under a Bayesian phylogenetic approach, the eggshell breaking strength and stiffness were positively associated with the interaction between the palisade layer thickness and LAB density, and the palisade layer thickness and TAB density. Three equally parsimonious PGLMMs best explained the interspecific variation in the ability of the eggshell material to resist the propagation of small cracks (fracture toughness): positive associations with eggshell ratio, and PL ratio, and negative association with the grain size ratio. Finally, the parsimonious PGLMMs that best explained the interspecific variation in the eggshell puncture energy included the addition of eggshell ratio and PL ratio, and eggshell ratio and relative HAB. For model summaries, see Tables S3 and S4.

DISCUSSION

Published literature has long suggested that eggshell strength is greater in avian obligate brood parasites than in their respective hosts. Stronger parasitic eggshells are indirectly suggested by regressions or comparisons that mainly include eggshell thickness, egg shape and/or allometric deviations (Brooker and Brooker, 1991; Picman and Pribil, 1997; Spaw and Rohwer, 1987; Spottiswoode and Colebrook-Robjent, 2007; Spottiswoode, 2010), microhardness (Igic et al., 2011) or microstructure (Soler et al., 2019). In contrast, few studies have only estimated eggshell breaking strength directly on intact eggs in parasites and hosts (Picman, 1989; Picman and Honza, 2021). For the first time in an analysis of a congeneric parasite–diverse host avian system, we jointly investigated the most important egg and shell mechanical and structural features, analyzed under a phylogenetic comparative approach to test alternative adaptive biological hypotheses on select hosts and parasite cowbirds that, despite belonging to the same genus (*Molothrus*), possess different egg destructive behaviors.

The reduction of the host's clutch by brown-headed cowbirds in both eventually parasitized and non-parasitized nests is mainly achieved through egg removal by grasp-ejection, although some studies also reported egg puncture-ejection, i.e. brown-headed cowbirds may carry a host egg in their open mandibles after having pierced it, and this egg is typically replaced by the female cowbird on a one-to-one numerical basis between the already present host eggs and new parasite eggs laid (Hauber, 2003; see López et al., 2021). In turn, the host's eggs are frequently punctured by shiny and screaming cowbirds in both parasitized and non-parasitized nests, and this egg-pecking behavior is well documented and has been mentioned by several authors (e.g. Carter, 1986; Fraga, 1978, 1985, 1998; Hoy and Ottow, 1964; Lichtenstein, 1998; Mermoz and Reboreda, 1994; Nakamura and Cruz, 2000; Post and Wiley, 1977; Sackmann and Reboreda, 2003), who reported that it is one of the most important costs of brood parasitism, leading to reduced host clutch and brood sizes, and also reduced numbers of fledglings.

López et al. (2021) found support for the hypothesis that egg geometric (size, shape) and shell mechanical (strength, stiffness) features in host species co-vary with the parasitic cowbird's own strategy of reducing the host's clutch size. These interspecific host egg and shell features were also positively affected by the frequency of parasitism, indicating that species-specific patterns of parasitic costs select for respective anti-parasitic defenses in hosts. In

