



# How to Make a Mimic? Brood Parasitic Striped Cuckoo Eggs Match Host Shell Color but Not Pigment Concentrations

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## Abstract

Hosts of avian brood parasites often use visual cues to reject foreign eggs, and several lineages of brood parasites have evolved mimetic eggshell coloration and patterning to circumvent host recognition. What is the mechanism of parasitic egg color mimicry at the chemical level? Mimetic egg coloration by Common Cuckoos *Cuculus canorus* is achieved by depositing similar concentrations of colorful pigments into their shells as their hosts. The mechanism of parasitic egg color mimicry at the chemical level in other lineages of brood parasites remains unexplored. Here we report on the chemical basis of egg color mimicry in an evolutionarily independent, and poorly studied, host-parasite system: the Neotropical Striped Cuckoo *Tapera naevia* and one of its hosts, the Rufous-and-white Wren *Thryophilus rufalbus*. In most of South America, Striped Cuckoos lay white eggs that are identical to those of local host species. In Central America, however, Striped Cuckoos lay blue eggs that match those of the Rufous-and-white Wren, suggesting that blue egg color in these cuckoo populations is an adaptation to mimic host egg appearance. Here we confirm that Striped Cuckoo eggs are spectrally similar to those of their hosts and consistently contain the same major eggshell pigment, biliverdin. However, wren eggshells lacked protoporphyrin, which was present in the parasitic cuckoo eggshells. Furthermore, biliverdin concentrations were significantly lower in cuckoo eggshells than in host eggshells. Similarity of host-parasite eggshell appearance, therefore, need not always be paralleled by a quantitative chemical match to generate effective visual mimicry in birds.

**Keywords** Biliverdin · Coevolution · Host-parasite interactions · Mimicry · Protoporphyrin IX

## Introduction

Obligate brood parasitic birds lay their eggs into the nests of other species and the hosts pay the fitness costs of caring for the unrelated parasitic young (Davies 2000). To circumvent

these costs, many hosts have evolved the ability to recognize and reject foreign offspring from the nests. In response, several lineages of parasites have evolved visual mimicry of the host offspring to prevent recognition and rejection (Grim 2005). Obligate brood parasitism has evolved independently in at least 7 lineages of extant birds (Sorenson and Payne 2005), and visual mimicry between host and parasitic offspring is present in most of these lineages (Hauber 2014; Stoddard and Hauber 2017).

Visual mimicry may occur at one or more of several developmental stages, including coloration and patterning of the eggshell (e.g., Common Cuckoo *Cuculus canorus* host-races in Eurasia; Stoddard et al. 2014, New World Striped Cuckoos *Tapera naevia* of their Rufous-and-white Wren *Thryophilus rufalbus* hosts; Mark 2013), at the nestling phase (e.g., *Vidua* finches of estrildid finch hosts in Africa: Schuetz 2005, several *Chalcites* bronze cuckoos of their diverse hosts in Australia: Langmore et al. 2011), or following fledging (e.g., Screaming Cowbirds *Molothrus rufoaxillaris* of their Grayish

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Baywing *Agelaioides badius* hosts in South America: de Marsico et al. 2012). The genetic, structural, and chemical bases of visual mimicry in these independent lineages remain largely unknown (but see Igc et al. 2012, Fossøy et al. 2016).

Obligate brood parasitic Common Cuckoos provide the best-studied example of the evolution of mimetic eggs: different host-races (called *gentes*) of cuckoos lay eggs that closely match the eggshell color and patterning of their respective songbird hosts (Stoddard et al. 2014). In these host-parasite systems, visual mimicry by the parasite is achieved chemically through the deposition of quantitatively similar concentrations of the two known avian eggshell pigments, biliverdin, which is responsible for the blue-green color in avian eggs, and protoporphyrin IX, which is responsible for the rusty colors and markings of avian eggs (Igc et al. 2012). These pigments combine with the calcite shell matrix to generate color and maculation patterns (Hanley et al. 2015) that are perceptually similar to those of the host egg as assessed by the avian visual system (Igc et al. 2012). Several other *Cuculus* species also closely mimic their respective host species' eggs (Payne 2005) and host-parasite visual eggshell mimicry has evolved independently in both of the two other parasitic Cuculiformes lineages (Sorenson and Payne 2005). However, the mechanism of egg color mimicry in those and other, non-cuckoo lineages of brood parasites remains unexplored. Here we asked: what is the chemical basis of egg color mimicry in an avian host-parasite system that is evolutionarily independent of the Common Cuckoo?

