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Avian eggshell coloration predicts shell-matrix protoporphyrin content

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Abstract: Avian eggshell pigmentation may provide information about a female's physiological condition, in particular her state of oxidative balance. Previously we found that female house wrens (*Troglodytes aedon* Vieillot, 1809) with lighter, less-maculated, and redder ground-colored shells were older and produced heavier offspring than females laying darker, browner eggs. The strong pro-oxidant protoporphyrin is responsible for this species' eggshell pigmentation, so differences in pigmentary coloration may be related to eggshell protoporphyrin content and reflect female oxidative balance and condition during egg-formation. Therefore, we tested the assumption that egg-surface coloration is related to the amount of protoporphyrin in the shell matrix. We analyzed digital photographs of eggs to determine maculation coverage as a measure of the overall ground coloration of the egg and its red-, green-, and blue-channel pixel values. Pigments were then extracted from these same eggs and analyzed using high-performance liquid chromatography. There was a strong, positive relationship between eggshell redness and protoporphyrin content of eggshells, but no relationship between percent maculation and protoporphyrin content. Thus, when older, larger females deposit more protoporphyrin in their eggshells, this may reflect a tolerance for high levels of circulating protoporphyrin or an effective mechanism for off-loading protoporphyrin into the eggshell matrix.

Keywords: House wren, *Troglodytes aedon*, Eggs, Female quality, HPLC, Eggshell pigmentation

Introduction

Eggshell pigmentation is an extended phenotypic trait of female birds, one that may provide information about a female's underlying physiological condition (Moreno and Osorno 2003; Moreno et al. 2006; Hodges et al. 2020) as well as serve as an adaptation for camouflaging eggs (Poulton 1890: 61ff.; Lack 1958; Weidinger 2001; Westmoreland and Kilti 2007), strengthening eggshells (Underwood and Sealy 2002; Gosler et al. 2005), and, for the embryo, mediating light exposure and modulating the thermal environment (Bakken et al. 1978; Westmoreland et al. 2007; Mauer et al. 2015; Lahti and Ardia 2016). Two pigments predominate in avian eggshells, protoporphyrin, responsible for the pink, rusty-brown, and black hues, and biliverdin, for the blue-green hues (Mikšík et al. 1994, 1996; Gorchein et al. 2009; but see Hamchand et al. 2020). Protoporphyrin plays an important role in heme synthesis (Chiabrando et al. 2014) and, because it is a strong pro-oxidant (Afonso et al. 1999), its presence in eggshells has inspired a number of studies aimed at determining if variation in eggshell pigmentation reflects differences in females' condition, in particular their state of oxidative balance at the time of egg production (Moreno and Osorno 2003; de Coster et al. 2013; Holveck et al. 2019). Such studies are typically based on the stated or unstated assumption that differences in eggshell coloration are positively related to the amount of protoporphyrin contained in the eggshell matrix (see Wegmann et al. 2015). However, only a handful of studies have examined this assumption, typically finding, at best, a weak correlation between physical or perceptual measures of eggshell coloration and protoporphyrin content (Cassey et al. 2012; Brulez et al. 2014; Wegmann et al. 2015; Gómez et al. 2019).

In house wrens (*Troglodytes aedon* Vieillot, 1809), the ground (base) color of the eggshell varies from white to light tan or pink, and it is either sparsely or densely maculated with

small spots or larger blotches of reddish brown (Johnson 2020; Hodges et al. 2020). In a recent study relating protoporphyrin-based eggshell pigmentation to female condition and levels of male parental provisioning in our study population, we found that females laying eggs with more sparsely maculated and redder shells were older and produced heavier offspring than females laying more densely maculated and browner eggs (Hodges et al. 2020). Thus, the physically quantified differences in the coloration of house wren eggshells (Fig. 1), which vary greatly among females (see Fig. 1 in Hodges et al. 2020) and are highly repeatable within clutches (Hodges et al. 2020), provide visual information about a female's condition and, potentially, her protoporphyrin load and her state of oxidative balance at the time of egg-laying. What is not clear, however, is whether the differences in egg-surface coloration accurately reflect the amount of protoporphyrin deposited into the shell matrix, an important assumption when testing the different hypotheses that have been proposed to explain among-female variation in eggshell pigmentation (Wegmann et al. 2015). Therefore, in this study we tested the assumption that differences in physical metrics of eggshell pigmentation are positively correlated with the amount of protoporphyrin deposited in the eggshells of house wrens.