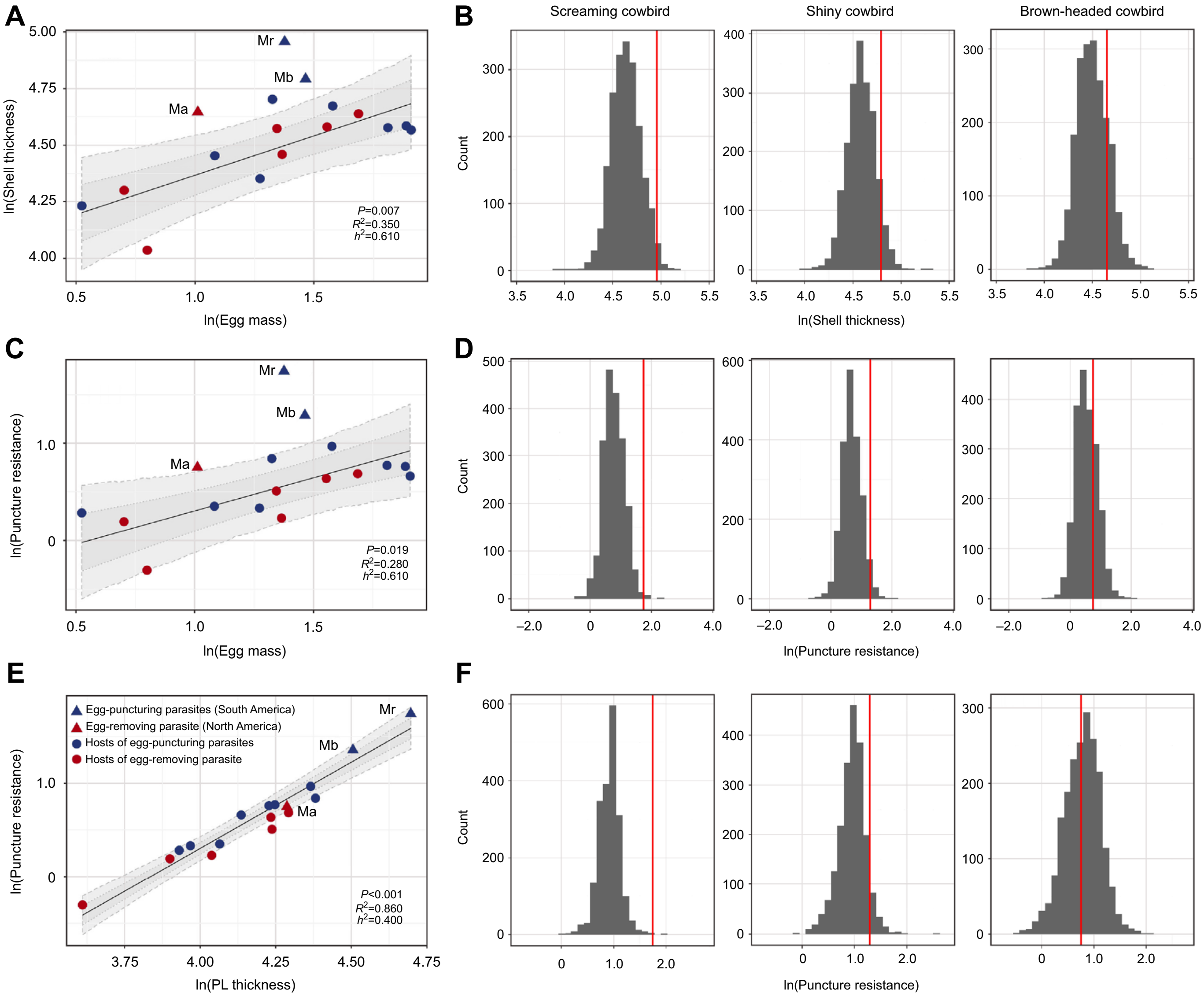

Fig. 8. Phylogenetic evolutionary association and evolutionary singularities. (A,C,E) Bayesian phylogenetic linear mixed-effect model (PGLMM) plots for (A) shell thickness (µm) against egg mass (g), (C) puncture resistance (N) against egg mass (g) and (E) puncture resistance (N) against palisade layer thickness (µm), showing the regression line with the standard error (dotted line) and 95% credible interval (dashed line) of the posterior distribution of the fitted values. Mean values for each species (circles and triangles) are shown. Ma, *Molothrus ater* (brown-headed cowbird); Mr, *Molothrus rufoaxillaris* (screaming cowbird); Mb, *Molothrus bonariensis* (shiny cowbird). *P*-values are from Markov chain Monte Carlo; h^2, Lynch's phylogenetic heritability. (B,D,F) Posterior distributions of predicted values for cowbird species. Vertical red lines indicate the observed values.

addition, selection pressures on parasites and hosts are asymmetric as parasites need a host for reproduction, but not all hosts will suffer from parasitism (Schmid-Hempel, 2011). Thus, selection pressures upon eggshell mechanical and structural shifts in eggs of both more frequently egg-puncturing parasites should usually be stronger than upon eggs of the hosts. Accordingly, our findings indicate the eggs of screaming and shiny cowbirds have exceptionally increased their shell mechanical properties not only compared with eggs of all hosts (regardless of relative egg dimensions) but also compared with eggs of the brown-headed cowbird. Breaking strength and stiffness were strongly associated with eggshell material ultrastructural and microstructural shifts (related to the increase in palisade layer thickness in cooperation with a greater complexity and density of the intercrystalline boundaries). Phylogenetic prediction analyses indicated that these mechanical and structural shifts have evolved exceptionally in the screaming and shiny cowbirds (evolutionary singularities), whereas in the egg-removing parasite, the brown-headed cowbird, they have not.

