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RESEARCH ARTICLE

High within-clutch repeatability of eggshell phenotype in Barn Swallows despite less maculated last-laid eggs

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ABSTRACT

Ecological and life-history variation and both interspecific and intraspecific brood parasitism contribute to diversity in egg phenotype within the same species. In this study, Barn Swallows (*Hirundo rustica erythrogaster*) laid eggs with high intraclutch repeatability in egg size, shape, and maculation. Despite this high intraclutch repeatability, last-laid eggs had consistently less of the eggshell covered in spots and fewer spots than earlier-laid eggs in the clutch. We examined sources of interclutch and intraclutch variation using both direct measurements and custom software (*SpotEgg, NaturePatternMatch*) that provide detailed information on egg characteristics, especially maculation measures. In addition to our main findings, maculation on different sides of the egg was highly repeatable; however, only shape, proportion of the eggshell maculated, and average spot size were repeatable between first and replacement clutches. Low intraclutch variation in maculation could allow females to recognize their clutch and this may be adaptive for colonial nesting species, such as the Barn Swallow. Characterizing intraspecific variation in egg size, shape, and maculation is the first step in understanding whether intraclutch variation is low enough—and interclutch variation high enough—such that eggs could serve as identity signals.

Keywords: egg shape, egg size, eggshell maculation, *Hirundo rustica*, intraclutch variation, lay order, repeatability

LAY SUMMARY

- Characterizing variation in avian egg size, shape, and speckling can help us understand the mechanistic basis of and functional outcomes for the patterns we see.
- Photographs of Barn Swallow (*Hirundo rustica erythrogaster*) eggs showed low within-nest and high between-nest variation in size, shape, and speckling.
- The most repeatable measure of egg appearance was egg shape, while the least repeatable was average spot size.
- First and replacement clutch eggs resembled each other only in shape, proportion of the eggshell speckled, and average spot size.
- The lay order of the eggs explained some of the within-nest variation in egg appearance; last-laid eggs were less spotted than earlier-laid eggs.

Alta repetibilidad dentro de la nidada del fenotipo de la cáscara del huevo en *Hirundo rustica erythrogaster*, a pesar de que los huevos del final de la puesta son menos maculados

RESUMEN

La variación ecológica y de la historia de vida, y el parasitismo de nidada tanto inter-específico como intra-específico, contribuyen a la diversidad en el fenotipo de los huevos dentro de la misma especie. En este estudio, individuos de *Hirundo rustica erythrogaster* pusieron huevos con alta repetibilidad dentro de la nidada en cuanto al tamaño, la forma y el maculado del huevo. A pesar de esta alta repetibilidad dentro de la nidada, los huevos del final de la puesta tuvieron consistentemente menos partes de la cáscara cubiertas de manchas y menos manchas que los huevos puestos al inicio de la puesta. Examinamos las fuentes de variación dentro de la nidada y entre nidadas utilizando mediciones directas y software personalizado (*SpotEgg, NaturePatternMatch*), que brindan información detallada sobre las características del huevo, especialmente las medidas de maculado. Además de nuestros hallazgos principales, el maculado en diferentes costados del huevo fue altamente repetible; sin embargo, solo la forma, la proporción de la cáscara del huevo maculada y el tamaño promedio de la mancha fueron repetibles entre la primera nidada y las de reemplazo. La baja variación dentro de la nidada en el maculado podría permitir que las hembras reconozcan su nidada y esto puede ser una adaptación para las especies que anidan en colonias, como *H. r. erythrogaster*. La caracterización de la variación intra-específica en el tamaño, la forma y el maculado de los huevos es el primer paso para comprender si la variación dentro de la nidada es lo suficientemente baja—y la variación entre nidadas lo suficientemente alta—como para que los huevos puedan servir como señales de identidad.

Palabras clave: forma del huevo, *Hirundo rustica*, maculado de la cáscara, orden de la puesta, repetibilidad, tamaño del huevo, variación dentro de la nidada

INTRODUCTION

Avian eggshells have a wide range of potential functions beyond the essential role of protecting developing embryos. Eggshell coloration and maculation in particular have been implicated in crypsis (e.g., Sánchez et al. 2004), thermoregulation (e.g., Westmoreland et al. 2007), sexual selection (e.g., Soler et al. 2005), and egg mimicry and antiparasitic recognition (e.g., Hanley et al. 2016). Despite a long history of studying the adaptive function of eggshells, there is a growing list of non-mutually exclusive explanations for interspecific and intraspecific patterns of egg shape, size, and maculation, with increasing evidence that many patterns are species- or site-specific. Recent work has highlighted the potential for aspects of eggshell phenotype to serve as identity signals (Birkhead et al. 2021, Quach et al. 2021, Šulc et al. 2021), which could be advantageous to colonially breeding birds or those affected by brood parasitism. To function as identity signals, traits are predicted to be highly variable, not necessarily condition-dependent or associated with fitness differences, and predicted to be highly repeatable within an individual (Dale et al. 2001, Quach et al. 2021). Characterizing repeatability of multiple measures of eggshell phenotype as well as understanding potential sources of phenotypic variation is a necessary step in determining whether eggshells could function as identity signals.

Egg size, shape, and maculation characteristics can vary substantially between females of the same species, while also having consistently high intraclutch repeatability values, particularly in size and shape metrics (Christians 2002; Table 1). Numerous studies have documented repeatable measures of egg phenotype (Table 1), but fewer studies report repeatability values across multiple measures of egg size (area, volume, length, breadth), shape, and maculation. Decreased intraclutch variation in eggshell maculation in particular may be due to brood parasitism in some species (Gosler et al. 2005, Kilner 2006, Cherry and Golser 2010). The signature hypothesis suggests that, in response to brood parasitism, host species have evolved the ability to produce “signature” maculation patterns (a potential identity signal) as a way to recognize their own eggs and eject parasitic eggs (Swynnerton 1918, Victoria 1972, Stokke et al. 1999, Cherry et al. 2007). Species not subject to brood parasitism could still benefit from egg recognition, particularly colonially nesting birds who might otherwise mislay eggs or misdirect incubation and nest defense behaviors (Brown 1984, Birkhead 2021, Quach et al. 2021; reviewed in Underwood and Seely 2002).