Specifically, we assessed the chemical constituents of eggshell color mimicry in the only New World lineage of obligate brood parasitic cuckoos, the Neomorphinae (Sorenson and Payne 2005). One of the brood parasitic species in this lineage, the Striped Cuckoo, lays blue eggs that accurately mimic the eggs of one of its hosts, the Rufous-and-white Wren, in Central America (Mark 2013). Previously, avian-visible spectrophotometric data showed patterns of similarity in reflectance between parasite and host eggshells; in turn, behavioral experimentation demonstrated the rejection of non-mimetically (and the acceptance of mimetically) colored model eggs, thus supporting the claim of evolved visual mimicry in this host-parasite system (Mark 2013). Here we used already collected egg specimens, calculated an additional spectral metric, and extracted, identified, and quantified colorful pigments from the blue eggshells of this parasite and host to determine whether the apparent visual mimicry is paralleled at the chemical level. To place the resulting patterns of host-parasite eggshell colors and pigment concentrations in an evolutionary context, we also opportunistically expanded our sampling to quantify the chemical basis of blue eggshell coloration in several non-parasitic members of New World cuckoo lineages (Hauber 2014),

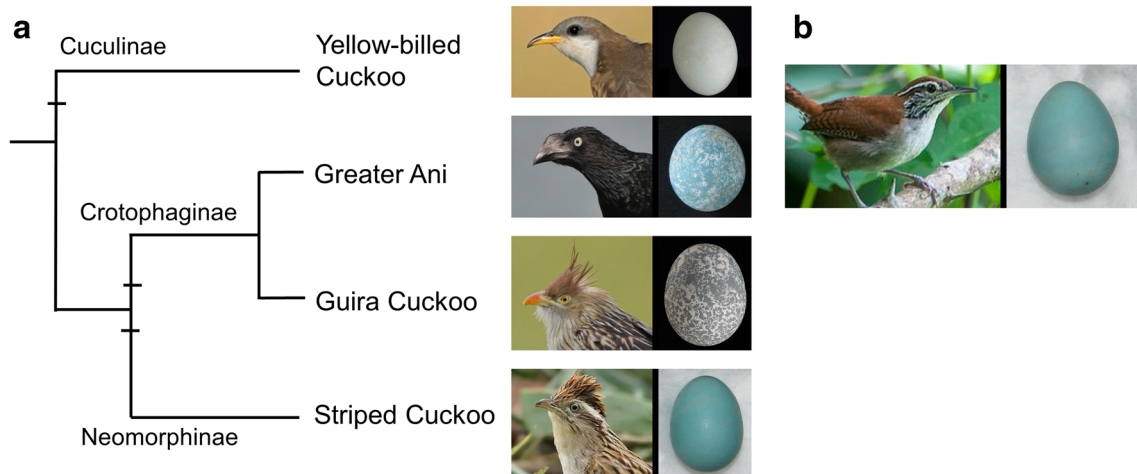
## Methods

### Host-Parasite Sampling and Field Work in Nicaragua

We sourced and analyzed the already collected eggshell samples from a previous study (Mark 2013) on the Striped Cuckoo, an obligate brood parasite, and one of its hosts, the Rufous-and-white Wren, conducted in the Miraflor Natural Reserve, Nicaragua; this resulted in a limited sample size for the current project but it also did not require the destructive sampling of additional viable eggs from the wild. Across its range, the Striped Cuckoo exhibits egg color polymorphism, laying unmarked white, bluish-white, or bluish-green eggs to match those of different local host species across their range from northern Argentina to southern Mexico (Mark 2013). In Nicaragua, Striped Cuckoos have been recorded to lay only blue-green eggs, which closely match the egg coloration of its most common local host, the Rufous-and-white Wren; Striped Cuckoo eggs are visually distinguishable from wren eggs by size and shape, but not in avian-visible color spectra (Mark 2013). We used eggs of Striped Cuckoos from parasitized nests, and eggs of Rufous-and-white Wrens from both parasitized and non-parasitized nests. All eggshells had been collected from partially incubated nests. The contents were removed and the eggshells were rinsed with ethanol then distilled water and stored at room temperature in a dark box.