Materials and methods

Species, study area and egg collection

The northern house wren is a small (≈ 11 g), secondary cavity-nesting songbird with a breeding range distributed across the mid-latitudes of North America (Johnson 2020). In our migratory study population in north-central Illinois, USA, the birds breed in nestboxes that we provide (see Lambrechts et al. 2010 for information on the nestboxes used). Males and females

locally return from the wintering grounds to the study site in late April and early May. Soon after arrival, males find and defend a nestbox in which they construct a crude, concave base of large sticks upon which females construct a cup-shaped nest using finer plant materials. Two broods are typically produced each breeding season with females laying clutches of 6-8 eggs in late April-early June and 5-7 eggs in late June-early July (Bowers et al. 2011, 2012, 2016). One egg, weighing ≈ 1.5 g, is laid each day, although occasionally a day is skipped, particularly during cold, wet weather in spring. The incubation period is ≈ 12 days (Baltz and Thompson 1988; Dobbs et al. 2006; Sakaluk et al. 2018) and the nestling period is 15-18 days (Bowers et al. 2016). More information on the house wren is found in Johnson (2020), which also contains additional data on our study population.

We analyzed eggs that were collected between 11-25 May 2017 from nests on the East Bay Study Area (40.655°N , 88.91°W), where there are 120 nestboxes (5.4/ha). Eggs were marked on the morning they were laid to indicate their position in the laying order. The eggs used in this study were collected on the morning they were laid and replaced with artificial eggs, which females readily accepted and continued to incubate. From 30 nests we selected 46 eggs that visually varied widely in amount of maculation and background color to obtain a set of eggs that represented the range of variation found in the study population (see Fig. 1, Hodges et al. 2020). From eight of these nests, we collected more than one egg (2 eggs from each of five nests, and 3, 4, and 6 eggs, respectively, from each of the remaining three nests). The sample comprised seven eggs each of eggs from positions 1, 3, 4, 5 in the egg-laying order; five eggs each from positions 2 and 7; four eggs from position 6; and two eggs from position 8. Two eggs were not included in the analysis because of missing metadata.

Eggshell coloration

Upon return to the laboratory on the day each egg was collected, we weighed them to the nearest 0.0001 g, measured their width and breadth to the nearest 0.01 mm using dial calipers, and photographed them from ≈ 15 cm away under standardized light conditions, after which they were frozen at -80°C and kept away from light until pigments were extracted in 2019. Digital photographs of eggs were captured as JPG files and analyzed using the software program ImageMagick (version 7.0.7-22, <http://www.imagemagick.org>). For image analysis, the digital photographs of eggs were centered, and a 10% crop was applied resulting in a central slice running from pole to pole to represent the entirety of the egg. A common threshold was applied to each image to differentiate maculation from background and to calculate percentage maculation. Images were also split into separate RGB channels to extract Red, Green, and Blue channel pixel values from the whole slice. Additional details of these procedures can be found in Hodges et al. (2020). We acknowledge that a digital camera-based quantification of the appearance of eggshells does not allow for modelling of all the avian-perceivable visual cues, including ultraviolet reflectance (Aidala et al. 2012), but physically quantified differences from RGB cues are typically matched by avian-perceivable differences (e.g., Hauber et al. 2019; Price-Waldman and Stoddard 2021).