Although low intraclutch variation can be beneficial to recognize the eggs of brood parasites or serve as an identity signal in some other context, variation within clutches can also have functional consequences. Egg size can change according to lay order; the brood survival hypothesis suggests that, due to hatching asynchrony, females may lay a larger last egg to increase the chances of nestling survival and provide more nutrients to eggs with the least incubation time (Slagsvold et al. 1984). Alternatively, the brood reduction hypothesis states that females may lay larger clutches than they can potentially provide high-quality care for, given that food availability can fluctuate over the time between laying and nestling parental care (Slagsvold et al. 1984). Therefore, any size hierarchy resulting from a more poorly provisioned, smaller, last-laid and last-hatching egg results in adaptive brood reduction if sufficient resources do not exist (Lack 1954, Slagsvold et al. 1984). There is mixed support for these 2 hypotheses (e.g., Amat et al. 2001, Dolenec 2004, Lifjeld et al. 2005, Gibson and Williams 2017), as fewer studies investigate how multiple aspects of egg phenotype change across the laying sequence.

Intraclutch variation in maculation may also be influenced by the order in which eggs are laid (e.g., López de Hierro and de Neve 2010, Hargitai et al. 2013, Poláček et al. 2017). A potential proximate explanation is that eggshell maculation may vary according to the calcium available to females across the laying sequence. Protoporphyrin IX, the molecule responsible for reddish-brown maculation, shares the same carrier protein as calcium (Kennedy and Vevers 1976, Solomon 1997). As a result, increased pigment may be deposited in calcium-depleted regions of the eggshell, and provide structural reinforcement to weaker portions of the eggshell (Gosler et al. 2011, Hargitai et al. 2013). This would generate a pattern of more pigment deposited in later-laid eggs if females become more calcium limited toward the end of the laying sequence (e.g., Gosler et al. 2005, De Coster et al. 2013). However, there is also evidence for pigment limitation or depletion throughout the laying sequence, producing patterns where later- or last-laid eggs are less maculated than the rest of the clutch

TABLE 1. Repeatability estimates in egg size, shape, and maculation from a variety of avian species, focused on studies that report within-clutch repeatability for more than 1 egg measure. *Indicates a range of *R* values across years and/or sites.

	Repeatability (*R* ± SE or CI if provided) of egg size, shape, and maculation					
Species	Length	Breadth	Volume (*V*), area (*A*), mass	Shape	Maculation	Reference
Branta bernicla			*V*: 0.51			Flint et al. (2001)
Anser canagicus	0.59 (0.42)	0.69 (0.24)	*V*: 0.62 (0.37) *A*: 0.57 (0.47)	Elongation: 0.73 (0.17) Sphericity: 0.73 (0.19)		Petersen (1992)
Fulica atra	0.67 (0.57–0.74)	0.68 (0.60–0.75)	*V*: 0.70 (0.61–0.77) *A*: 0.70 (0.62–0.76)		No. spots: 0.65 (0.56–0.72) Area spots: 0.61 (0.51–0.69) Spot size: 0.43 (0.33–0.52)	Gómez et al. (2021)
Chroicocephalus ridibundus	0.42–0.67*	0.45–0.64*	*V*: 0.37–0.66*			Bańbura and Zielińksi (1990)
Himantopus himantopus	0.63 (0.04)	0.49 (0.04)	*V*: 0.60 (0.04) *M*: 0.77 (0.02)			Adamou et al. (2009)
Vanellus vanellus	0.53 (0.06)	0.58 (0.05)	*V*: 0.58 (0.05)			Grønstøl (1997)
Charadrius semipalmatus	0.72	0.68	*V*: 0.67 *M*: 0.68			Nol et al. (1997)
Vanellus spinosus	0.60 (0.45–0.82)	0.72 (0.60–0.88)	*V*: 0.73 (0.62–0.89)	Elongation: 0.56 (0.40–0.80)		Ciach (2011)
Anthus rubescens	0.70–0.80*	0.57–0.73*	*V*: 0.60–0.82*	Elongation: 0.61–0.79*		Hendricks (1991)
Hirundo rustica	0.62 (0.09)	0.77 (0.06)	*V*: 0.71 (0.07)			Bańbura and Zielińksi (1998)
Troglodytes aedon			*V* early season: 0.60 (0.04); *V* late season: 0.69 (0.06) *M* early season: 0.69–0.74*; *M* late season: 0.65–0.73*			Styrsky et al. (2002)
Panurus biarmicus	0.50 (0.41–0.60)	0.48 (0.39–0.58)	*V*: 0.50 (0.41–0.60	Elongation: 0.47 (0.38–0.57)		Surmacki et al. (2003)
Cyanistes caeruleus	0.57–0.83*	0.42–0.77*	*V*: 0.25–0.67*			Chabi et al. (2000)
Cyanistes caeruleus			*V*: 0.78 (0.03)	Sphericity: 0.71 (0.03)		Bańbura et al. (2018)
Cyanistes caeruleus	0.83 (0.02)	0.91 (0.01)	*V*: 0.91 (0.01)	Sphericity: 0.66 (0.03)		Kouidri et al. (2015)
Ficedula hypoleuca	0.72	0.60	*V*: 0.62			Potti et al. (2008)
Lanius collurio	0.53 (0.48–0.59)	0.51 (0.46–0.56)	*V*: 0.55 (0.49–0.60)			Golawski and Mitrus (2018)
Cercotrichas galactotes	0.38 (0.06)	0.63 (0.05)	*V*: 0.63 (0.05) *M*: 0.71 (0.04	Sphericity: 0.40 (0.06)		Adamou et al. (2018)
Passerculus sandwichensis	0.79 (0.04)	0.74 (0.05)	*V*: 0.78 (0.04)	Sphericity: 0.72 (0.05)	Spotting: 0.46 (0.07)	Wheelwright et al. (2012)