Eggshell mechanical features

The force–displacement relationships are of paramount importance for estimating a variety of macro-mechanical features. These relationships allowed us to estimate the ability to resist breaking under tensile stress and to describe the general stiffness characteristics of the eggshell materials. According to Voisey and Hunt (1974), breaking strength does not describe any physical property of the material from which the eggshell is constructed, but this feature allowed us to describe the response of the more frequently egg-puncturing parasites' eggshells as a composite structure more complex than the eggshells of egg-removing parasite and hosts. Our

Table 1. Phylogenetic evolutionary associations: summary of the Bayesian phylogenetic linear mixed-effect models (PGLMMs)

	Posterior mean	Lower 95% CrI	Upper 95% CrI	*P*
Eggshell total thickness versus egg mass (R^2=0.35, h^2=0.61)				
Random effect:				
Phylogenetic	0.0281	0.0035	0.0620	–
Species	0.0152	0.0017	0.0366	–
Residual variance units	0.0019	0.0015	0.0023	–
Fixed effect:				
Intercept	4.0179	3.6568	4.3674	<0.0001
Egg mass (BS)	0.3491	0.1123	0.5882	0.0067
Egg mass (WS)	−0.0365	−0.0991	0.0273	0.2570
Eggshell puncture resistance versus egg mass (R^2=0.28, h^2=0.61)				
Random effect:				
Phylogenetic	0.1499	0.0023	0.3518	–
Species	0.0786	0.0018	0.2182	–
Residual variance units	0.0074	0.0059	0.0091	–
Fixed effect:				
Intercept	−0.3779	−1.2084	0.4371	0.3506
Egg mass (BS)	0.6793	0.1264	1.2240	0.0189
Egg mass (WS)	0.0343	−0.0852	0.1534	0.5674
Egg puncture resistance versus PL thickness (R^2=0.86, h^2=0.40)				
Random effect:				
Phylogenetic	0.0152	0.0018	0.0359	–
Species	0.0125	0.0021	0.0276	–
Residual variance units	0.0074	0.0059	0.0091	–
Fixed effect:				
Intercept	−7.0617	−8.4586	−5.7383	<0.0001
PL thickness (BS)	1.8412	1.5070	2.1649	<0.0001
PL thickness (WS)	0.1343	−0.2380	0.2633	0.9130

BS, between-species variability; WS, within-species variability; PL, palisade layer; CrI, credible interval. *P*-values are from Markov chain Monte Carlo. All the variables were log-transformed prior to analyses. In all these associations, the phylogenetic signals (h^2) did not take values close to the extremes 0 and 1, which implies that the data support models of evolution process that are somewhere between a ‘star’ phylogeny and Brownian motion.

elastic modulus (E) and fracture toughness (K_c) estimated values fell within the same order of magnitude as those reported by Mabe et al. (2003). K_c values have been estimated in previous papers using different methodologies (Mabe et al., 2003; Xiao et al., 2014; Gosler et al., 2011). However, Taylor et al. (2016) devised another novel way to measure K_c, obtaining values much lower than previous published values, which were considered as ‘incorrect and misleading’. Unlike in those papers, the current analyses are not intended to assess host and parasite eggshell quality for industrial and/or commercial purposes; our results are of ecological importance within a dynamic co-evolutionary process revealing eggshell mechanical behaviors for generating statistically comparable quantitative data, and to establish the ecologically relevant properties between parasites and hosts.