Eggshell protoporphyrin quantification

Pigments were extracted using ethylenediaminetetraacetic acid (EDTA) and acetonitrile–acetic acid following the detailed protocols of Mikšík et al. (1996) as modified by Dainson et al. (2018). In brief, we cleaned all of the eggshell fragments of each egg with a 70% ethanol

solution, air-dried them, and weighed them to the nearest 0.0001 g. Fragments were then rinsed with 1 mL milliQ H₂O followed by 1 mL of 70% ethanol. Fragments were again air dried for 10 min. We added 0.75 mL of EDTA (pH 7.2) and homogenized the mix for 1 min, which was then incubated at room-temperature for 5 min uncapped (to allow CO₂ to escape). We centrifuged the mix at 15000 g for 30 s and discarded the supernatant. We repeated the EDTA addition and centrifugation steps two more times, vortexing for 30 s between rinses instead of homogenizing. We then added 0.25 mL of acetic acid and 0.75 mL of acetonitrile and vortexed for 2 min in 30-s bursts, stopping to release pressure from CO₂ between bursts. Finally, we centrifuged the mix for 2 min at 15000 g, then removed the supernatant, which we transferred to a clean Eppendorf tube, typically ending up with $\approx$ 1 mL of pinkish liquid per egg. This was stored in -20 °C in a light-tight box for no more than 2 weeks prior the next step.

Samples were sent to the University of Illinois at Urbana-Champaign for quantification by high-performance liquid chromatography (HPLC) at the School of Chemical Sciences; we analyzed the amounts of eluted Protoporphyrin IX (562.658 g/mol) at 377 nm at 6.1 min. We quantified the resulting concentration on a μ Mol pigment/g shell matrix weight basis.

Statistics

We used SAS statistical software (version 9.4; SAS Institute, Cary, NC, USA), two-tailed tests with $\alpha = 0.05$ and the Satterthwaite's degrees-of-freedom approximation, which can result in non-integer denominator degrees of freedom. We included nest identity as a random effect to account for the statistical nonindependence of eggs laid by the same female within the same clutch. To evaluate the relationship between egg coloration and the concentration of

protoporphyrin in the eggshell matrix, we used a generalized linear mixed model with a lognormal response distribution and an identity link function (PROC GLIMMIX), with protoporphyrin concentration as the response variable and percent maculation (eggshell darkness); egg order; and red-, green-, and blue-channel pixel values of the shells' ground coloration included as explanatory variables.

Results

The mean protoporphyrin concentration ($\pm$ SE) of house wren eggshells, irrespective of clutch or laying order (Table 1), was 0.415 ± 0.017 μ Mol/g. Protoporphyrin concentration increased significantly with the red channel pixel value (estimate $\pm$ SE = 0.0938 ± 0.0271 ; Fig. 1) but was not significantly associated with the blue or green values (Table 1). There was no significant association between percent maculation and protoporphyrin concentration (Table 1). Within females, protoporphyrin concentration declined over the laying order (estimate $\pm$ SE = -0.0316 ± 0.0185), but this trend was not significant (Table 1).

Discussion

We found a strong, positive relationship between eggshell redness and the protoporphyrin content of eggshells but no relationship between percent maculation and protoporphyrin content. The former is not surprising as reflectance of protoporphyrin falls within the red and near-infrared portion of the human-visible spectrum (Bakken et al. 1978), but the latter is contrary to our expectation because one might expect that the greater dispersion of protoporphyrin over the eggshell's surface would be associated with more maculation coverage of the shell itself.

Although it can be argued that these results may pertain only to our study population of house wrens (but, see Gómez et al. 2019), we consider it appropriate to caution against the assumption that measures of total maculation coverage, whether assessed human-visually or digitally using computational methods, are always and strongly correlated with eggshell protoporphyrin content. Nonetheless, the results demonstrate that, at least in our study population, an objective measure of color (viz., shell ground coloration redness) is a good and positive predictor of eggshell protoporphyrin content.