(Nice 1937, Lowther 1988, López de Hierro and de Neve 2010). Distinctive last-laid eggs may have adaptive value and serve as a signal to brood parasites that the clutch is complete and incubation has already begun (Yom-Tov 1980). However, it is possible that increased variation in egg phenotype within clutches due to lay order (or other sources) might mean that eggs are less useful as identity signals, especially if that variation is spread across the laying sequence rather than restricted to 1 egg that differs in appearance.

Eggs laid by the same female can also vary in size, shape, and maculation between breeding attempts. Because of the challenge of following females of known identity across multiple breeding attempts, we know less about how egg phenotype differs between subsequent clutches, during the same breeding season or between consecutive years (but see Styrsky et al. 2002, Sanz and García-Navas 2009, Honza et al. 2012, Wheelwright et al. 2012, Birkhead et al. 2021). Differences in egg characteristics between multiple clutches laid by the same female could further our understanding of eggs as identity signals and indicate the degree of heritable variation, as repeatability in phenotype serves as an upper limit for heritability estimates.

In this study, we investigated sources of interclutch and intraclutch variation in the size, sh ape, and maculation of North American Barn Swallow (*Hirundo rustica erythrogaster*) eggs. North American Barn Swallows are not subject to interspecific brood parasitism (Kilner 2006); however, intraspecific brood parasitism has been observed in European populations (*H. r. rustica*) (Møller et al. 1987, Czechowski and Zduniak 2005), and confirmed via extra-pair maternity (Petrželková et al. 2015). Furthermore, due to high nesting density breeding colonies of many North American Barn Swallows, females have been observed mislaying eggs in nests other than their own (Turbek et al. 2019). Both intraspecific brood parasitism and high-density breeding colonies may lead to decreased intraclutch variation in Barn Swallow eggs.

We predicted that the majority of the variation in egg size, shape, and maculation would be between clutches laid by different females rather than within those clutches. Furthermore, we predicted that aspects of egg phenotype would be highly repeatable, including between different sides of the same egg, and, based on previously reported egg repeatability values (Table 1), we expected that measures of size and shape would be more repeatable than maculation. We predicted that egg size, shape, and maculation would also be repeatable between first and replacement clutches laid by the same female. Finally, we examined whether lay order explained any of the variation within clutches in egg size, shape, and maculation. We predicted that egg size would increase with lay order to compensate for hatching asynchrony, as has often been found in open nesting passerine birds (Slagsvold 1984). Although less is known about how maculation changes with lay order, we predicted that the last-laid egg in a clutch would differ in maculation from the rest of the clutch, but made no a priori prediction about whether it would be more or less maculated.

METHODS

Study System

North American Barn Swallows are migratory aerial insectivores that are often found breeding in colonies inside structures such as barns or under the eaves of buildings. In most of the eastern USA, Barn Swallows breed between April and August, raising 2 or more broods of offspring (Brown and Brown 1999). Clutch sizes are typically 3–6 eggs, with females laying 1 egg per day (Brown and Brown 2020). The eggs are ovate or elliptical ovate, with a pale white ground color, and small darker brown spots (maculation), which tend to be more highly concentrated around the crown of the egg (corona ring), and are typically more sparsely spotted toward the pointed end.

Study Sites

We studied Barn Swallows breeding at 15 different sites in 2 geographic locations, Georgia (7 sites, 2018–2019) and Ohio (8 sites, 2019–2021). In Georgia, birds were studied at 1 farm near Danielsville (34°15.25′N, 83°19.32′W) and several pairs breeding in a rural residential community, Serenbe (33°33.04′N, 84°42.52′W). Study populations in Ohio included farms located in rural, agricultural areas, including 1 large colony near Fredericktown (40°27.39′N, 82°38.24′W), another large colony near Gambier (40°20.26′N, 82°22.47′W), and several smaller colonies within 5 miles of the Gambier site. The majority of the eggs (84%) in our sample come from Ohio.

Egg Photographs

Eggs (n = 705) were digitally photographed at study sites following clutch completion and returned to their nests. In Georgia, first clutches were photographed in May (2019), and second clutches in June and July (2018). In Ohio, first clutches were photographed in May and June (2020, 2021), and second clutches in June and July (2019, 2021). In 2021, eggs (n = 54) at 1 site were collected from 12 nests to initiate replacement clutches (IACUC approval from Kenyon College; Ohio scientific collections permit 23-134), and first and replacement eggs were photographed. These eggs were collected close to hatching for an unrelated study that required synchronization of breeding. Therefore, relaying of replacement clutches generally occurred 2–3 weeks after the first-clutch eggs were laid. A subset of eggs (n = 74, 1 site in 2021) from 16 nests were photographed on one side and then rotated 180° and photographed on the opposite side to analyze similarity of maculation patterns on different sides of the egg. Additionally, 191 eggs from 43 nests at 1 site in 2020 were marked each day with a small number in permanent marker (black ultrafine point Sharpie) to establish a record of lay order. These nests were checked daily between 0800 and 1100 hours using a mirror on the end of an extendable pole to identify and mark newly laid eggs. In all cases, a Nikon D3300 camera was used to photograph eggs (32-mm lens, shutter speed: 1/60 s, Aperture: F5, ISO: 200, all photos shot in RAW format using the flash). An 18% reflectance gray card and ruler were used to standardize for light and scale (but not for color, which we did not quantify here). Fieldwork was done in accordance with the Ornithological Council's Guidelines for the Use of Wild Birds in Research (Fair et al. 2010).

