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Rozprawa doktorska

Rola pigmentacji skorupek jaj w doborze płciowym gąsiorka *Lanius collurio*

Klaudia Szala

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PhD thesis

The function of eggshell pigmentation in the sexual selection of the red-backed shrike *Lanius collurio*

Klaudia Szala

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Rozprawa doktorska została wykonana w Zakładzie Biologii i Ekologii Ptaków, w Instytucie Biologii Środowiska, na Wydziale Biologii Uniwersytetu im. Adama Mickiewicza w Poznaniu.

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Zmienność wyglądu jaj gąsiorka *Lanius collurio*. Rycina przedstawia po jednym jaju z każdego lęgu sfotografowanego w trakcie badań. Fot. Klaudia Szala.

The variability of appearance of eggs of the red-backed shrike *Lanius collurio*. The figure shows one egg from every clutch photographed during this study. Photo: Klaudia Szala.

There is probably no one quality of natural objects from which we derive so much pure and intellectual enjoyment as from their colors. The "heavenly" blue of the firmament, the glowing tints of sunset, the exquisite purity of the snowy mountains, and the endless shades of green presented by the verdure-clad surface of the earth, are a never-failing source of pleasure to all who enjoy the inestimable gift of sight.

– Alfred Russel Wallace (1877), *The colors of animals and plants*, The American Naturalist

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Podziękowania | Acknowledgements

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A list of other papers that I co-authored during my PhD:

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Gruszka D., Kaczmarek J.M., **Szala K.**, Kaczmarski M. (2024) Estimation of green toad *Bufotes viridis* population size based on photo-identification at two urban sites with different management histories. North-Western Journal of Zoology 20(1): 50-57.

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Szala K., Dylewski Ł., Tobółka M. (2020) Winter habitat selection of Corvids in an urban ecosystem. Urban Ecosystems 23: 483–493.

Kaczmarski M., **Szala K.** (2020) Shift in the timing of mating period of the green toad *Bufotes viridis* – a case study from Cytadela city park in Poznań. Przegląd Przyrodniczy 31 (4): 83-88 [in Polish with English summary].

Streszczenie

Skorupki jaj ptaków charakteryzują się dużą zmiennością wyglądu. Różne gatunki różnią się między sobą jasnością i kolorem skorupki oraz obecnością, ilością, kolorem i rozmieszczeniem plamkowania. Na przestrzeni lat narodziło się wiele hipotez dotyczących funkcji ubarwienia skorupek jaj. Jedną z nowszych hipotez jest zaproponowana w 2003 roku hipoteza SSEC (ang. *sexually selected eggshell coloration hypothesis*) o roli pigmentacji w po-kopulacyjnym doborze płciowym ptaków. Zgodnie z nią pigmentacja skorupek jaj jest sygnałem kondycji samicy lub sygnałem wielkości jej inwestycji w jaja w po-kopulacyjnym doborze płciowym, który wpływa na wielkość inwestycji rodzicielskiej samca. W przypadku biliwerdyny, barwnika odpowiedzialnego za niebieskozielone zabarwienie, wiele badań rzeczywiście pokazało, że samice w lepszej kondycji i samice więcej inwestujące w jaja, składały bardziej pigmentowane jaja, co – przynamniej w niektórych przypadkach – wpływało na wielkość wysiłku rodzicielskiego samca mierzonego najczęściej jako liczbę wizyt z pokarmem dla piskląt. Natomiast wyniki dotyczące protoporfiryny IX, barwnika nadającego skorupkom jaj czerwonobrązowe zabarwienie, są niejednoznaczne. U wielu gatunków znaleziono negatywną zależność pomiędzy intensywnością pigmentacji skorupek jaj a kondycją samicy. Z drugiej strony jaja w bardziej pigmentowanych lęgach często miały większą objętość, większe żółtko i wyższy sukces klucia, a wykluwające się z nich pisklęta miały wyższą masę. Większość prac badających zależność pomiędzy pigmentacją skorupek jaj a inwestycją samca nie znalazła żadnej zależności, a wyniki dwóch prac, które taką zależność znalazły, były przeciwstawne. Ponadto większość z tych badań była prowadzona na gatunkach ptaków gniazdujących w dziuplach, gdzie może być zbyt ciemno, żeby ptaki były w stanie rzetelnie ocenić kolor lub jasność jaj.

Głównym celem projektu doktorskiego było przetestowanie hipotezy SSEC na gatunku, który składa jaja wybarwione protoporfiryną i który buduje gniazda o otwartej konstrukcji, gdzie docierające światło pozwala na ocenę wyglądu jaj przez samce. Jako gatunek modelowy wybrałam gąsiorka *Lanius collurio*, a badania terenowe prowadziłam przez trzy sezony lęgowe w zachodniej Wielkopolsce. Fotografowałam lęgi wraz ze standardami szarości i wykorzystując modele wizualne, zmierzyłam różne aspekty wyglądu jaj z perspektywy ptaków, a więc odbiorców sygnału. Kondycję samic oraz piskląt określałam jako wyskalowany współczynnik masy ciała na podstawie zebranych pomiarów biometrycznych, a w przypadku samic dodatkowo zmierzyłam szerokość dziennych prążków wzrostu na sterówkach. Inwestycję samca określałam jako liczbę wizyt z pokarmem przypadającą na jedno pisklę na

jednostkę czasu. Dodatkowo próbka 43 zebranych jaj gąsiorka (pojedyncze niewyklute jaja lub porzucone lęgi) pozwoliła mi stwierdzić, czy zewnętrzny wygląd jaja jest dobrym wskaźnikiem zawartości pigmentu w skorupce, co jest ważnym założeniem hipotezy SSEC. W końcu sprawdziłam, czy pomiary skorupek jaj ptaków wykonane przy użyciu fotografii cyfrowej w zróżnicowanym naturalnym oświetleniu dają powtarzalne wyniki.