### Non-Parasitic Cuckoo Egg Samples

We also opportunistically sourced and analyzed eggshell samples from three species of parental, non-parasitic New World cuckoo lineages (Fig. 1): wild Yellow-billed Cuckoos *Coccyzus americanus*, in California and Arizona, USA; wild Greater Anis *Crotophaga major*, in Panama; and captive Guira Cuckoos *Guira guira* from the Wildlife Conservation Society's Bronx Zoo breeding colony (native to South America). These three species all lay eggs with blue shells and represent two subfamilies within the Cuculidae family; Greater Anis and Guira Cuckoos belong to the subfamily Crotophaginae (the sister clade of the Neomorphinae, which contains the parasitic Striped Cuckoo; Sorenson and Payne 2005). In turn, the Yellow-billed Cuckoo belongs to a different lineage, the Cuculinae. Therefore, our sampling encompassed three separate evolutionary origins of blue-green eggshell coloration within the Cuculidae (in the subfamilies Crotophaginae, Neomorphinae, and Cuculinae each; Fig. 1). Eggshell samples were sourced from partially incubated clutches of the Yellow-billed Cuckoo, whereas all Guira Cuckoo and Greater Ani eggs were unincubated. The blue-green calcite shell of the eggs of Guira Cuckoos and Greater Anis is covered with vaterite, a white, chalky polymorph of calcium carbonate (Portugal et al. 2018). Since our goal was to characterize the chemical basis of the blue-green



**Fig. 1** **a** Simplified phylogeny of the cuckoos (Cuculidae) showing relationships of the 4 species sampled in this study (non-parasitic Yellow-billed Cuckoo [YBCU] *Coccyzus americanus*, Greater Ani [GRAN] *Crotophaga major*, and Guira Cuckoo [GUCU] *Guira guira*; and brood-parasitic Striped Cuckoo [STCU] *Tapera naevia*). Blue-green eggshell coloration has evolved independently in the three subfamilies

represented here. **b** Rufous-and-white Wren [RWWR] *Thyrophilus rufalbus* and egg, host of the obligate brood parasitic Striped Cuckoo. Photo credits: Joe Overcash (YBCU), Kamiel Spoelstra (GRAN), and Macaulay Library of the Cornell Laboratory of Ornithology (GUCU, STCU, RWWR)

calcite coloration, we removed the vaterite layer from these shells prior to spectral and pigmentary analyses. The chemical composition and concentration of the vaterite vs. calcite egg pigments in Greater Anis and Guira Cuckoos are reported separately (Hauber et al. 2018).

### Spectral and Chemical Analyses

Eggshells were measured for their reflectance between 300 and 700 nm using an Ocean Optics Spectrometer, following Igic et al. (2012). To calculate blue-green chroma (BGC), we followed the ratio proposed by Honza et al. (2007) and applied by Cassey et al. (2008): we calculated the area under the curve for all the avian visible wavelengths (300–700 nm) and used this to divide the area under the reflectance curve obtained for wavelengths 400–575 nm.

For the pigment analyses, we first measured the area of each shell fragment using photographs in JPEG format (by a Nikon Coolpix 8700) under controlled light conditions. We placed a size standard within each photograph and used ImageJ 1.40 (National Institute of Health, USA) to size-calibrate each image and measure the surface area. In addition, the mass of each fragment was measured with 1 mg precision (Mettler Toledo XS403S), and the thickness measured to an accuracy of 0.01 mm with a point micrometer (Series 112; Mitutoyo Corp., Kawasaki, Japan). Then, all eggshells were cleaned with 70% ethanol and manually pulverized. We used an ethylenediaminetetraacetic acid (EDTA) extraction protocol (Gorchein et al. 2009; Verdes et al. 2015) to extract biliverdin and protoporphyrin IX for pigment analysis from eggshell fragments (weight range: 40–500 mg). Ultimately,

this resulted in 1 ml of dissolved sample in acetonitrile-acetic acid (4:1 v/v). Dissolved samples were measured in a Cary 300 UV-Vis spectrophotometer for UV absorbance, with biliverdin and protoporphyrin absorbance observed at 377 nm and 405 nm, respectively (Igic et al. 2010).