Brown-headed cowbirds parasitize small hosts more often (Davies, 2000), and although they also parasitize hosts of similar or larger size, they do it at a lower frequency relative to the screaming, shiny and bronzed (*Molothrus aeneus*) cowbirds’ parasitism patterns (e.g. Astié and Reboreda, 2006, 2009; Carter, 1986; Mason, 1986; Peer and Sealy, 1999). Although eggs of the brown-headed cowbird and their larger hosts showed similar values of eggshell strength, stiffness and shearing stress, these mechanical features are still sufficient as a counter-defense against small hosts that would attempt to puncture-eject parasite eggs, as grasp-ejection for these hosts by their limited beak-gape size is impossible (Spaw and Rohwer, 1987). In turn, even though the shiny and screaming cowbirds use hosts that mostly do not puncture-eject parasitic eggs (e.g. Reboreda et al., 2013), the laying parasites often do attempt, in the frequently multiply parasitized nest by different females, to pierce the already laid parasitic eggs (see above). These high rates of multiple parasitism and parasitic egg piercing do not apply to most brown-headed cowbirds and their hosts (e.g. Hauber, 2001). Accordingly, our new data allow us to re-assess the suggestion that an extremely strong and stiff eggshell protects the parasite eggs of the screaming and shiny cowbirds from damage during the laying of parasitic eggs and from other parasite females during multiple parasitism events. The mechanical properties found in our study, which are shared by the three parasitic cowbirds, and that stood out over those of their hosts (mainly those of larger size) were the greater ability of the shell material to resist the propagation of small cracks and absorb energy during elastic deformation. These features may be ubiquitously beneficial during the ‘collisions between eggs’ as a result of all cowbirds laying eggs from an elevated position (López et al., 2018), and may mainly be beneficial in resisting egg damage when parasite eggs are jostled with other eggs in a host nest when the parasites are violently attacked by larger hosts during laying (Neudorf and Sealy, 1994; Gloag et al., 2013) because parasite and host eggs can be jostled into one another and differences in the ability to deform elastically and resist crack propagation may help them to resist damage. López et al. (2018) found no evidence to support the latter hypothesis; however, they suggested that their own damage estimates should be viewed as conservative because it is possible that more violent jolts may occur at the nests (e.g. supplementary movies in Gloag et al., 2012, 2014).

Table 2. Fitted values by Bayesian linear models, and predicted mean values by evolutionary associations and phylogenetic predictions

	Mean posterior density [2.5% and 97.5% quantile]		
	Screaming cowbird	Shiny cowbird	Brown-headed cowbird
Eggshell thickness (μm) based on egg mass (g)			
Fitted by Bayesian linear model	142.29 [134.85, 149.80]	120.65 [115.75, 125.98]	104.21 [98.79, 109.39]
Predicted by evolutionary associations	89.95 [76.91, 104.81]	92.79 [79.04, 109.20]	79.16 [66.62, 94.56]
Predicted by phylogenetic prediction	104.12 [78.01, 143.12]	98.29 [72.24, 131.58]	90.03 [65.39, 124.61]
Egg puncture resistance (N) based on egg mass (g)			
Fitted by Bayesian linear model	5.71 [5.17, 6.32]	3.63 [3.32, 3.96]	2.12 [1.92, 2.33]
Predicted by evolutionary associations	1.75 [1.21, 2.50]	1.85 [1.27, 2.69]	1.36 [0.95, 1.95]
Predicted by phylogenetic prediction	2.12 [1.20, 4.12]	1.95 [1.09, 3.56]	1.65 [0.89, 3.29]
Egg puncture resistance (N) based on PL thickness (μm)			
Fitted by Bayesian linear model	5.71 [5.17, 6.32]	3.63 [3.32, 3.96]	2.12 [1.92, 2.33]
Predicted by evolutionary associations	4.85 [3.92, 6.13]	3.40 [2.89, 4.13]	2.31 [2.03, 2.64]
Predicted by phylogenetic prediction	2.49 [1.64, 3.65]	2.58 [1.36, 4.90]	2.37 [1.34, 4.96]

PL, palisade layer.

Eggshell material ultrastructural and microstructural features

The results of both eggshell characterization and comparisons between egg groups indicated that the eggshell feature shared by the three parasites, and that stood out relative to the host eggshells, was the greater shell mass relative to the egg full mass (eggshell ratio). Eggshell ratio was moderately and positively correlated with eggshell total thickness (r=0.61, P<0.001); therefore, other features (not studied here) such as eggshell density (Picman and Pribil, 1997) or mammillary cone density (López, 2021; see below) may also be related to the greater mass of the parasitic eggshells. This specific feature explained (sum with PL ratio) interspecific variation in the eggshell puncture energy (by multimodel-inference analysis). However, the eggshell ratio and total thickness did not explain the extreme mechanical features of the egg-puncturing parasitic cowbirds.