Quantifying Aspects of Eggshell Phenotype

SpotEgg is an image processing program that provides detailed information about egg phenotype including egg volume, area, length, breadth, number of spots, and total area of spots, among other measures which were not analyzed here, including color variables (Gómez and Liñán-Cembrano 2017). We used the breadth and length data to calculate egg sphericity (breadth/length) as a proxy of egg shape (Hoyt 1976). To obtain these egg measures using *SpotEgg*, we used a linearization process in which the program uses areas of known pixel values and known scale to extract the most detail from areas in the egg image. The image then goes through normalization, in which an area of known reflectance is selected in order to scale pixel values, verifying the overall reflectance value for the image (Gómez and Liñán-Cembrano 2017). After linearization and normalization, *SpotEgg* uses Region of Interest definition to define the shape and size of the egg for analysis. To do this, the egg was first manually outlined before the program generated a polygon matching the edge of each egg. Lastly, we used the spot detection function to count the number of spots and calculate the total area of the eggshell covered in spots (Gómez and Liñán-Cembrano 2017). We obtained the number of spots using either *SpotEgg*'s automatic spot detection (where only minimum spot size is specified) or by specifying parameters for the radius filter, minimum spot size, sensitivity, and background fill threshold, evaluating the performance of 5 different spot detection configurations for every egg (see Supplementary Material for details). The detection results from each configuration file were then visually inspected and compared for every egg, and the most accurate configuration file was selected. File selection was based on accuracy of spot detection, which was indicated on an output image (detected spots outlined; Supplementary Material Figures 1 and 2). High accuracy meant that the program did not substantially over or under detect spots on the eggshell.

Quantifying Variation in Maculation Pattern

NaturePatternMatch is a pattern recognition and quantification tool that uses scale-invariant-feature-transform (SIFT) to identify and recognize significant features of egg maculation patterns (Stoddard et al. 2014). *NaturePatternMatch* uses a model of vertebrate vision that imitates neural responses in the brain responsible for object recognition, to assess pattern recognizability in bird eggs. We used *NaturePatternMatch* to compare similarity in maculation pattern between first and replacement clutches and across the laying sequence. Prior to any analysis with *NaturePatternMatch*, we used the photo editor, GIMP, to outline the egg and remove the background, as any shadow from the egg could interfere with maculation pattern characterization. Because *NaturePatternMatch* was not used to quantify egg dimensions, this photo manipulation process had no influence on our measures of egg size and shape. The program generates similarity scores between all eggs in the dataset, and then uses this matrix to quantify how well each egg matches back to eggs from every nest in the dataset, producing a ranked list of nests. We processed the images using a Gaussian filter to generate SIFT features that determine the specific signature of each egg's maculation. After *NaturePaternMatch* had generated SIFT features for each egg, all images were compared to each other, to generate similarity ranks between all eggs. Examining differences in maculation pattern complements the detailed description of eggshell phenotype obtained via *SpotEgg*, as different patterns can be formed from similar number of spots (Supplementary Material Figure 3).

Statistical Analyses

Depending on the distribution of the data, we used analysis of variance (ANOVA) or Kruskal–Wallis tests to evaluate variance partitioning for egg size, shape, and maculation measures from *SpotEgg* within and between nests. Number of spots and average spot size were not normally distributed and required a nonparametric approach. We tested whether the measures of egg size, shape, and maculation within and between (first vs. replacement) clutches were repeatable, as well as the repeatability of different sides of the same egg, using the R package *rptR* (Stoffel et al. 2017). Repeatable measures within the same clutch, for different sides of the egg, and between first and replacement clutch means were identified by comparison to a null distribution of 2,000 permuted samples, and 95% confidence intervals (CIs) were estimated using 2,000 bootstrap samples. A Poisson distribution was specified for repeatability of the number of spots and average spot size instead of the Gaussian distribution used for other measures. Except for the 12 nests where we collected eggs and compared first vs. replacement clutch eggs, we did not know the identity of the female. In some cases, we photographed eggs at the same site in consecutive years, which could have resulted in an estimated 29 clutches laid by the same female. This estimation is based on the number of nests photographed at the same sites across years (n = 8 in Georgia, n = 49 in Ohio) and the 50% return rate of adults in our populations. To ensure that any potential pseudoreplication did not affect our results, we ran the ANOVAs or Kruskal–Wallis tests and repeatability analyses on a reduced dataset (454 eggs) that only included data from any 1 site in 1 year.

While we do not have sufficient geographic sampling to formally ask whether Barn Swallow eggs differ by location, we did investigate whether egg phenotype differed between Georgia and Ohio to account for the fact that we combined these samples together in repeatability analysis. We used the measures generated by *SpotEgg* (area, volume, width, length, sphericity, number of spots, total area of the eggshell spotted, and average spot size) as response variables in linear mixed models with location as a fixed effect and nest ID as a random effect. Linear mixed models were run using the R packages *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017) and residuals were checked for normality and homoscedasticity. Site was not included in these models as nests were all unique to location and sites in Georgia were sparsely sampled. However, we did investigate whether there were site-level differences in eggshell phenotype in Ohio where the bulk of the data were collected. Here we fitted a linear mixed model for each of the same *SpotEgg* variables listed above with a random effect of nest ID nested within site.

NaturePatternMatch ranks were used in conjunction with *SpotEgg* repeatability measures to analyze if maculation characteristics differed on 2 sides of the same egg. We used a chi-squared test to calculate the frequency in which the patterns of different sides of the same egg matched back to each other rather than to other eggs in the dataset (n = 73, each with photos of 2 sides).