Wyniki badań pokazały, że samice w lepszej kondycji składały lęgi o mniej czerwonych plamach na skorupkach, ale z drugiej strony pisklęta z lęgów o bardziej czerwonych plamach były w lepszej kondycji. Chociaż samce gąsiorka były w stanie dostrzec różnice w kolorze i jasności jaj pochodzących z różnych lęgów w badanej populacji, to inwestycja samca w potomstwo nie była związana z kolorem ani z plamkowaniem skorupek jaj. Co więcej, zewnętrzny wygląd jaja okazał się być u gąsiorka słabym wskaźnikiem koncentracji protoporfiryny w skorupce. To oznacza, że chociaż kondycja samicy była powiązana z wyglądem jaja, to niekoniecznie była związana z ilością pigmentu zawartego w skorupce, co może wyjaśniać brak odpowiedzi samca na ten sygnał. Ponadto pomiary tego samego zestawu 36 jaj przepiórki japońskiej *Coturnix japonica* w różnych warunkach świetlnych wykazały, że obecność chmur oraz wysokość słońca nad horyzontem wpływają na pomiary wyglądu jaj. Zmienne oświetlenie wprowadzało stosunkowo dużo zmienności w pomiary jasności jaj oraz kontrastu pomiędzy plamami a tłem jaja, natomiast pomiary koloru i wielkość plamek były dużo bardziej powtarzalne pomimo zmiennego oświetlenia, szczególnie w przypadku pomiarów wykonanych przy jednolitym zachmurzeniu. To wskazuje na konieczność wzięcia pod uwagę zmiennego oświetlenia podczas planowania prac terenowych i wykonywaniu pomiarów przy jednolitej pogodzie i w ograniczonych godzinach.

Słowa kluczowe: kontrast chromatyczny, skalibrowana fotografia cyfrowa, standardy szarości, sygnały wizualne, wysiłek reprodukcyjny, wysokociśnieniowa chromatografia cieczowa

Abstract

There is a great diversity in the appearance of avian eggshells across species. Eggshells of different species can vary in brightness and colour, as well as in the presence, amount, colour, and distribution of maculation. Over years, many hypotheses have been proposed to explain functions of eggshell pigmentation. One of the most recent hypotheses is the sexually selected eggshell coloration (SSEC) hypothesis proposed in 2003. According to the SSEC hypothesis, eggshell pigmentation acts as a signal of female condition or of her investment in eggs in post-mating sexual selection which elicits higher parental investment of her partner. In case of biliverdin, the pigment responsible for the blue-green coloration, many studies have indeed shown that females in better condition and females investing more in eggs had laid more pigmented eggs. In some cases, it was reflected in the higher investment of males, usually quantified as the number of provisioning visits. On the other hand, in case of protoporphyrin IX, the pigment responsible for reddish-brown coloration, results are equivocal. In many species, a negative relationship between eggshell pigmentation and female condition was found. Conversely, eggs from more pigmented clutches often had higher volume, higher mass of yolk, and had higher hatching success. Likewise, chicks originating from more pigmented clutches were heavier. Most studies exploring the relationship between eggshell pigmentation and investment of males did not find any relationship and two studies that found such a relationship provided opposing results. Furthermore, most of these studies were carried out on cavity-nesting species and cavities can be too dark for birds to properly assess colour or brightness of the eggs.

The main aim of the doctoral project was to test the SSEC hypothesis on a species with protoporphyrin-based eggshell pigmentation that builds cup-nests where the lack of light does not limit males' ability to assess the appearance of eggs. I selected red-backed shrike *Lanius collurio* as a model species and carried out fieldwork in western Poland for three years. I photographed clutches alongside grey standards and used visual models to measure different aspects of eggshell appearance from the perspective of the relevant receivers of the signal – birds. I measured the condition of females and chicks using biometric measurements to calculate scaled mass index. In case of females, I additionally measured average width of growth bars in tail feathers. I quantified the investment of males as the number of provisioning visits standardised for one chick and one time unit. Moreover, a sample of 43 unhatched eggs of the red-backed shrike (hatching failure or abandoned clutches) allowed me to check if external appearance of the egg is a good predictor of the concentration of the pigment in the

eggshell which is an important assumption of the SSEC hypothesis. Finally, I explored whether digital photography measurements of eggshells taken in different natural light conditions yield repeatable results.

Analyses revealed that females in better condition laid clutches with less reddish spots, but on the other hand, chicks originating from clutches with redder spots were in superior condition. Although red-backed shrike males were able to perceive differences in colour and brightness between eggs from different clutches in the studied population, their parental effort was not linked to eggshell colour or patterning. Moreover, external appearance of the egg turned out to be a poor predictor of the concentration of protoporphyrin in the eggshell. This means, even though female condition was related to eggshell appearance, it was not necessarily related to the amount of the pigment contained in the eggshell. This can explain the lack of the male's response to this signal. Furthermore, measurements of the same set of 36 eggs of Japanese quail *Coturnix japonica* in variable natural light conditions showed that the presence of the cloud cover and elevation of the sun affect measurements of the appearance of eggs. Variable illumination introduced relatively much noise in the measurements of eggs' brightness and of contrast between spots and eggshell background, while measurements of colour and size of spots were much more repeatable despite variable illumination, especially when there was a uniform cloud cover. This indicates that it is necessary to pay attention to the variable natural illumination when planning of the fieldwork and to take measurements in uniform weather conditions and in a restricted timeframe.

Keywords: calibrated digital photography, chromatic contrast, grey standards, high performance liquid chromatography, provisioning effort, visual signals

Wprowadzenie

Budowa skorupek ptasich jaj i ich ubarwienie

Skorupki ptasich jaj są zbudowane z soli nieorganicznych, węglanów i fosforanów wapnia i magnezu, wśród których największą część stanowi węglan wapnia ($CaCO_3$) oraz macierzy organicznej składającej się z włókien białkowych, na której osadzają się sole nieorganiczne (Romanoff & Romanoff, 1949; Board, 1982). U wielu gatunków skorupka posiada dwie warstwy różniące się budową. Warstwa wewnętrzna, która przylega do błon pergaminowych i w przekroju poprzecznym przypomina zaokrąglone stożki, posiada większą zawartość substancji organicznych niż warstwa zewnętrzna, a sole nieorganiczne są tutaj ułożone nieregularnie. Warstwa zewnętrzna składa się głównie z węglanu wapnia, którego kryształy są zorientowane długą osią prostopadle do powierzchni skorupki, co zapewnia skorupce twardość i wytrzymałość (Romanoff & Romanoff, 1949; Board, 1982). Powierzchnia jaja może być dodatkowo pokryta kutykulą o różnym składzie i grubości w zależności od taksonu (Mikhailov, 1997; D'Alba *et al.*, 2016).