Ultra high performance liquid chromatography (UHPLC) analysis was performed in a method similar to that described in our previous avian eggshell extraction studies (Verdes et al. 2015; Dearborn et al. 2017). Briefly, samples were run with a flow rate of 0.4 mL/min using water with 0.01 formic acid and acetonitrile with 0.1 formic acid as solvents A and B, respectively, with the linear gradient set to 2% A and 98% B at 6.5 min. Absorbance was monitored at 377 nm and 405 nm. Biliverdin eluted at ~3.5 min and protoporphyrin at ~5.6 min (for representative traces, see Verdes et al. 2015 and Hauber et al. 2018). Pigment presence or absence was also independently confirmed through mass spectrometry (following Verdes et al. 2015). Biliverdin and protoporphyrin will sometimes elute at both wavelengths (377 and 405 nm), though at a lower abundance. To account for this we measured the area of each pigment peak at each wavelength and then input their relative proportions into Beer Lambert's law ( $A = \epsilon lc$ ) in order to calculate each pigment's concentration. Samples were then standardized in three different ways: by the weight of the initial eggshell sample ( $\mu\text{M/g}$ ), by its surface area ( $\mu\text{M/mm}^2$ ), and by its volume ( $\mu\text{M/mm}^3$ ) (following Igic et al. 2012, Verdes et al. 2015). These different metrics allowed us to account for and contrast the impact of the known variation of pigment presence throughout the different layers of the avian eggshell matrix in other species (Sparks 1994; Miksik et al. 2007).

## Predictions and Statistical Analyses

Greater Ani ( $n = 10$ ) eggs were collected from different nests. Yellow-billed Cuckoo ( $n = 9$ ) eggs were collected from different nests ( $n = 7$ ) except for 3 sourced from the same abandoned nest. Striped Cuckoo eggs ( $n = 5$ ) and Rufous-and-white Wren eggs ( $n = 7$ ) were collected from different nests, except for 3 matched host-parasite samples sourced from the same nests. The captive Guira Cuckoo eggs ( $n = 6$ ) were sourced from a communal nest attended by several females, but we did not conduct genetic analyses to identify the laying females. Given that for most species the majority of eggs were sourced from different nests, in our statistical analyses we treated each egg as an independent data point. Given the very limited species-sampling of the cuckoo lineages with a total of  $n = 4$  species from 3 lineages, we did not control for phylogenetic positions of these taxa in our analyses but consider our results informative for future, expanded sampling approaches.

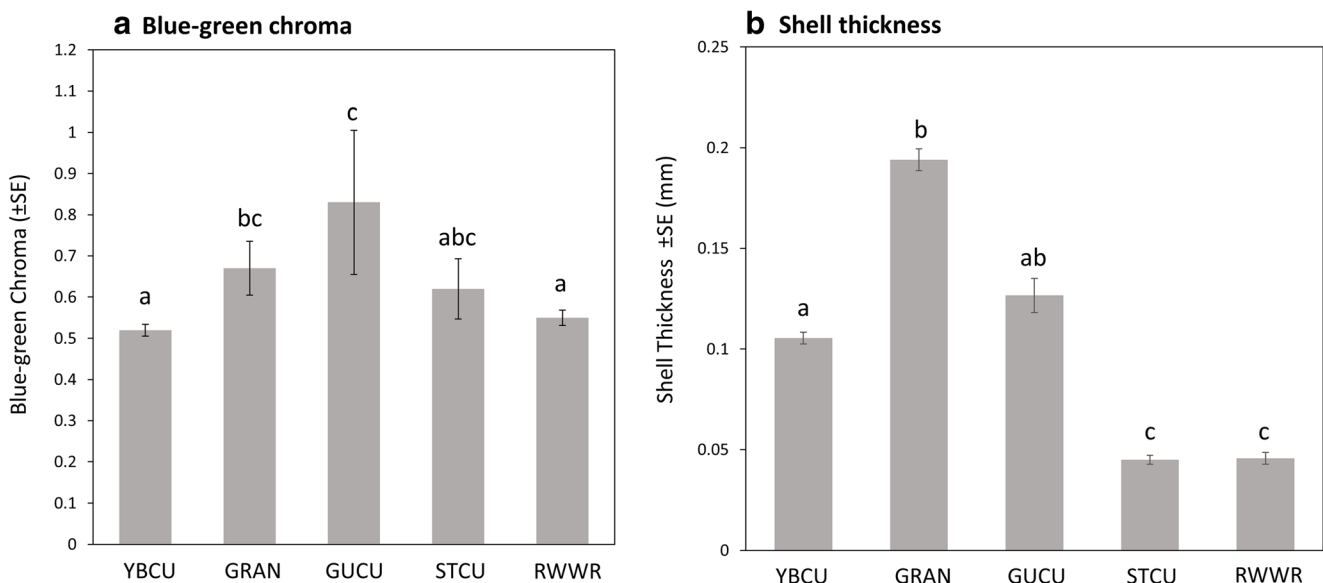
Based on the hypothesis that Striped Cuckoo eggshell coloration has evolved to mimic that of Rufous-and-white Wrens, we predicted that (1) blue-green chroma (BGC) should be more similar between these two species than across the other species in our data set; (2) the same pigments should be present in both host and parasite eggshells; and (3) the concentrations of shell pigments should be more similar between these two species than across the other species in our data set. We examined all trait differences across species with the conservative Kruskal-Wallis test. In order to identify significant differences between pairs of species, we used post hoc Dunn's tests with Bonferroni's adjustment for multiple hypothesis testing. Significance levels for all analyses were set at  $\alpha \leq 0.05$  and significant *post-hoc* differences are indicated in each figure with different letters.