To examine the effects of lay order on egg size, shape, and maculation measures obtained from *SpotEgg*, we analyzed a dataset restricted to 5-egg clutches (the most common size, n = 110 eggs from 22 clutches) with known lay order, and used repeated-measures ANOVAs or Friedman tests to ask how position in the lay order influenced the egg phenotype. *NaturePatternMatch* ranks were used in a complementary way, asking how well first, middle, or last eggs matched back to other eggs in the clutch, using data from clutches of 3–6 eggs. We analyzed the *NaturePatternMatch* output using sign tests, comparing first vs. middle eggs, last vs. middle eggs, and last vs. first eggs. Middle eggs used the average rank of the second, third, fourth, and fifth eggs in cases of 6-egg clutches, and fewer in cases of smaller clutches. Unless specified, values reported are means ± standard error (SE). An alpha value of 0.05 was used to determine significance. All statistical analyses were completed using R 4.1.1 (R Core Team 2021).

RESULTS

Descriptive Statistics for Eggshell Phenotype

Eggs varied substantially in size, shape, and maculation characteristics (Table 2). In this sample of 705 eggs, there was a wide range in egg volume and length; the egg with the greatest volume was 73% larger than the egg with the smallest volume, with a mean volume of 2,035.80 ± 185.97 mm^3. Mean egg length was 19.92 ± 0.99 mm, and the longest egg was 37% longer than the shortest egg. Eggs also ranged in sphericity with the general shape being ovate (mean sphericity = 0.72 ± 0.04); however, some eggs approached round (maximum sphericity = 0.85, 1 is perfectly spherical), while others were narrow for their length (minimum sphericity = 0.61). The number of spots on the eggshells was the most variable characteristic measured, with a minimum of 6 spots, a maximum of 502 spots, and a median of 152 spots.

Geographic and Site-Level Variation in Eggshell Phenotype

Eggs from Ohio (n = 621) were more ovate ($\beta = -0.03 \pm 0.01$, $P < 0.001$), had fewer spots ($\beta = -60.15 \pm 17.17$, $P < 0.001$), and larger spots ($\beta = 0.03 \pm 0.02$, $P = 0.03$) than eggs from Georgia (n = 84). Within Ohio, there were minimal to no site effects on any measure of egg phenotype; site explained no proportion of variation in all but 2 measures of eggshell phenotype (number of spots, total area of eggshell spotted), where the proportion of the variation explained was 3.07% and 2.73%, respectively.

Interclutch Variation in Eggshell Phenotype

Most of the variation in egg characteristics occurred between rather than within clutches. ANOVAs/Kruskal–Wallis tests were highly significant for all variables (Table 2). The findings were very similar using the reduced dataset where there was no possibility of inclusion of eggs laid by the same female.

Intraclutch Variation in Eggshell Phenotype

We found high intraclutch repeatability for all egg size, shape, and maculation characteristics across 705 eggs from 139 nests (Table 2). Females laid eggs that were highly consistent in size and shape characteristics, with sphericity ($R = 0.77 \pm 0.03$) and length ($R = 0.72 \pm 0.03$) being the most repeatable variables. Maculation was also repeatable within clutches; however, some measures were less consistent within clutch than size and shape characteristics, as the average size of spots ($R = 0.25 \pm 0.08$) and total area spotted ($R = 0.57 \pm 0.03$) was the least repeatable aspects of egg phenotype (Table 2). In contrast, the number of spots was as repeatable within the clutch ($R = 0.71 \pm 0.03$) as our egg size measures. Repeatability values were very similar when calculated from the reduced dataset (n = 454 eggs)

TABLE 2. Descriptive statistics, repeatability (*R*), and ANOVA/Kruskal–Wallis results for Barn Swallow egg size, shape, and maculation measures. All within-clutch measures are highly repeatable (all $P < 0.001$ by permutation); asterisk (*) indicates significant repeatability between first and replacement clutches. Majority of the variation in these measures is found between rather than within nests (all ANOVA/Kruskal–Wallis tests, df = 158, $P < 0.001$).

Egg measure	Mean (SD)/ median (IQR) ($n = 705$ eggs)	Range ($n = 705$ eggs)	*R* within clutches (SE) ($n = 705$ eggs)	*R* between clutches (SE) ($n = 104$ eggs)	*F*/KW χ^2 ($n = 705$ eggs)
Length (mm)	19.94 (0.99)	16.91–23.16	0.72 (0.03)	0.43 (0.22)	12.71
Breadth (mm)	14.27 (0.50)	12.73–15.92	0.69 (0.03)	0.33 (0.22)	10.80
Volume (mm^3)	2035.80 (185.99)	1513.61–2630.18	0.67 (0.03)	0.22 (0.21)	9.79
Sphericity (*B/L*)	0.72 (0.04)	0.61–0.85	0.77 (0.02)	0.48 (0.22)*	16.16
Number of spots	152 (154)	6–502	0.71 (0.03)	0.44 (0.22)	546.02
Total area spotted (%)	23.82 (7.78)	2.80–51.32	0.57 (0.04)	0.46 (0.22)*	6.66
Average spot size (%)	0.14 (0.09)	0.05–0.88	0.25 (0.08)	0.56 (0.21)*	551.03

that excluded any eggs that could be laid by a female already represented in the dataset, with *R* values increasing or decreasing by a maximum of 0.04. The reduced dataset included 454 eggs from 82 nests, and excluded any possibility than a female's eggs could have been included in this analysis more than once.

Egg maculation characteristics (e.g., number of spots) and the patterns they formed were similar between opposite sides of the same egg when compared to maculation characteristics of other eggs. Number of spots on the egg and the total area of spots were highly repeatable on both sides of the egg ($R = 0.89 \pm 0.02$, $R = 0.91 \pm 0.02$, respectively). However, average spot size was not repeatable on opposite sides of the same egg ($R = 0.11 \pm 0.15$). Using *NaturePatternMatch*, we found that eggs matched back to a corresponding side of the same egg at a higher rate than they did to other eggs in the dataset ($\chi^2 = 67.343$, df = 1, $P < 0.001$, $n = 148$).