Skorupki ptasich jaj charakteryzują się dużą zmiennością ubarwienia (Kennedy & Vevers, 1976). Prawdopodobnie wspólny przodek ptaków posiadał białą skorupkę (Kilner, 2006), choć istnieją przesłanki, że pigmentowane skorupki występowały wcześniej w drzewie rodowym gadów, już u dinozaurów Dinosauria (Wiemann *et al.*, 2018). Istnieją różne mechanizmy produkcji barwnych skorupek jaj. Jednym z nich jest wykorzystanie pigmentów, które są odkładane na powierzchni lub wewnątrz struktury skorupki. Dotychczas w skorupkach ptasich jaj wyróżniono pięć pigmentów: niebieskozieloną biliwerdynę, czerwonobrązową protoporfirynę IX, żółtobrązową bilirubinę, czerwonopomarańczową uroerytrynę oraz fioletową mezobiliwiolinę. Dwa pierwsze barwniki występują w skorupkach wielu gatunków ptaków z różnych grup systematycznych (Kennedy & Vevers, 1976), natomiast bilirubinę i uroerytrynę znaleziono do tej pory tylko u kusaczy Tinamiformes (Hamchand *et al.*, 2020), a mezobiliwiolinę wyłącznie u dropika czarnobrzuchego *Lissotis melanogaster* (Kennedy & Vevers, 1976). Co ciekawe zdarza się, że skorupki jaj o pozornie białym wyglądzie zawierają niewielkie, choć wykrywalne ilości barwników, np. u bociana białego *Ciconia ciconia*, sowy jarzębatej *Surnia ulula*, albo grzywacza *Columba palumbus* (Kennedy & Vevers, 1976). Wszystkie wspomniane pigmenty są powiązane ze szlakiem metabolicznym hemu i mają strukturę tetrapirolu: posiadają cztery pięcioczłonowe pierścienie, z których każdy zawiera cztery atomy węgla i jeden atom azotu. Układ naprzemiennie występujących wiązań

pojedynczych i podwójnych (zwany chromoforem) pochłania określone długości fal świetlnych, co sprawia, że postrzegamy te związki chemiczne jako kolorowe (McGraw, 2006; Sparks, 2011; Hamchand *et al.*, 2020).

Do naniesienia pigmentów na skorupkę jaja dochodzi w ostatnim odcinku jajowodu, odcinku macicznym, a więc w tej samej części, w której powstaje sama skorupka. Jajo spędza tam najdłuższy czas podczas swojej wędrówki wzdłuż światła jajowodu – około 20 z 24 godzin (Sparks, 2011). W tym czasie gruczoły w ścianie jajowodu wydzielają sole mineralne i kolejne ich warstwy są nanoszone na zewnętrzną część jaja tworząc coraz grubszą skorupkę. W zależności od momentu, w którym pracę rozpoczną gruczoły wydzielające pigmenty, warstwy pigmentu mogą znajdować się wewnątrz struktury skorupki, lub na samej jej powierzchni. Niewiele jest badań na temat lokalizacji barwników w przekroju poprzecznym skorupki, ale wynika z nich, że miejsce występowania pigmentów w skorupce jest specyficzne dla gatunku lub grupy gatunków (Harrison, 1966; Mikhailov, 1997). U kury domowej *Gallus gallus domesticus* pigment jest rozproszony w całym przekroju skorupki (Baird *et al.*, 1975), choć u niektórych ras z Chin znajduje się wyłącznie w najbardziej zewnętrznych warstwach (Wang *et al.*, 2007). Podobnie jest w przypadku jaj przepiórek japońskich *Coturnix japonica*, gdzie duża część pigmentu jest zdeponowana w kutykuli (Tamura & Fujii, 1967). Duża zmienność występuje również u ptaków wróblowych Passeriformes. Przykładowo, niektóre gatunki z rodziny pokrzewkowatych Sylviidae wykazują silną pigmentację w całym przekroju skorupki, podczas gdy u wierzbówek z rodzaju *Cettia* pigment zawiera tylko zewnętrzna część skorupki, a głębsze warstwy są jego zupełnie pozbawione (Harrison, 1966). W jajach krogulca *Accipiter nisus* warstwy protoporfiryny występują zarówno na powierzchni, jak i głębiej w strukturze skorupki (Jagannath *et al.*, 2008). Natomiast u pustułki *Falco tinnunculus* większość pigmentu znajduje się na samej powierzchni i możliwe jest starcie części pigmentu wilgotną ściereczką z powierzchni świeżo złożonych jaj (Fargallo *et al.*, 2014).

Wszystkie wyżej wymienione pigmenty mogą być odkładane w skorupce w formie jednolitej warstwy i w zależności od koncentracji barwnika – oraz wzajemnych proporcji, jeśli barwników jest więcej – skorupki jaj mogą przybierać całą paletę barw (Hanley *et al.*, 2015; Hamchand *et al.*, 2020). Dodatkowo protoporfiryna może występować w postaci plam albo smug o różnym kształcie, wielkości i rozmieszczeniu (Kennedy & Vevers, 1976; Pike, 2015). Smugi powstają prawdopodobnie w wyniku obracania się jaja w momencie, gdy pigment jest nanoszony (Pike, 2015). W efekcie tworzy to dużą zmienność wzorów ubarwienia, którą obserwujemy u ptaków.