In the contemporaneous study of a different set of eggs from the study population, older, larger female house wrens produced lighter, redder eggshells as well as nestlings with greater size-corrected mass than younger, smaller females (Hodges et al. 2020). These traits are known proxies of individual maternal quality (Barnett et al. 2015) in house wrens (Bowers et al. 2014, 2017), indicating that older females were in better condition than younger ones. Thus, based on the positive relationship between protoporphyrin content and eggshell redness in the current analysis, older, larger females deposited more protoporphyrin into their eggshells than younger, smaller females, a result consistent with the ‘good-quality’ hypothesis that high-quality females produce eggshells with more protoporphyrin than low-quality females (Moreno and Osorno 2003). A number of prior studies, using a variety of ways to estimate protoporphyrin content based on visible characteristics of the eggshell, have reported a positive correlation between measures of female quality and estimates of protoporphyrin (e.g., Martínez-de la Puente et al. 2007; Minias et al. 2020), whereas others have not found such statistical linkages (e.g., Sanz and García-Navas 2009). We note that one consistent conclusion emerging from studies comparing measures of eggshell pigmentation and putative measures of female quality is that the relationship varies not only among species (Sanz and García-Navas 2009; Poláček et al. 2017)

but also even within species and their populations (Corti et al. 2018). Combining these disparate results with our current findings, namely that the measure of shell redness, but not maculation coverage, was positively correlated with eggshell protoporphyrin content, prompts us to suggest that many of these conflicting results may come about because the oft-used visual assessment of various aspects of eggshell pigmentation simply does not fully reflect protoporphyrin content, one cause of which may be that visual measures only inspect the egg's surface and protoporphyrin may be distributed throughout the eggshell in diverse species (Jagannath et al. 2008).

It remains unclear exactly the source of the protoporphyrin deposited into the forming eggshell, but it is generally thought to be derived *de novo* from the shell gland (Samiullah et al. 2015). Being a strong pro-oxidant, high levels of circulating protoporphyrin should lead to high levels of reactive oxygen species (ROS) and substantial oxidative stress (Afonso et al. 1999). It is not clear, however, whether the deposition of more protoporphyrin in the eggshells by good-condition females than by poor-condition females can be attributed to (i) a better antioxidant response coupled with tolerance of high levels of circulating protoporphyrin or (ii) a more efficient mechanism of (presumably) active transport to off-load protoporphyrin to the eggshell (Moreno and Osorno 2003; De Coster et al. 2013). These hypotheses can be tested in future works by (i) comparing direct measures of protoporphyrin-based eggshell pigmentation with female protoporphyrin and oxidative stress levels, (ii) assessing damage to proteins (carbonylation) caused directly or indirectly by reactive oxygen species, and (iii) measuring the expression of genes known to play important roles in breaking down ROSs or handling ROS-damaged proteins (Monaghan et al. 2009; Ghezzi 2020).

Competing interests

The authors declare there are no competing interests.

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References

- Aidala, Z., Huynen, L., Brennan, P.L.R., Musser, J., Fidler, Chong, N., Machovsky Capuska, G.E., Anderson, M.G., Talaba, A., Lambert, D., and Hauber, M.E. 2012. Ultraviolet visual sensitivity in three avian lineages: paleognaths, parrots, and passerines. *J. Comp. Physiol. A.* **198**: 495-510.
- Afonso, S., Vanore, G., and Batlle, A. 1999. Protoporphyrin IX and oxidative stress. *Free Radical Res.* **31**: 161-170.
- Bakken, G.S., Vanderbilt, V.C., Buttemer, W.A., and Dawson, W.R. 1978. Avian eggs: thermoregulatory value of very high near-infrared reflectance. *Science* **200**: 321-323.
- Baltz, M.E., and Thompson C.F., 1988. Successful incubation of experimentally enlarged clutches by house wrens. *Wilson Bull.* **100**: 70-79.
- Barnett, C.A., Suzuki, T.N., Sakaluk, S.K., and Thompson, C.F. 2015. Mass-based condition measures and their relationship with fitness: in what condition is condition? *J. Zool.* **296**: 1-5.
- Bowers, E.K., Sakaluk, S.K., and Thompson, C.F. 2011. Adaptive sex allocation in relation to hatching synchrony and offspring quality in house wrens. *Am. Nat.* **177**: 617-629.
- Bowers, E.K., Sakaluk, S.K., and Thompson, C.F. 2012. Experimentally increased egg production constrains future reproduction of female house wrens. *Anim. Behav.* **83**: 495-500.
- Bowers, E.K., Hodges, C.J., Forsman, A.M., Vogel, L.A., Masters, B.S., Johnson, B.G.P., Johnson, L.S., Thompson, C.F., and Sakaluk, S.K. 2014. Neonatal body condition, immune