Differences in Eggshell Phenotype Between First and Replacement Clutches

In the subset of 104 eggs from 24 (12 first, 12 replacement) nests where we could compare eggshell phenotype between a female's first and replacement clutches, we found that females laid eggs with more variation in size, shape, and maculation between breeding attempts compared to eggs within the same breeding attempt (Supplementary Material Figure 4). Mean values of egg characteristics for first and replacement clutches laid by the same female were less repeatable than intraclutch repeatability within the same breeding attempt, with only shape (sphericity), total area of the eggshell spotted, and average spot size significantly repeatable (Table 2). Of all the variables analyzed, the volume of eggs was the least repeatable between clutches laid by individual females ($R = 0.22 \pm 0.21$). In general, eggs from first clutches were wider than those from replacement clutches (paired *t*-test: $t = 2.77$, df = 46, $P = 0.01$, $n = 104$ eggs from 24 clutches), which meant that replacement clutch eggs were more ovate compared to first clutches (paired *t*-test: $t = 4.32$, df = 46, $P < 0.001$, $n = 104$ eggs from 24 clutches; Figure 1). Comparisons of egg maculation patterns using *NaturePatternMatch* revealed that patterns of maculation differed between first and replacement clutches (sign test, $S = 31$, $P < 0.001$, $n = 104$). A female's eggs were no more likely to match an egg from her other clutch than an egg laid by a different female.

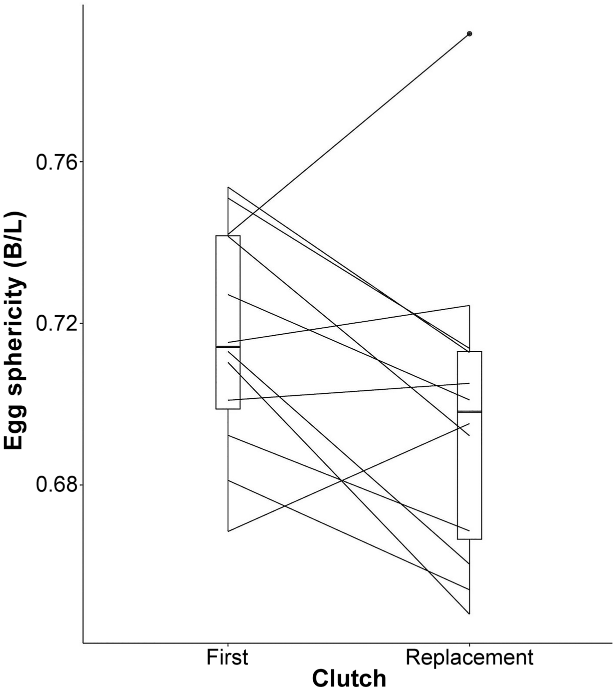

FIGURE 1. Paired mean egg sphericity (breadth/length) from first vs. replacement clutch eggs for 12 female Barn Swallows. On average, replacement clutch eggs were less spherical and therefore more ovate compared to first-clutch eggs (paired *t*-test: $t = 4.32$, df = 46, $P < 0.001$, $n = 104$ eggs from 24 clutches).