Poza pigmentami, na wygląd jaja może również wpływać obecność i budowa kutykuli, która oddziałuje na odbicie fal świetlnych o długości pomiędzy 300 a 400 nm (ultrafioletowych, UV) (Fecheyr-Lippens *et al.*, 2015). Światło UV jest niewidoczne dla ludzi, ale oczy wielu gatunków ptaków są na nie wrażliwe (Cuthill *et al.*, 2000). To oznacza, że dwa jaja o różnym odbiciu w zakresie fal UV mogą wyglądać różnie dla ptaków, choć dla ludzi będą jednakowe. Ponadto niezwykle regularna i gładka nanostruktura kutykuli u kusaczy jest powodem wyjątkowego połysku oraz niewielkiej iryzacji (efektu zmiany barwy w zależności od kąta patrzenia), które można zaobserwować na skorupkach tej grupy ptaków (Igic *et al.*, 2015). Natomiast stopniowo ścierająca się w trakcie inkubacji gruba warstwa białej kutykuli jest powodem nietypowego wyglądu skorupek jaj guiry *Guira guira* oraz kleszczojada gładkodziobego *Crotophaga ani* (D'Alba *et al.*, 2016). Kolor skorupki może również się zmieniać na skutek pokrycia jaja wydzielinami gruczołu kuprowego. Taka sytuacja ma miejsce u dudka *Upupa epops*, gdzie samica regularnie nakłada i rozsmarowuje wydzielinę na skorupkach jaj, co sprawia, że stopniowo zmieniają one ubarwienie z jasnoniebieskiego tuż po złożeniu na zielonoszare pod koniec inkubacji. Wydzielina posiada właściwości antybakteryjne, dzięki czemu zabezpiecza wnętrze jaja przed zakażeniem (Díaz Lora *et al.*, 2020).

Funkcje pigmentów

Różnorodność ubarwienia skorupek ptasich jaj prowadzi do pytania o funkcje pełnione przez pigmenty. Na przestrzeni lat narodziło się wiele hipotez na ten temat. Część z nich skupia się na mechanicznej lub strukturalnej funkcji barwnika, inne na funkcji sygnalizacyjnej. Większość hipotez nie wyklucza się wzajemnie i jest możliwe, że różne czynniki wspólnie kształtują wygląd jaja u różnych gatunków ptaków (Kilner, 2006). Poniżej prezentuję krótki przegląd hipotez dotyczących funkcji pigmentacji skorupek jaj ptaków.

Wzmocnienie skorupki

Okres składania jaj to olbrzymi wysiłek dla samic, które muszą wygospodarować duże ilości zasobów z własnych organizmów, aby użyć ich do produkcji jaj. Szczególnie dużym wyzwaniem jest zgromadzenie odpowiedniej ilości wapnia do budowy wystarczająco grubej skorupki (Graveland & Drent, 1997). Według jednej z hipotez protoporfiryna służy jako strukturalne wzmocnienie skorupki w przypadku deficytu wapnia (Gosler *et al.*, 2005). Przykładowo, u bogatek *Parus major* zaobserwowano, że skorupka jest cieńsza w miejscu występowania plamy, niż w pozbawionym pigmentu sąsiadującym miejscu (Gosler *et al.*,

2005). Podobną zależność znaleziono u krogulca, gdzie dodatkowo wykazano, że jaja samic narażonych na większą ekspozycję na dichlorodifenylodichloroetylen (DDE – produkt rozpadu dichlorodifenylotrichloroetanu, DDT) zaburzający gospodarkę wapniową organizmu, miały cieńszą, ale bardziej pigmentowaną skorupkę (Jagannath *et al.*, 2008). Natomiast u przepiórki japońskiej znaleziono odwrotną zależność: plamkowane regiony miały grubszą skorupkę i większą zawartość wapnia w stosunku do regionów pozbawionych pigmentów (Orłowski *et al.*, 2017). Ta pozorna rozbieżność może wynikać z różnego miejsca depozycji pigmentu w przekroju poprzecznym skorupki i różnych funkcji pigmentu w zależności od miejsca jego położenia (Jagannath *et al.*, 2008). Podczas gdy u przepiórki większość protoporfiryny jest odkładana pod koniec procesu budowy skorupki, więc pigment znajduje się w dużej mierze blisko powierzchni skorupki (Tamura & Fujii, 1967), u krogulca i bogatki warstwy protoporfiryny są obecne również głębiej w strukturze skorupki (Gosler *et al.*, 2005; Jagannath *et al.*, 2008). Prawdopodobnie to właśnie warstwy pigmentu występujące głębiej wewnątrz skorupki odpowiadają za jej wzmocnienie (Jagannath *et al.*, 2008).

Termoregulacja

Pigmenty absorbują część promieniowania słonecznego (włączając w to fale podczerwone, a więc promieniowanie cieplne), co prowadzi do nagrzewania się pigmentowanych powierzchni. Zarodek potrzebuje do rozwoju odpowiedniej temperatury nieprzekraczającej 43°C (Bakken *et al.*, 1978). Długi okres inkubacji sprawia, że nieuniknione są krótsze lub dłuższe przerwy w wysiadywaniu, na przykład podczas obracania jaj, kiedy jeden z rodziców zmienia drugiego, kiedy oboje rodzice odlatują, aby się pożywić, albo kiedy zostaną spłoszone przez drapieżnika. W takiej sytuacji, w zależności od klimatu, w którym znajduje się lęg, jaja stopniowo się wychładzają lub nagrzewają. Zaobserwowano, że w przeciwieństwie do melanin, biliwerdyna i protoporfiryna odbijają stosunkowo dużą część promieniowania podczerwonego, dzięki czemu jajo może pozostać dłużej wyeksponowane na promieniowanie słoneczne zanim się przegrzeje, niż gdyby do pigmentacji skorupek jaj były wykorzystane melaniny (Bakken *et al.*, 1978). Z drugiej strony mocno pigmentowane jaja mimo wszystko absorbują część promieniowania cieplnego. Jest to korzystne w przypadku przerw w inkubacji w chłodniejszym klimacie, ponieważ jajo pozbawione ciepła inkubującego rodzica wolniej się wychładza. Badania porównawcze na dużej liczbie gatunków z rożnych regionów świata wykazały, że rzeczywiście istnieje tendencja do występowania mocniej pigmentowanych protoporfiryną skorupek jaj w gradiencie od równika w kierunku bieguna północnego, podczas

gdy jaja w cieplejszych i suchszych regionach są jaśniejsze i wybarwione głównie biliwerdyną (Wisocki *et al.*, 2020).