responsiveness, and hematocrit predict longevity in a wild bird population. *Ecology* **95**: 3027–3034.

Bowers, E.K., Grindstaff, J.L., Soukup, S.S., Drilling, N.E., Eckerle, K.P., Sakaluk, S.K., and Thompson, C.F. 2016. Spring temperatures influence selection on breeding date and the potential for phenological mismatch in a migratory bird. *Ecology* **97**: 2880–2891.

Bowers, E.K., Sakaluk, S.K., and Thompson, C.F. 2017. Interactive effects of parental age on offspring fitness and age-assortative mating in a wild bird. *J. Exp. Zool.* **327A**: 302–310.

Brulez, K., Cassey, P., Meeson, A., Mikšík, I., Webber, S.L., Gosler, A.G., and Reynolds, S.J. 2014. Eggshell spot scoring methods cannot be used as a reliable proxy to determine pigment quantity. *J. Avian Biol.* **45**: 94–102.

Cassey, P., Mikšík, I., Portugal, S.J., Maurer, G., Ewen, J.G., Zarate, E., Sewell, M.A., Karadas, F., Grim, T., and Hauber, M.E. 2012. Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two *Turdus* thrushes. *J. Avian Biol.* **43**: 503–512.

Chiabrando, D., Mercurio, S., and Tolosano, E. 2014. Heme and erythropoiesis: more than a structural role. *Haematologia* **99**: 973–983.

Corti, M., Romano, A., Costanzo, A., Bentz, A.B., Navara, K.J., Parolini, M., Saino, N., and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. *J. Avian Biol.* **49**: 1–17.

- Dainson, M., Mark, M., Hossain, M., Yoo, B., Holford, M., McNeil, S.E., Riehl, C., and Hauber, M.E. 2018. How to make a mimic? Brood parasitic striped cuckoo eggs match host shell color but not pigment concentrations. *J. Chem. Ecol.* **44**: 940-946.
- De Coster, G., De Neve, L., and Lens, L., 2013. Intra-clutch variation in avian eggshell pigmentation covaries with female quality. *J. Ornithol.* **154**: 1057-1065.
- Dobbs, R.C., Styrsky, J.D., and Thompson, C.F., 2006. Clutch size and the costs of incubation in the house wren. *Behav. Ecol.* **17**: 849–856.
- Ghezzi, P. 2020. Environmental risk factors and their footprints in vivo—A proposal for the classification of oxydative stress biomarkers. *Redox Biol.* **34**: 101442.
- Gómez, J., Liñán-Cembrano, G., Castro, M., Pérez-Hurtado, A., Ramo, C., and Amat, J.A. 2019. The protoporphyrin content of Kentish plover *Charadrius alexandrinus* eggshells is better predicted by the fractal dimension of spottiness than by colour. *J. Ornithol.* **160**: 409-415.
- Gorchein, A., Lim, C.K., and Cassey, P. 2009. Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **23**: 602-606.
- Gosler, A.G., Higham, J.P., and Reynolds, S.J. 2005. Why are birds' eggs speckled? *Ecol. Lett.* **8**: 1105-1113.
- Hamchand, R., Hanley, D., Prum, R.O., and Brückner, C. 2020. Expanding the eggshell colour gamut: uroerythrin and bilirubin from tinamou (*Tinamidae*) eggshells. *Sci. Rep.* **10**: 11264.