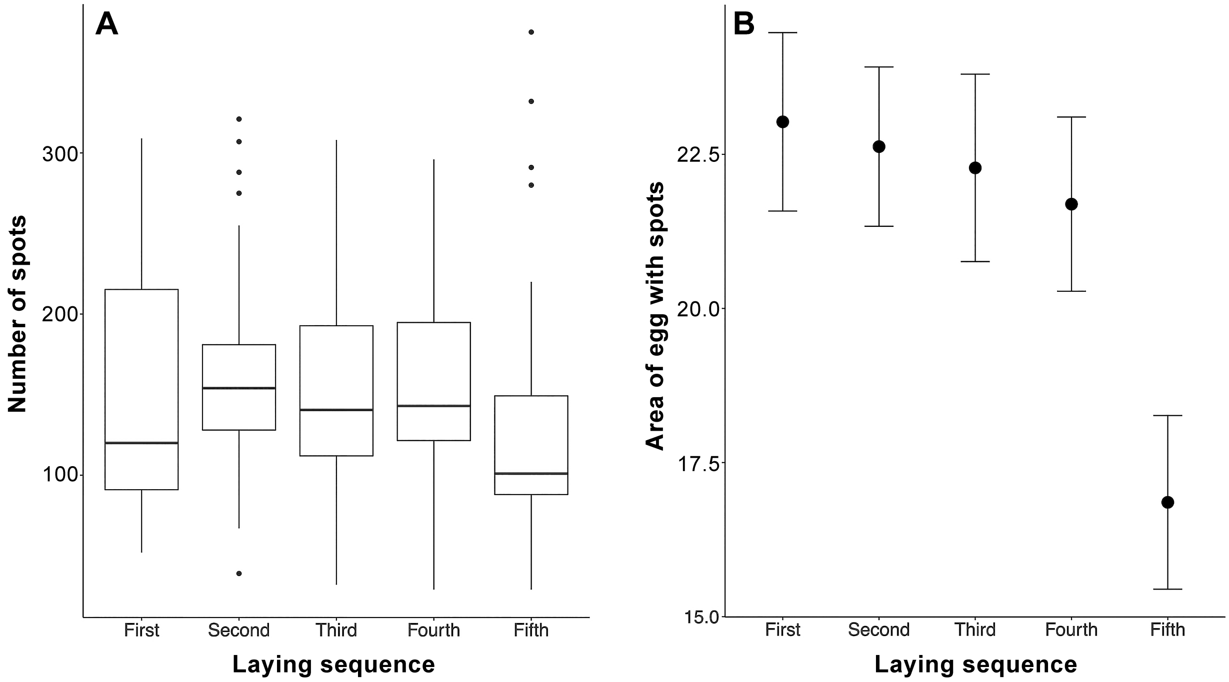

FIGURE 2. (A) Number of spots on eggs across the laying sequence. Last-laid eggs had fewer spots than earlier-laid eggs (Friedman test: $\chi^2 = 12.27$, df = 4, $P = 0.02$, $n = 22$ eggs from 22 clutches). Plot shows medians (line) and quartiles (boxes). (B) Percent of eggshell covered in spots across the laying sequence. Last-laid eggs we less maculated than earlier-laid eggs (repeated-measures ANOVA: $F = 6.20$, df = 4, $P = 0.002$, $n = 110$ eggs from 22 clutches). Plot shows means and standard errors.

Intraclutch Variation in Eggshell Phenotype Due to Lay Order

Lay order accounted for some of the intraclutch variation in number of spots (Friedman test: $\chi^2 = 12.273$, df = 4, $P = 0.02$, $n = 110$ eggs; Figure 2A), and within clutches, last-laid eggs had a lower total area of the eggshell covered in spots (repeated-measures ANOVA: $F = 6.20$, df = 4, $P < 0.001$, $n = 110$ eggs; Figure 2B). *NaturePatternMatch* analysis of eggs with known lay order indicated that specific maculation patterns also varied consistently with lay order. Maculation of last-laid eggs was distinctive, and was less likely to match back to the rest of the clutch when compared to the patterns of first- and middle-laid eggs (sign test, $S = 23$, $P = 0.02$, $n = 33$ nests). First-laid eggs, however, were not different in maculation from the rest of the clutch (sign test, $S = 14$, $P = 0.85$, $n = 33$ nests), and we did not find any pattern in size or shape variation across the laying sequence.

DISCUSSION

The high intraclutch repeatability of egg size, shape, and maculation measures suggests that Barn Swallow eggs could be effective identity signals. However, we found greater variation in egg phenotype between first and replacement clutches laid by the same female than within either of those clutches, which could diminish the identity signal value if females do not also adjust any recognition accordingly. Some intraclutch variation in maculation was related to lay order; the last-laid egg tended to have a distinctive maculation pattern, and less of the eggshell was covered in spots compared to eggs laid earlier in the sequence. Because only the last egg differed, the clutches could still bear high identity information, and we suggest alternative signaling value of the differentially maculated, last-laid egg.

We found that the laying sequence of eggs contributed to some of the intraclutch variation in maculation measures (e.g., number of spots) and maculation patterns, which have been examined less frequently in this context. Last-laid eggs were significantly different from the rest of the clutch in maculation pattern; they had fewer spots and thus less of the eggshell surface maculated. Notably, we did not find that egg size or shape changed predictably with lay order, which is in contrast to the more commonly reported trend of eggs increasing in size across the laying sequence (e.g., Dolenec 2004, Lifjeld et al. 2005, Orłowski et al. 2016; but see Gibson and Williams 2017). Distinctive maculation patterns on last-laid eggs have been observed in other species where last-laid eggs are often paler in color or have less pigmentation than the rest of the clutch (Lowther 1988, López de Hierro and de Neve 2010, Huo et al. 2018). Yom-Tov (1980) suggests that conspicuous last-laid eggs may signal to brood parasites that the clutch is complete, and therefore deter parasites from laying eggs in a clutch that has already begun incubation. Although Barn Swallows are not parasitized by other species, laying distinctive last-laid eggs could serve a similar purpose in signaling clutch completion to intraspecific brood parasites, which have been found in European Barn Swallow colonies (Møller et al. 1987, Czechowski and Zduniak 2005, Petrželková et al. 2015).

Poláček et al. (2017) found that females laid significantly paler last-laid eggs and placed darker eggs in central incubation positions, indicating that laying eggs with distinctive coloration and maculation may be a way females recognize higher-quality eggs and place them in priority incubation positions. Female Barn Swallows may lay less maculated last eggs due to a decrease in the availability of protoporphyrin IX across the laying sequence. López de Hierro and de Neve (2010) observed a pattern of reduced maculation in later-laid eggs, and proposed that this result may be related to short-term limitation of protoporphyrin IX. It is unclear whether pigment limitation could result in less maculation on the last-laid egg as we found rather than a gradual decline in maculation over the laying sequence.

Within clutches, egg size and shape measures were highly repeatable, with shape (sphericity) being the most repeatable variable examined. Similarly high repeatability has been observed in egg size variables (Valkama et al. 2002, Svagelj and Quintana 2011) including breadth (Nol et al. 1997), volume, and mass (Styrsky et al. 2002) among a wide range of species (Table 1). High repeatability in egg shape is consistent with numerous findings that egg size and shape characteristics tend to be strongly related to female identity. Wheelwright et al. (2012) found that similarity in size and shape of eggs laid by individual females suggests high heritability of these egg traits. However, as noted by Wheelwright et al. (2012) and Styrsky et al. (2002), some of the decreased intraclutch variation in egg size and shape may also result from consistent environmental conditions—including food availability—during laying. Previous work has found greater variation between rather than within breeding seasons across multiple years, indicating that local ecological dynamics may influence egg phenotype (Honza et al. 2012). Additional work is needed to further understand the degree of plasticity in egg phenotype and the relative role of genetic vs. environmental constraints.