Ochrona przed UV

Promieniowanie UVB (290-320 nm) jest szkodliwe dla rozwijającego się zarodka, ponieważ powoduje mutacje. Węglan wapnia będący głównym składnikiem skorupek jaj pochłania część szkodliwego promieniowania UVB, jednak pigmenty mogą stanowić dodatkową ochronę, co jest szczególnie istotne w przypadku gatunków posiadających cieńsze skorupki (Maurer *et al.*, 2015). Taka ochrona jest ważna zwłaszcza dla ptaków gniazdujących w miejscach bezpośrednio eksponowanych na promieniowanie słoneczne, np. na ziemi w miejscach pozbawionych drzew, choć może być korzystna również dla ptaków gniazdujących w innych miejscach. Przykładowo, badania na wikłaczu zmiennym *Ploceus cucullatus*, który buduje gniazda o zamkniętej konstrukcji, wykazały, że populacje tego gatunku żyjące w miejscach o większym nasłonecznieniu posiadają jaja o ciemniejszym i bardziej intensywnym niebieskozielonym kolorze (Lahti, 2008). Zaobserwowano również, że skorupki jaj gatunków o dłuższym czasie inkubacji przepuszczają mniej promieniowania UV do wnętrza jaja (Maurer *et al.*, 2015).

Pozytywna rola światła

Pigmentacja skorupek jaj może również pełnić ważną rolę w procesach foto-reaktywacji (naprawie uszkodzeń DNA spowodowanych promieniowaniem UVB) oraz foto-akceleracji (przyśpieszeniu rozwoju zarodka pod wpływem działania światła) modulując ilość oraz spektrum światła docierającego do wnętrza jaja (Coleman & McNabb, 1975; Thoma, 1999). Jednakże większość badań na ten temat dotyczy udomowionych gatunków ptaków trzymanych w warunkach sztucznych, które nie odzwierciedlają warunków świetlnych doświadczanych przez dzikie ptaki w ich naturalnym środowisku. Niewiele więc wiadomo na temat działania mechanizmów foto-reaktywacji i foto-akceleracji w naturalnych warunkach ani na temat roli pigmentacji w tych procesach (Maurer *et al.*, 2011).

Funkcja antybakteryjna

Protoporfiryna pod wpływem światła wytwarza reaktywne formy tlenu. Badania laboratoryjne japońskiego zespołu wykazały, że ta właściwość protoporfiryny zawartej w skorupkach jaj kury domowej przyczynia się do znacznego zmniejszenia ilości dwóch gatunków bakterii gram dodatnich: *Staphylococcus aureus i Bacillus cereus* (Ishikawa *et al.*,

2010). Stąd wysnuto wniosek, że protoporfiryna działa antybakteryjnie zabezpieczając powierzchnię jaja przed nadmiernym rozwojem szkodliwych bakterii, które poprzez pory mogłyby zainfekować wnętrze jaja (Ishikawa *et al.*, 2010). Jednak dalsze badania, które przeprowadzono w warunkach środowiska naturalnego, nie znalazły poparcia dla tej hipotezy: nie znaleziono różnic w ilości bakterii gram dodatnich na powierzchni pigmentowanych jaj wystawionych na działanie światła słonecznego i pozbawionych dostępu do światła ani pomiędzy pigmentowanymi i białymi jajami (Dearborn *et al.*, 2017).

Kamuflaż

Już wiele lat temu zaobserwowano, że ptaki gniazdujące w ukrytych miejscach np. dziuplach, norach lub budujące gniazda o zamkniętej konstrukcji, często składają jaja o jednolicie białej skorupce (Wallace, 1889). Wallace (1889) zaproponował, że pierwotnie jaja ptaków były białe, a pigmenty wyewoluowały, aby utrudnić wykrycie jaj przez drapieżniki, gdy ptaki zaczęły składać lęgi w bardziej odkrytych miejscach. Kolejne prace dostarczyły poparcia dla tej hipotezy. Lack (1958) znalazł zależność pomiędzy miejscem, w którym składane są jaja a kolorem ich tła i obecnością plamek u drozdowatych Turdidae. Podobnie praca porównawcza Kilner (2006) pokazała, że lokalizacja gniazda wyjaśnia dużo zmienności w pigmentacji skorupek jaj ptaków i że ptaki gniazdujące na ziemi często znoszą brązowe i plamkowane jaja. Kolejne badania porównawcze znalazły zależność pomiędzy poziomem drapieżnictwa doświadczanym przez dany gatunek a odbiciem światła przez skorupki, ale wyłącznie w zakresie długości fal UV, nie było natomiast różnic w jasności ani w odbiciu światła w zakresie fal widzialnych (Hanley *et al.*, 2013). Natomiast w przypadku gatunków ptaków budujących gniazda o otwartej konstrukcji na gałęziach drzew i krzewów pokazano, że kolor jaj nie ma takiego znaczenia, ponieważ drapieżniki wizualne skupiają się w swoich poszukiwaniach raczej na gnieździe, niż na jajach (Götmark, 1992).

Inaczej ma się sytuacja w przypadku ptaków gniazdujących bezpośrednio na ziemi (Kilner, 2006). Kolor oraz plamkowanie jaj utrudniają ich wykrycie przez drapieżniki wizualne, ale każdy z tych elementów wyglądu jaja wykorzystuje inny mechanizm zapewniający skuteczny kamuflaż. Podobieństwo koloru jaja do koloru podłoża zmniejsza kontrast pomiędzy nimi, dzięki czemu jajo wtapia się w tło, na którym leży. Natomiast obecność kontrastowych plam sprawia, że mniej widoczny jest charakterystyczny kształt jaja, co może być szczególnie ważne na heterogenicznym podłożu (Cuthill *et al.*, 2005; Lovell *et al.*, 2013). Badania na przepiórce japońskiej wykazały, że samice nie składają jaj w przypadkowych miejscach, tylko wybierają miejsce złożenia lęgu w zależności od wyglądu ich jaj, co zwiększa stopień

kamuflażu (Lovell *et al.*, 2013). Również dzikie ptaki potrafią wybierać mikrosiedliska, które maksymalizują kamuflaż ich lęgów (Stevens *et al.*, 2017; Hancock *et al.*, 2023), a niektóre gatunki, na przykład szablodziób *Recurvirostra avosetta i* sieweczka morska *Charadrius alexandrinus* dodają do zagłębienia z lęgiem kamyki, muszle i gałązki, które – zwiększając heterogeniczność podłoża – utrudniają wykrycie jaj (Gómez *et al.*, 2018). Duże znaczenie ma też wzajemna zależność pomiędzy stopniem kamuflażu lęgu oraz upierzenia inkubującego rodzica. Wiele gatunków sieweczkowatych Charadriidae znosi jaja o znakomitym kamuflażu, znacznie lepszym niż upierzenie dorosłych ptaków, natomiast u lelkowatych Caprimulgidae sytuacja jest odwrotna – inkubujący rodzic jest znacznie lepiej zakamuflowany, niż jego jaja (Troscianko *et al.*, 2016). Jest to powiązane z dystansem ucieczki: podczas gdy sieweczkowate zrywają się z gniazda, gdy drapieżnik jest kilkadziesiąt metrów od nich i polegają na kamuflażu jaj, lelki siedzą nieruchomo na gnieździe prawie do ostatniej chwili i uciekają dopiero, gdy drapieżnik zbliży się na odległość około dwóch metrów (Wilson-Aggarwal *et al.*, 2016).