Hauber, M.E., Luro, A., McCarty, C.J., Barateli, K., Cassey, P., Hansen, E., and Dale, J. 2019.

Interannual repeatability of eggshell phenotype in individual female common murrelets (*Uria aalge*). *Can. J. Zool.* **97**: 385-391.

Hodges, K.E., Mortimer, N.T., Vrailas-Mortimer, A.D., Sakaluk, S.K., and Thompson, C.F.

2020. Connecting the dots: avian eggshell pigmentation, female condition and paternal provisioning effort. *Biol. J. Linn. Soc.* **130**: 114-127.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C., and Grégoire, A. 2019. Eggshell coloration

indicates female condition during egg-laying: a field experiment in blue tits. *Biol. J. Linn. Soc.* **128**: 181-200.

Jagannath, A., Shore, R.F., Walker, L.A., Ferns, P.N., and Gosler, A. G. 2008. Eggshell

pigmentation indicates pesticide contamination. *J. Appl. Ecol.* **45**: 133-140.

Johnson, L.S. 2020. House Wren (*Troglodytes aedon*), version 1.0. *In* Birds of the World. *Edited*

by A.F. Poole. Cornell Lab of Ornithology, Ithaca, NY, USA.

Lack, D., 1958. The significance of the colour of turdine eggs. *Ibis* **100**: 145-166.

Lambrechts, M.M., Adriaensen, F., Ardia, D.R., Artemyev, A.V., Atiénzar, F., Bañbura,

J., Barba, E., Bouvier, J.-C., Camprodon, J., Cooper, C.B., Dawson, R.D., Eens, M., Eeva,

T., Faivre, B., Garamszegi, L.Z., Goodenough, A.E., Gosler, A.G., Grégoire, A., Griffith,

S.C., Gustafsson, L., Johnson, L. S., Kania, W., Keiřs, O., Llambias, P.E., Mainwaring,

M.C., Mänd, R., Massa, B., Mazgajski, T.D., Møller, A.P, Moreno, J., Naef-Daenzer,

B., Nilsson, J.-Å., Norte, A.C., Orell, M., Otter, K.A., Park, C.R., Perrins, C.M., Pinowski,

J., Porkert, J., Potti, J., Remes, V., Richner, H., Rytkönen, S., Shiao, M.-T., Silverin,

B., Slagsvold, T., Smith, H.G., Sorace, A., Stenning, M.J., Stewart, I., Thompson,

- C.F., Tryjanowski, P., Török, J., van Noordwijk, A.J., Winkler, D.W., and Ziane, N. 2010. The design of artificial nestboxes for the study of secondary hole-nesting birds: a review of methodological inconsistencies and potential biases. *Acta Ornithol.* **45**: 1-26.
- Lahti, D.C., and Ardia, D.R. 2016. Shedding light on bird egg color: pigment as parasol and the dark care effect. *Am. Nat.* **187**: 547-563.
- Martínez-de La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S., and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? *J. Avian Biol.* **38**: 377-384.
- Maurer, G., Portugal, S.J., Hauber, M.E., Mikšík, I., Russell, D.G.D., and Cassey, P. 2015. First light for avian embryos: eggshell thickness and pigmentation mediate variation in development and UV exposure in wild bird eggs. *Funct. Ecol.* **29**: 209-218.
- Mikšík, I., Holáň, V., and Deyl, Z. 1994. Quantification and variability of eggshell pigment content. *Comp. Biochem. Physiol. Part A.* **109A**: 769-772.
- Mikšík, I., Holáň, V., and Deyl, Z. 1996. Avian eggshell pigments and their variability. *Comp. Biochem. Physiol. Part B.* **113**: 607-612.
- Minias, P., Gómez, J., and Włodarczyk, R. 2020. Egg spottiness reflects female condition, physiological stress, and ornament expression in a common rallid species. *Auk: Ornitholog. Adv.* **137**: 1-13.
- Monaghan P., Metcalfe, N.B., and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**: 75-92.
- Moreno, J., and Osorno, J.L. 2003. Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? *Ecol. Lett.* **6**: 803-806.

- Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-de la Puente, J., Sanz, J.J., Mateo, R., and Soler, J.J. 2006. Experimental evidence that egg color indicates female condition at laying in a songbird. *Behav. Ecol.* **17**: 651–655.
- Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M., and Hoi, H. 2017. Eggshell coloration and its importance in postmating sexual selection. *Ecol. Evol.* **7**: 941–949.
- Poulton, E.B. 1890. The colours of animals, their meaning and use, especially considered in the case of insects. D. Appleton and Company, New York, NY, USA.
- Price-Waldman, R., and Stoddard, M.C. 2021. Avian coloration genetics: recent advances and emerging questions, *J. Hered.* **2021**, esab015.
- Sakaluk, S.K., Thompson, C.F., and Bowers, E.K. 2018. Experimental manipulation of incubation period reveals no apparent costs of incubation in house wrens. *Anim. Behav.* **137**: 167–177.
- Samiullah, S., Roberts, J.R., and Chousalkar, K. 2015. Eggshell color in brown-egg laying hens—a review. *Poultry Sci.* **94**: 2566–2575.
- Sanz, J.J., and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits *Cyanistes caeruleus*. *J. Anim. Ecol.* **78**: 31–41.
- Underwood, T.J., and Sealy, S.G. 2002. Adaptive significance of egg coloration. *In* Avian Incubation. *Edited by* D.C. Deeming. Oxford University Press, Oxford, U.K. pp. 280–298.
- Wegmann, M., Vallat-Michel, A., and Richner, H. 2015. An evaluation of different methods for assessing eggshell pigmentation and pigment concentration using great tit eggs. *J. Avian Biol.* **46**: 597–607.

- Weidinger, K. 2001. Does egg colour affect predation rate on open passerine nests? *Behav. Ecol. Sociobiol.* **49**: 456-464.
- Westmoreland, D., Schmitz, M., and Burns, K.E. 2007. Egg color as an adaptation for thermoregulation. *J. Field Ornithol.* **78**: 176-183.
- Westmoreland, D., and Kilti, R.A. 2007. Egg coloration and selection for crypsis in open-nesting blackbirds. *J. Avian Biol.* **38**: 682-689.

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Fig. 1. Representative photographs of house wren (*Troglodytes aedon* Vieillot, 1809) eggs showing (A) light, (B) intermediate, and (C) dark coloration. Please refer to Figures 1 and 2 in Hodges et al. (2020) for additional details.

Fig 2. Eggshell protoporphyrin content in relation to house wren (*Troglodytes aedon* Vieillot, 1809) eggshell redness. Predicted protoporphyrin concentration (back-transformed from the natural log) in relation to each eggshell's red-channel pixel value after controlling for clutch effects, egg-laying order, and other coloration traits. See Table 1 and text for the description of the generalized linear mixed model.

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Table 1. Coloration and protoporphyrin concentration of house wren (*Troglodytes aedon* Vieillot, 1809) eggshells. Significant effect bolded.

Trait	<i>F</i>	<i>df</i>	<i>p</i>
Protoporphyrin			
Red	11.96	1, 36.22	0.0014
Green	0.55	1, 37.38	0.4621
Blue	2.63	1, 37.79	0.1135
Percent maculation	1.24	1, 35.4	0.2735
Egg-laying order	2.90	1, 37.65	0.0970

Figure 1

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671x376mm (38 x 38 DPI)

Figure 2