We found consistently high intraclutch repeatability of all maculation variables, which corroborates previous findings that female Barn Swallows show decreased intraclutch variation in maculation (scored by eye) when compared to interclutch variation within the same females (Brown and Sherman 1989). With the exception of average spot size, maculation patterns and spotting measures were also similar on different sides of the same egg, indicating that utilizing 1 photograph of a random side of an egg is a reliable method for characterizing maculation. Far fewer studies have looked at repeatability of eggshell maculation. Of the studies that do, we observed similar repeatability of the number of spots, and total area of spots on eggs to eggs from Eurasian Coots (*Fulica atra*) (Gómez et al. 2021; Table 1), but greater repeatability than the total area of spots on eggs laid in a population of House Wrens (*Troglodytes aedon*) (Hodges et al. 2020). We found that the average spot size of eggs in our study was less repeatable than in the work of Gómez et al. (2021); however, in both studies, average spot size was the least repeatable measure of egg phenotype. High repeatability of maculation characteristics may provide further support for the signature hypothesis (Swynnerton 1918). Decreased intraclutch variation in maculation within other species has been previously suggested as a mechanism for females to recognize their own eggs and eject eggs laid by both interspecific and intraspecific brood parasites (e.g., Pike 2011, Hauber et al. 2019, Gómez et al. 2021). Although North American Barn Swallows are not a host species for interspecific brood parasites, their high-density nesting colonies contribute to the 8% incidence of mislaid eggs (Turbek et al. 2019). In general, we found higher repeatability values than other studies examining maculation variables (Wheelwright et al. 2012, Hargitai et al. 2016, Hodges et al. 2020), and high repeatability in maculation pattern is a prerequisite for egg recognition and discrimination. However, whether Barn Swallows can recognize their own eggs is still unknown, and further experiments with model eggs (e.g., Hauber et al. 2021) are necessary to begin to understand whether the low intraclutch variation in egg phenotype aids in egg and clutch recognition.

Females in this study laid eggs with more variation between first and replacement clutches than within their individual breeding attempts. Inconsistency of egg phenotype between subsequent clutches suggests that some variation in Barn Swallow eggs is likely related to local environmental conditions and/or changes in female physiological state between the 2 bouts of laying. We observed that all egg size, shape, and maculation variables were less repeatable between subsequent clutches compared within breeding attempts, which is consistent with the work of Grønstøl (1997), in which egg volume and length were less repeatable between rather than within clutches laid by the same female. Interestingly, Grønstøl (1997) found that egg breadth was repeatable between first and replacement clutches, which is not consistent with our findings. Other studies have found consistently high repeatability of egg size and shape measures between subsequent clutches, both within and between breeding seasons (Styrsky et al. 2002, Wheelwright et al. 2012), which would suggest that egg size and shape characteristics are likely heritable (Christians 2002), and that less of the variation is due to environmental conditions. Alternatively, because of the tendency for birds to be breeding site philopatric, similarity of eggs across multiple breeding seasons could reflect similar environmental conditions within the same breeding location, as factors such as diet (e.g., Karell et al. 2008, Hargitai et al. 2013, Duval et al. 2016) and temperature (Bańbura and Zieliński 1995) have previously been found to affect egg characteristics. Fewer studies have examined variation in maculation between clutches laid by the same female. Prior work in Eurasian Barn Swallows found that females laid eggs with consistent maculation patterns between breeding seasons (Corti et al. 2018). However, Corti et al. (2018) also found that eggs varied in the intensity and size of spots between breeding locations, indicating that some variation in maculation may in fact be related to local conditions at breeding sites.

We found that eggs in the replacement clutch were on average more narrow and therefore more ovate in comparison to eggs laid by those same females during the first clutch. The overall pattern of decreased egg sphericity may be related to seasonal changes in environmental conditions across the breeding season. Barn Swallows are aerial insectivores, and variation in temperature and precipitation between first and replacement clutch timing may indirectly affect female nutritional status by influencing insect abundance. For example, food-supplemented Pied Flycatchers (*Ficedula hypoleuca*) laid heavier and more intensely blue-green eggs compared to control females (Moreno et al. 2006). In contrast to our results, Lifjeld et al. (2005) found that egg size increased in replacement clutches; however, this work was conducted in a subalpine region where temperature and food abundance increased between the 2 bouts of laying. We need more long-term studies to understand how eggs laid by the same female vary within and between breeding seasons, as findings so far have been mixed.

The high intraclutch repeatability of egg size and shape characteristics we found in this study is consistent with other published accounts, indicating that most of the variation in birds' eggs exists between, rather than within clutches. However, it is more challenging to contextualize our eggshell maculation findings, as there is a clear need for more standardized methods to compare maculation measures. Researchers utilize several different methods (e.g., principal component analysis (PCA), scoring by eye, spectroscopy, different methods for spot detection or maculation description), which each have varying degrees of accuracy in quantifying eggshell traits (Wegmann et al. 2015). We observed that egg size, shape, and maculation characteristics were rarely consistent between first and replacement clutches, indicating a need for experimental work to identify the extent to which this variation in egg phenotype is heritable. Some of our analyses (between clutches laid by the same females, lay order effects) are based on smaller sample sizes and therefore the results must be interpreted with caution. Finally, future work is needed to clearly understand whether and to what degree North American Barn Swallows are affected by intraspecific brood parasitism. High rates of extra-pair maternity have been observed in European populations of Barn Swallows (Petrželková et al. 2015), suggesting that intraspecific brood parasitism may play a greater role in shaping egg maculation characteristics than previously understood, but experimental egg recognition studies are still needed.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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Author contributions: I.I.L., T.T., E.B.S., and A.-R.F.B. formulated the questions; I.I.L., T.T., E.B.S., M.C.S., and Y.L. collected the data; E.B.S., B.W.B., A.-R.F.B., M.C.S., and Y.L. did the photo analysis; A.-R.F.B. and I.I.L. analyzed the data; and A.-R.F.B. wrote the paper with input from I.I.L and T.T.

Data deposits: Analyses reported in this article can be reproduced using the data provided by Beech et al. (2022).

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