Ubarwienie aposemantyczne

Interesującym wątkiem w historii badań nad ptasimi jajami były badania nad potencjalną aposemantyczną funkcją ubarwienia skorupek jaj. Swynnerton (1916) zaproponował, że skorupki jaj niektórych gatunków ptaków są ubarwione jaskrawo, ponieważ w ten sposób sygnalizują drapieżnikom, że ich jaja są niesmaczne lub niejadalne. Swynnerton przetestował swoją hipotezę serwując jaja różnych gatunków ptaków galago gruboogonowemu *Otolemur crassicaudatus*, manguście małej *Urva edwardsii* oraz szczurowi *Rattus* sp. skrupulatnie notując ich reakcje i preferencje. Nie znalazł wsparcia dla swojej hipotezy: chociaż zwierzęta wykazywały preferencje smakowe, to nie były one związane z kolorem skorupki (Swynnerton, 1916). Podobne badania zostały przeprowadzone kilkadziesiąt lat później przez Cotta (1948), który wykorzystał panel ekspertów smakowych testujących przydatność do spożycia różnych posiłków w trakcie drugiej wojny światowej. Cott twierdził, że znalazł poparcie dla hipotezy Swynnertona, jednak jego badania nasuwają szereg zastrzeżeń metodycznych. Przykładowo, jaja nie były surowe (a w takim przecież stanie spożywa je drapieżnik), tylko rozmieszane i ścięte na parze. Ponadto wątpliwości budzi jego definicja jaskrawości kolorów, na przykład wszystkie testowane jaja ptaków wróblowych zaklasyfikował jako jaskrawo ubarwione i aposemantyczne, pomimo ogromnej zmienności ubarwienia w tej grupie. Powtórna analiza tych danych wykonana z wykorzystaniem bardziej obiektywnej klasyfikacji kolorów nie potwierdziła jego konkluzji o aposemantycznej roli ubarwienia jaj (Lack, 1958).

Rozpoznawanie własnych jaj

Podczas gdy niektóre gatunki ptaków składają stosunkowo jednolicie ubarwione jaja w obrębie gatunku, inne prezentują ogromną zmienność ubarwienia i plamkowania jaj. Sztandarowym przykładem takiego gatunku jest nurzyk *Uria aalge*. Nurzyki składają pojedyncze jaja bezpośrednio na ziemi i gniazdują w ogromnym zagęszczeniu sięgającym nawet do 30 inkubujących ptaków na 1 m^2 (Birkhead, 1978). W tak wielkim zagęszczeniu istnieje ryzyko przypadkowej zamiany jaj z sąsiadem, co mogłoby spowodować, że ptaki wychowywałyby obce potomstwo. Tschanz (1959), a za nim Birkhead (1978) zaproponowali, że duża zmienność wyglądu jaj u nurzyka ułatwia parom ptaków rozpoznawanie ich własnych jaj, dzięki czemu nie dochodzi do takich pomyłek. Rzeczywiście nurzyki potrafią rozpoznać swoje jajo nawet, gdy zostanie zamienione z innym, sąsiednim jajem, natomiast bliskie filogenetycznie nurzykom alki *Alca torda*, które gniazdują w mniejszym zagęszczeniu, nie są do tego zdolne (Birkhead, 1978). Jeśli wzory na jajach tego gatunku wyewoluowały, aby ułatwić rozpoznawanie przez pary ich własnych jaj, taki sygnał wizualny powinien spełnić szereg warunków: być bardzo zmienny w populacji, a równocześnie charakterystyczny dla pojedynczych samic i stosunkowo jednolity w ciągu ich życia (Quach *et al.*, 2021). Ponadto aby nieść jak najwięcej informacji o tożsamości właściciela, taki sygnał powinno tworzyć wiele niezwiązanych ze sobą elementów (Caves *et al.*, 2015). W przypadku skorupek jaj takimi niezależnymi elementami mogą być na przykład kolor tła, kolor plam, wielkość, kształt i kontrast plam oraz ich rozmieszczenie (Stoddard & Stevens, 2010, 2011). Zgodnie z tymi przewidywaniami, ilościowe badania wyglądu jaj nurzyków wykazały, że wzory na jajach tych ptaków niosą w sobie informację o tożsamości właściciela równie niepowtarzalną, jak odcisk palca u człowieka (Quach *et al.*, 2021).

Podobnej presji są poddawani gospodarze pasożytów lęgowych, lecz w tym przypadku umiejętność rozpoznawania własnych jaj nie wynika z gniazdowania w dużym zagęszczeniu, tylko z obecności obcych jaj we własnym gnieździe. Obligatoryjne pasożyty lęgowe to grupa gatunków ptaków, które wykorzystują opiekę rodzicielską innych ptaków podrzucając im do gniazd swoje własne jaja. W toku ewolucji tak bardzo wyspecjalizowały się w wykorzystywaniu innych gatunków do własnego rozrodu, że nie są w stanie dłużej same opiekować się swoim potomstwem (Stevens, 2013). Jednym z najlepiej poznanych i przebadanych pasożytów lęgowych jest kukułka *Cuculus canorus*. Interakcja pomiędzy kukułką i jej gospodarzami to klasyczny przykład koewolucji (Swynnerton, 1918; Rothstein, 1990). Kukułka jest większym ptakiem niż większość jej gospodarzy, co oznacza, że ptaki,