Parasitic cowbirds are known for their greater eggshell total thickness beyond what is predicted by allometry (see Introduction), and the greater eggshell total thickness does not imply a proportional increment of each shell layer. Eggshells of the two egg-puncturing cowbirds had a greater proportion of palisade layers compared with the hosts and brown-headed cowbird. For example, the host-specialist parasite, the screaming cowbird, and its main host, the grayish baywing, have similar egg size (~3.9 g; Table S1), egg shape (López et al., 2021) and mammillary layer thickness (~31 µm), but they showed differences in palisade layer thickness (~110 versus ~80 µm, respectively). The mammillary layer does not contribute differentially to the eggshell strength properties (Bain, 1990); however, screaming and shiny cowbirds' eggs have a higher density of mammillary cones (Fig. S2; López, 2021), indicating a higher density of nucleation sites on the outer membranes. Eggshell calcification originates at nucleation sites, and the density and distribution of these sites critically influence shell structure, affecting the crystals' size during the palisade layer formation (Dunn et al., 2011). The palisade layer was one of the predictors selected by us to partially explain the interspecific variation of each mechanical feature analyzed, strongly supporting that it is indeed more critical to measure the palisade layer when ascribing eggshell mechanical properties.

Soler et al. (2019) showed that parasitic eggshells of the great spotted cuckoo (*Clamator glandarius*) were composed of smaller calcite crystal units, and that these had a lower degree of crystal orientation than in two of its host species (*Corvus monedula* and *Pica pica*). Similar to this previous example, both egg-puncturing parasitic cowbirds (but not the egg-removing parasite) had smaller crystal units making up the palisade layers. However, we found no evidence of differences in the preferred crystal orientation between cowbirds and hosts. The crystal size was negatively associated with fracture toughness. This result is consistent with those found by Lv et al. (2015) and Vavro and Sou-iek (2013), where the calcite mineral fracture toughness was measured in various samples, being higher in materials with small crystal size. We found that other eggshell characteristics, such as the calcite crystal size, increase the fracture toughness of the more frequently egg-puncturing cowbird eggs.

For the first time in avian host–parasite interactions, we studied the complexity of calcite crystal boundary networks by quantifying the densities of low-, high- and total-angle boundaries. Despite the egg-removing cowbird showing a greater relative length of low-angle borders, we did not find sufficient evidence to identify it as an evolutionary singularity. However, other specific results may explain the greater eggshell breaking strength and stiffness of the egg-removing cowbird relative to their small hosts. In turn, both more frequently egg-puncturing cowbirds were identified as positive outliers, resulting in evidence of exceptional evolution in terms of their TAB density. In addition, the screaming and shiny cowbirds were positive outliers according to their LAB density and HAB density, respectively. The greater complexity and density of boundary networks create many intercrystalline paths, which consume energy by debonding and cause a growing crack or fissure to deflect from side to side, even in an opposite direction, greatly increasing the energy consumed in crack or fissure propagation. These particular microstructure characteristics act cooperatively with the palisade layer thickness on the eggshell breaking strength and stiffness. We also found novel eggshell microstructural characteristics that might explain the extreme strength and stiffness of the two egg-puncturing cowbirds' eggs.

Influence of shell material structure on the egg mechanical features

A few words are required on the different evolutionary strategic paths followed, with the goal of achieving failure resistance. Increasing the stiffness may work for a while, but only until the breaking strength (F) is reached. Elastic energy accumulation allows higher F to be sustained with smaller displacements under load. However, that strategy requires the simultaneous increment of the stress concentration factor K_c (fracture toughness) for increasing F. Besides the corrections introduced by shape-dependent parameters (Eqns 5–7), the stiffness grows as T^2, and meanwhile F grows as $T^{3/2}$ (where T is eggshell thickness), which seems to be a dead end for the combined strategy of increasing the two properties simultaneously by increasing the thickness. A large thickness without a simultaneous increase of the size of the egg is clearly not efficient as the eggshell volume would take the most of the egg volume, and the energy spent making such large eggs might become unaffordable. The elastic constants (E) are barely dependent on the microstructure features, where the measured variations around pure calcite values might be consequence of the composite nature of the eggshell. However, K_c is dependent on microstructure, which determines the increase of low- and high-angle boundaries revealed in the present study as a way of increasing energy dissipation during fracture.

Avian eggshell has an unusual combination of mechanical properties (high Young's modulus combined with low fracture toughness), making it ideally suited as a container for the developing chick, which must be strong and stiff but also brittle enough to fracture when required (Taylor et al., 2016). Our analyses regarding the benefits and constraints on parasitic eggshell strength, stiffness and toughness metrics have implications for the hatching process too, which presents a shell strength fragility asymmetry that is not always well understood developmentally.