## Results

The spectral assessment of the blue eggshell coloration revealed significant species-level variation of BGC in our samples (Kruskal-Wallis test;  $df = 4$ ,  $\chi^2 = 27.04$ ,  $p = 0.0001$ ). The post hoc Dunn's comparisons revealed that, as predicted, there was no significant difference in BGC between the parasitic Striped Cuckoo and its Rufous-and-white Wren host eggshells ( $p = 0.73$ ; Fig. 2a). Similarly, eggshell samples also varied in thickness across species (Kruskal-Wallis test;  $df = 4$ ,  $\chi^2 = 32.40$ ,  $p = 0.0001$ ), but post hoc values were not significantly different between Striped Cuckoos and Rufous-and-white Wrens ( $p = 0.99$ ; Fig. 2b).

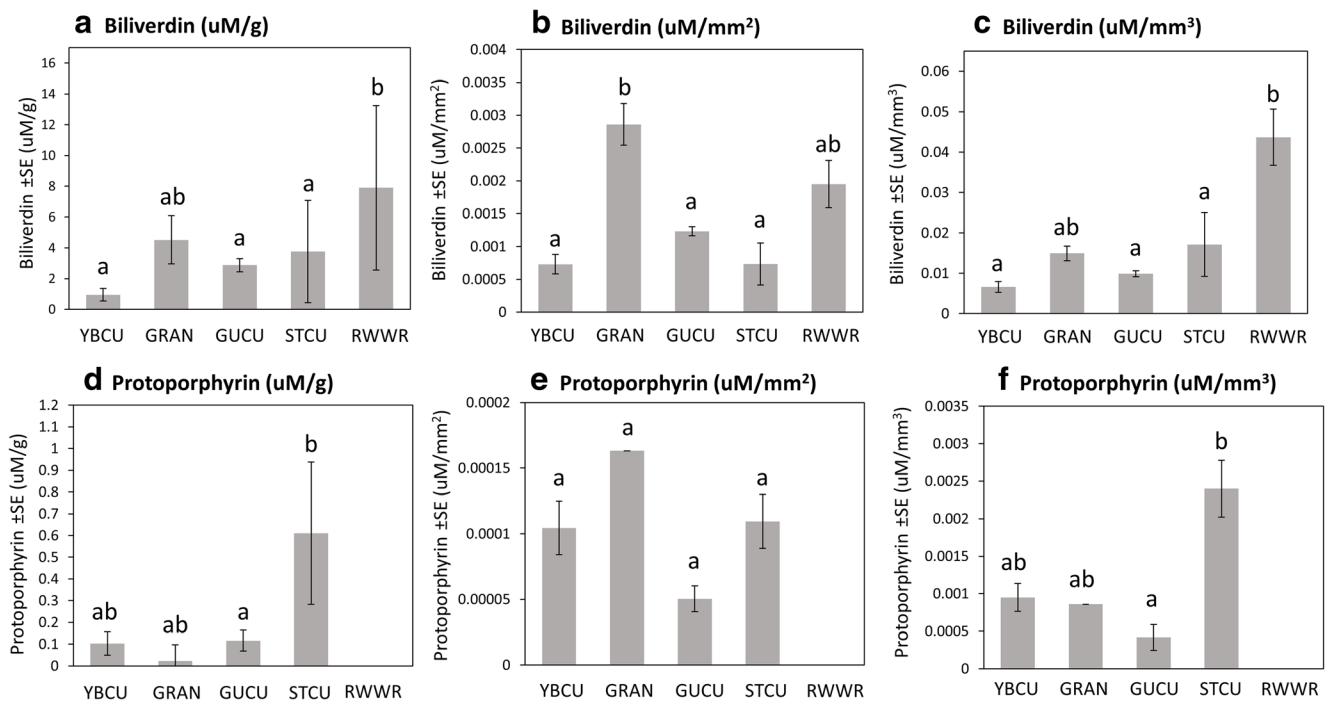
We detected biliverdin in eggshell samples from all of the species in our sampling, including 4 of 9 Yellow-billed Cuckoo, 6 of 7 Rufous-and-white Wren, and in all shells of all the remaining species. There was significant species-level variation in the detectable levels of biliverdin concentration in our samples irrespective of the pigment concentration metric used (Kruskal-Wallis tests; all  $df = 4$ ,  $\chi^2 \geq 15.20$ ,  $p \leq 0.0032$ ; Fig. 3a–c). Contrary to our predictions, post hoc Dunn's comparisons revealed that Rufous-and-white Wren eggs had significantly higher biliverdin concentrations than the Striped Cuckoo samples when concentration was measured by mass or by volume ( $\mu\text{M/g}$ :  $p = 0.04$ , Fig. 3a;  $\mu\text{M/mm}^3$ :  $p = 0.04$ , Fig. 3c). When standardized by surface area, Rufous-and-white Wren samples also had higher biliverdin concentrations than did Striped Cuckoo samples, but the difference was not statistically significant ( $\mu\text{M/mm}^2$ :  $p = 0.16$ ; Fig. 3b).