które zaakceptują jajo będą musiały zainwestować dużo czasu i wysiłku w opiekę nad obcym pisklęciem, które ma ogromne zapotrzebowanie energetyczne. Dodatkowo pisklę kukułki tuż po wykluciu wyrzuca z gniazda jaja lub pisklęta gospodarza, co oznacza, że sukces reprodukcyjny gospodarza jest zredukowany do zera. To nakłada dużą presję selekcyjną na gospodarzy – jedynie osobniki, które potrafią rozpoznać i odrzucić obce jajo z własnego gniazda, będą w stanie wyprowadzić lęgi. To sprawia, że na przestrzeni kolejnych pokoleń zdolność gospodarzy do rozpoznawania obcych jaj rozprzestrzenia się w populacji. To z kolei nakłada presję selekcyjną na kukułkę sprawiając, że sukces reprodukcyjny będą miały tylko te osobniki, których jaja będą najbardziej przypominały jaja gospodarza, co zapobiegnie wykryciu jaja i wyrzuceniu go z gniazda. To znów sprawia, że gospodarze potrafiący rozpoznać coraz subtelniejsze różnice w wyglądzie jaj mogą uzyskać przewagę nad innymi osobnikami w populacji, które tego nie potrafią. W toku koewolucji z różnymi gatunkami gospodarzy, kukułka wykształciła szereg linii genetycznych specjalizujących się w wybranych gatunkach ptaków, gdzie samica z poszczególnych linii składa jaja o skorupkach podobnych do jaj jej gospodarza (Antonov *et al.,* 2010). Jednak pasożyty lęgowe nie zawsze specjalizują się w wybranych gatunkach gospodarzy. Przykładowo, starzyk brunatny *Molothrus ater*, północnoamerykański obligatoryjny pasożyt lęgowy wykorzystuje ponad 200 różnych gatunków gospodarzy. U starzyka nie wykształciły się odrębne linie genetyczne, a wygląd jego jaj nie jest dopasowany do jednego gatunku gospodarza, ale stanowi kompromis w podobieństwie do kilku różnych gatunków i zależy od obecności poszczególnych gatunków gospodarzy w okolicy, ich liczebności oraz zdolności do rozpoznawania obcych jaj (Hanley *et al.*, 2021).

Pigmentacja skorupek jaj to ważny element „wyścigu zbrojeń" pomiędzy gospodarzami i pasożytami lęgowymi. Zaobserwowano, że gatunki, które są gospodarzami pasożytów lęgowych, podobnie jak ptaki gniazdujące w dużym zagęszczeniu, składają jaja o wielu różnych niepowiązanych ze sobą elementach (np. kolorze, wielkości plamek, ich rozmieszczeniu i kształcie) tworzących wspólnie niepowtarzalny wzór plamkowania, co pomaga ptakom rozpoznać ich własne jaja (Caves *et al.,* 2015) oraz że zmienność wyglądu jaj w obrębie lęgu jest mała (Stokke *et al.,* 2002). Ponadto gatunki o długiej wspólnej historii z pasożytem lęgowym rozwijają duży polimorfizm plamkowania i koloru jaj pomiędzy samicami w obrębie populacji (Stokke *et al.*, 2002; Spottiswoode & Stevens, 2012).

Obce jajo może też zostać podrzucone przez inną samicę tego samego gatunku (wewnątrzgatunkowe pasożytnictwo lęgowe), jednak dotychczas niewiele zostało przeprowadzonych badań na ten temat (Stevens, 2013).

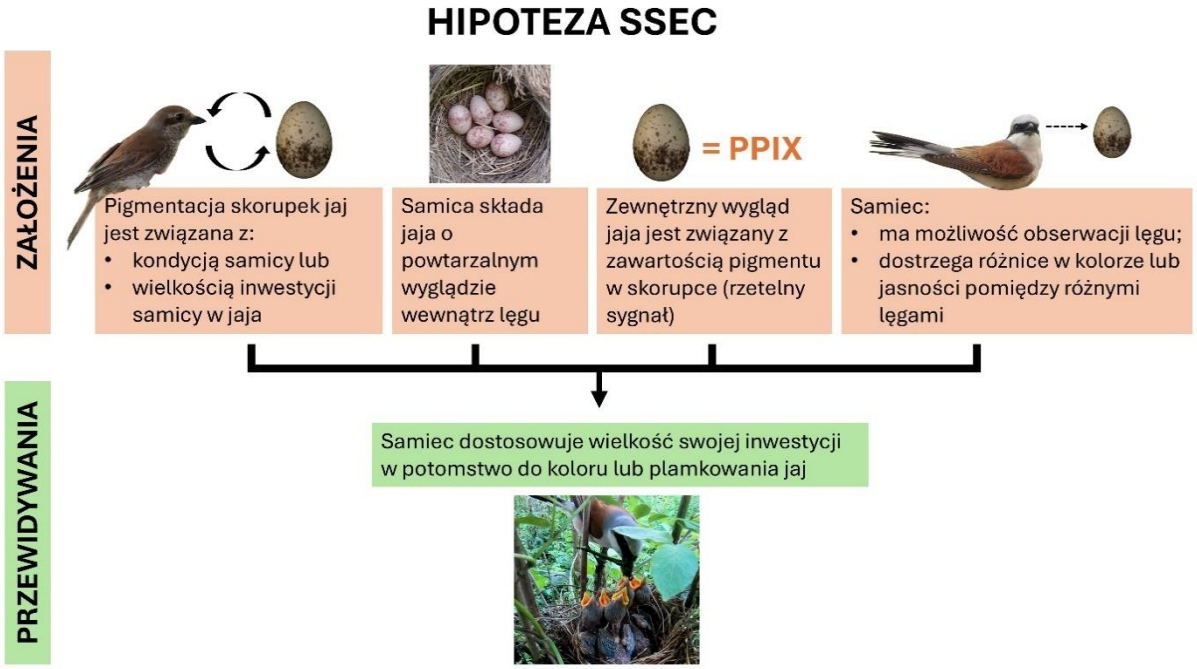
Pigmentacja skorupek jaj i dobór płciowy