Recently, López (2021) demonstrated that incubated eggs of both egg-puncturing parasitic cowbirds show a stronger loss of their eggshell strength, shear stress, stiffness and mainly fracture toughness, including a greater degradation of the mammillary cones, compared with the incubated eggs of their main and frequent hosts, strongly supporting that eggshells of both egg-puncturing cowbirds have a highly anisotropic property and that the parasitic embryos need not engage greater effort during hatching, relative to their hosts. On the one hand, using the materials provided in abundance by nature, including the chemical components of calcite, birds have evolved to generate an eggshell as strong and stiff as required for incubation (Huynen et al., 2010) and, sometimes, extraordinarily stronger and stiffer, because of the need to withstand damaging pecks and piercings. In turn, any external attack is actually combined with compression pressures, in an attempt to create fissures in the bio-ceramic material. On the other hand, no avian eggshell can exceed a limit so as to preclude a successful embryonic hatching process. The result is asymmetric in this sense, because as the chick tries to break through the shell, it does so by imposing expansion-inducing forces through internal shell pressure, which tends to open the small cracks produced by the beak's pressure points.

Conclusions

By applying a multidisciplinary approach and analyses using a phylogenetic comparative integral framework, our results show strong differences between parasitic species-specific patterns in mechanical and structural features. In the context of adaptations to the parasitic lifestyle, the reported patterns of all these diverse eggshell features support a new set of interpretations, confirming several hypotheses regarding the impact of both reproductive strategies (parasitic versus parental) and parasitic behavior of egg destruction (more versus less frequently puncturing). (1) The greater ability of the shell material to resist the propagation of small cracks and absorb energy during elastic deformation in parasitic cowbirds eggs provides adaptive advantages over their hosts, mainly larger hosts, as a result of their laying behavior from an elevated position and when parasite eggs collide with another eggs in a nest during violent attacks by larger hosts. (2) Brown-headed cowbird eggshells showed consistent increases in the density of the intercrystalline border network, which moderately affected the breaking strength, shear stress and stiffness, but still provided an adaptive advantage over those small hosts that puncture-eject parasite eggs. (3) Congeneric screaming and shiny cowbirds' eggs showed greater increases in eggshell thickness, resulting in a greater proportion of the palisade layer thickness, accompanied by an increase in the density of the mammillary cones. The latter affects the crystal morphology during the palisade layer formation. All these characteristics strongly increase eggshell mechanical features; therefore, they provide an adaptive advantage over all the tested host's eggs, regardless of relative egg dimensions. (4) Our new data also allowed us to re-assess that increased eggshell mechanical features protect the eggs of the screaming and shiny cowbirds from damage during the laying of parasitic eggs and from puncture and breakage by other parasitic females that peck eggs during their own parasitism attempts.

Acknowledgements

We appreciate the suggestions of Dr Pablo Leal and Alec Luro about using EBSD and Bayesian Analyses. We are grateful to Prof. Charles Nunn for his comments about Phylogenetic Prediction analyses. Miri Dainson assisted with fieldwork in the USA. Danielle Allen provided linguistic assistance. A.V.L. is very grateful to Gunther Keil, Geraldine Keil, Joan Ormondroyd (neighbors in NY state), Héctor R. López, Nora M. Rego and Fabiana Graff (neighbors in Buenos Aires Province) for their hospitality during the fieldwork. Copious EBSD data were obtained at the Laboratorio de Microscopía Electrónica, Centro Científico Tecnológico Rosario, Argentina.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.V.L., R.E.B., J.C.R., V.D.F., M.E.H.; Methodology: A.V.L., R.E.B., M.A., L.N.G., V.T., P.R.; Software: A.V.L.; Validation: A.V.L., R.E.B., M.A., L.N.G., M.E.H.; Formal analysis: A.V.L.; Investigation: A.V.L., R.E.B., L.N.G., M.E.H.; Resources: A.V.L., R.E.B., M.A., L.N.G., M.E.H.; Data curation: A.V.L.; Writing - original draft: A.V.L.; Writing - review & editing: A.V.L., R.E.B., M.A., L.N.G., J.C.R., V.D.F., V.T., P.R., M.E.H.; Visualization: A.V.L., M.E.H.; Supervision: M.E.H.; Project administration: M.E.H.; Funding acquisition: J.C.R., V.D.F., M.E.H.

Funding

A.V.L. was supported by a postgraduate scholarship from the University of Buenos Aires. This work was supported by grants of the Agencia Nacional de Promoción Científica y Tecnológica and the University of Buenos Aires (to V.D.F. and J.C.R.), and funds from the Human Frontier Science Program (to M.E.H.). Additional support was provided by the Hanse-Wissenschaftskolleg, Germany (to M.E.H.).

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