We identified protoporphyrin IX from all of the Striped Cuckoo eggs, all of the Guira Cuckoo eggs, 3 of 9 Yellow-billed Cuckoo eggs, and 1 of 10 Greater Ani eggs. Contrary to



**Fig. 2** Comparisons of (a) blue-green chroma (mean  $\pm$  SE) recorded by spectrophotometry and (b) shell thickness taken from the eggshell samples of our study species. For species codes, please see Fig. 1. legend; different letters indicate significant *post-hoc* differences





**Fig. 3** Different metrics of concentrations of (a–c) biliverdin and (d–e) protoporphyrin IX (mean  $\pm$  SE) detected from eggshell samples of our study species; note that RWWR yielded no detectable pigment in (d–e).

For species codes, please see Fig. 1. legend; different letters indicate significant *post-hoc* differences

our predictions, no protoporphyrin was detected in the host Rufous-and-white Wren eggs. The variation in the detectable levels of this pigment's concentration was significant across the cuckoo species (Kruskal-Wallis tests; all  $df = 4$ ,  $\chi^2 \geq 8.02$ ,  $p \leq 0.045$ ; Fig. 3d–f), irrespective of the concentration metric analyzed.

## Discussion

Prior analysis of avian-visible spectral data concluded that the coloration of the immaculate blue-green eggs of the parasitic Striped Cuckoo are similar to those of their hosts, the Rufous-and-white Wren (Mark 2013); furthermore, behavioral experiments from the same study showed that this visual mimicry is adaptive for the cuckoo because it reduces the hosts' rejection of mimetic foreign eggs in the nest (Mark 2013). Here, we replicated the spectral mimicry result by focusing on the BGC metric of host and parasite eggshells and showed that, as predicted, these are statistically indistinguishable between Rufous-and-white Wrens and their Striped Cuckoo parasites (Fig. 2a).

This visual mimicry, however, was not paralleled at the chemical level for either eggshell color pigment. Biliverdin was present in both Striped Cuckoo and Rufous-and-white Wren eggs, but its concentrations, using the weight or volume based metrics, were significantly lower in parasitic than in host eggs (Fig. 3a,c). Thus, chromatic (and perceptual)

mimicry by the Striped Cuckoo is achieved despite the lower levels of biliverdin compared to its host. Critically, the mass and volume based concentration metrics are useful to standardize across potential biases in the relative eggshell thickness between parasites and hosts, as diverse lineages of parasitic cuckoos, as well as cowbirds, have been found to have consistently thicker eggs than their respective hosts (e.g., Brooker and Brooker 1991; Yang et al. 2018). In our small sample, however, this was not the case as Striped Cuckoo and host wren eggshells were similar in thickness (Fig. 2b).

Furthermore, the relative pigment concentration patterns did not remain consistent when analyzing the data using a concentration metric standardized by surface area of the eggshell sample. In fact, as predicted, biliverdin concentrations ( $\mu\text{M}/\text{mm}^2$ ) were not statistically different between Striped Cuckoos and Rufous-and-white Wrens (Fig. 3b). However, when we look in more detail, we also see that wren (host) biliverdin concentrations were also statistically similar to Greater Anis, which in turn were significantly higher than those of Striped Cuckoos, implying an intermediate level of biliverdin in the host species. Furthermore, pigments, proteins, and other non-calcite compounds are known to be distributed across the width of the eggshell matrix of avian eggs (Sparks 1994; Miksik et al. 2007), therefore we recommend that only shell mass or volume based standardization of the pigment metrics should be used to generate interspecifically comparable concentration measurements.

Using these three metrics of pigment concentration, biliverdin was not only also present in eggshells of all of the other New World cuckoos also sampled here, but these levels were statistically similar for parasitic and non-parasitic species, indicating that biliverdin concentrations are shared across this taxonomically diverse set of cuckoo lineages overall.

Biliverdin is hypothesized to be a potentially costly antioxidant to produce (Moreno et al. 2006), and it may be that Striped Cuckoos are limited by biliverdin for their increased parasitic egg production rates, and deposit only the minimum concentrations sufficient to achieve spectral mimicry. However, the non-parasitic cuckoos also deposit similarly low levels of biliverdin in their blue eggshells, suggesting that greater parasitic egg production rate is not the (sole) cause of lower biliverdin concentrations in Striped Cuckoo eggs.

The other eggshell pigment, protoporphyrin IX, was present in the eggs of all Striped and Guira Cuckoo eggs, some of the Yellow-billed Cuckoos and Greater Ani eggs, and, contrary to our expectations, none of the Rufous-and-white Wren eggs. The lack of protoporphyrin in Rufous-and-white Wren eggs implies again that visual mimicry by Striped Cuckoo eggs can be achieved without a quantitative match of eggshell pigment concentrations. However, the lack of this second pigment in wren eggs was not surprising, as this species lays unmarked blue-green eggs (Mark 2013, Fig. 1). In turn, the presence of protoporphyrin in the immaculate eggs of several cuckoo species may instead indicate that this pigment may serve another, non-signaling function. Specifically, protoporphyrin IX has a putative photoactivated antimicrobial function in eggshells (Ishikawa et al. 2010, but see Dearborn et al. 2017) as well as a structural strengthening function (Gosler et al. 2011), and microbial loads as well as physical stresses are predicted to be more unpredictable for eggs of parasitic cuckoos laying into nests of many different hosts and of communally-breeding cuckoos with multiple breeding pairs laying many eggs in the same nest. The Striped and Guira Cuckoo may therefore require stronger antimicrobial defenses or greater structural eggshell strength compared to parental, solitary nesting species (Soler et al. 1999). However, contrary to this prediction, protoporphyrin was mostly absent in the eggs of Greater Anis, another non-parasitic but communally nesting species in our sample implying that communal breeding per se is not necessarily associated with the presence of antimicrobial pigments in the eggshells.

Finally, the Striped Cuckoo as a species displays egg color polymorphism between blue and white egg morphs (Mark 2013), likely in response to selection by rejecter hosts that lay one or more of these egg morphs themselves. Coevolutionary arms races between mimetic parasites and hosts has led to the evolution of host egg color polymorphism in other cuckoo-host (Yang et al. 2010) and in cowbird-host systems (de la Colina et al. 2012), too, but the chemical basis of mimicry in these systems remains to be studied in detail.

Overall, we demonstrate that perceptual mimicry and foreign-egg acceptance need not always be caused by parallel chemical composition between the eggshells of host and parasite avian species. Our results here are in contrast with patterns from the Common Cuckoo host-race chemical and visual mimicry system (Igic et al. 2012), but support previous general findings across birds that there is often not a one-to-one relationship between eggshell pigment concentrations and the resulting eggshell color and pattern appearance (Brulez et al. 2016; Cassey et al. 2012). Finally, it remains to be explored which pigments and what biochemical processes generate avian host-brood parasite mimicry at the other developmental stages of offspring development, including hatchling skin coloration (e.g., Langmore et al. 2011), and fledgling plumage patterning (e.g., de Marsico et al. 2012).

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