Według kolejnej hipotezy (ang. *sexually selected eggshell coloration hypothesis*, hipoteza SSEC) pigmentacja skorupek jaj stanowi sygnał kondycji samicy w po-kopulacyjnym doborze płciowym. Bardziej intensywna pigmentacja skorupek jaj świadczy o lepszej kondycji samicy i na podstawie tego sygnału samiec dostosowuje wielkość swojej inwestycji rodzicielskiej w ich wspólne potomstwo (Moreno & Osorno, 2003). Hipoteza SSEC opiera się na hipotezie zróżnicowanej alokacji (ang. *differential-allocation hypothesis*), według której osobniki dostosowują wielkość wysiłku rodzicielskiego do atrakcyjności swojego partnera – partnerzy bardziej atrakcyjnych samic zwiększają swój wysiłek rodzicielski i odwrotnie (Burley, 1988). Zgodnie z hipotezą SSEC w przypadku gatunków poligynicznych inwestycja samca w lęgi o bardziej pigmentowanych skorupkach (a więc lepszej kondycji samicy) powinna być większa w stosunku do jego inwestycji w lęgi o mniej pigmentowanych jajach (Moreno & Osorno, 2003). Zgodnie z tym przewidywaniem zaobserwowano zależność pomiędzy intensywnością niebieskozielonego ubarwienia jaj a stopniem poligynii u europejskich ptaków wróblowych (Soler *et al.*, 2005). Natomiast w przypadku gatunków monogamicznych samiec w parze z samicą składającą bardziej pigmentowane jaja może zwiększyć wielkość swojej aktualnej inwestycji w lęg kosztem przyszłych prób reprodukcji (Cherry & Gosler, 2010).

Hipoteza SSEC opiera się na założeniu, że pigmentacja skorupek jaj stanowi rzetelny sygnał kondycji samicy. Zarówno biliwerdyna, jak i protoporfiryna są związane z metabolizmem hemu: protoporfiryna to bezpośredni prekursor w syntezie hemu, natomiast biliwerdyna jest bezpośrednim produktem jego rozpadu (Sparks, 2011). Biliwerdyna posiada właściwości przeciwutleniające, a jej ilość w skorupce może odzwierciedlać zdolność organizmu samicy do walki z wolnymi rodnikami (Moreno & Osorno, 2003). Natomiast protoporfiryna posiada właściwości pro-oksydacyjne, a jej podniesiony poziom w wątrobie zwiększa aktywność enzymów antyoksydacyjnych (Afonso *et al.*, 1999), dlatego większa pigmentacja może świadczyć o lepszej zdolności organizmu do radzenia sobie ze stresem oksydacyjnym (Moreno & Osorno, 2003). Oprócz tego wybarwienie skorupek protoporfiryną może też służyć jako wskaźnik poziomu anemii u samicy, gdzie samice składające jaja o mniejszej ilości protoporfiryny bardziej cierpią z powodu anemii (de Coster *et al.*, 2012). Z drugiej strony złożenie jaj o mocno pigmentowanych skorupkach to okazja dla samicy na pozbycie się tego pro-oksydantu ze swojego organizmu, co może sygnalizować wysoki poziom stresu oksydacyjnego w jej organizmie, a więc jej gorszą kondycję (Sanz & García-Navas,

2009). Zatem przewidywania dotyczące kierunku zależności pomiędzy kondycją samicy a ilością protoporfiryny w skorupkach składanych przez nią jaj nie są jednoznaczne (Moreno & Osorno, 2003).

Poza związkiem pomiędzy kondycją samicy a ilością pigmentu w skorupkach składanych przez nią jaj, hipoteza SSEC posiada też szereg innych założeń (Rycina 1). Pigmentacja skorupek jaj może świadczyć nie tylko o kondycji samicy, ale również o wielkości jej inwestycji w jaja, co przekłada się na późniejszą kondycję piskląt (Moreno *et al.*, 2005). Zaproponowano również, że o lepszej kondycji samicy może świadczyć nie tyle intensywność pigmentacji jaj, co większa jednolitość ubarwienia wszystkich jaj w lęgu, ponieważ oznacza to, że metabolizm samicy pracuje na równym poziomie (Reynolds *et al.*, 2009). Ponadto aby działać jako sygnał, jaja składane przez pojedynczą samicę w tym samym lęgu powinny być stosunkowo równomiernie ubarwione (Moreno *et al.*, 2004). Następnie hipoteza SSEC zakłada, że zewnętrzny wygląd jaja odzwierciedla zawartość pigmentu w skorupce (Moreno *et al.*, 2006a), że samiec ma możliwość porównania wyglądu skorupek jaj z różnych lęgów (Holveck *et al.*, 2010; Riehl, 2011) oraz że jest on w stanie ocenić wygląd jaj, co nie zawsze jest oczywiste – na przykład w przypadku ptaków gniazdujących w ciemnych dziuplach (Holveck *et al.*, 2010). Niniejszy projekt skupia się na hipotezie SSEC, dlatego poniżej przedstawiam bardziej szczegółowy przegląd stanu wiedzy na temat roli barwników w po-kopulacyjnym doborze płciowym ptaków.



Rycina 1. Schemat przedstawiający założenia i przewidywania hipotezy SSEC (ang. *sexually selected eggshell coloration hypothesis*) o roli pigmentacji skorupek jaj w po-kopulacyjnym doborze płciowym ptaków. Fot. Klaudia Szala.

Stan badań na temat hipotezy SSEC

Dotychczasowe badania nad hipotezą SSEC przyniosły niejednoznaczne rezultaty. W przypadku biliwerdyny, najbardziej zbadanym systemem są muchołówki żałobne *Ficedula hypoleuca*. Wiele założeń hipotezy SSEC jest spełnionych u tego gatunku: samice składają jaja o stosunkowo jednolitym ubarwieniu w lęgu (Moreno *et al.*, 2004), samice składające bardziej pigmentowane jaja mają silniejszy układ immunologiczny (Moreno *et al.*, 2005) i wyższy wskaźnik masy ciała (Morales *et al.*, 2006), a bardziej pigmentowane jaja są większe (Moreno *et al.*, 2004) i mają więcej immunoglobulin (Morales *et al.*, 2006). Natomiast odpowiedź samca, którą zwykle mierzono jako liczbę wizyt z pokarmem, różni się pomiędzy populacjami muchołówki. W populacji hiszpańskiej (Moreno *et al.*, 2004, 2006b) znaleziono odpowiedź samca zgodną z hipotezą SSEC (a więc samce inwestowały więcej w lęgi o intensywniejszym ubarwieniu), natomiast w populacji norweskiej (Johnsen *et al.*, 2011) i fińskiej (Mari *et al.*, 2024) nie znaleziono zależności pomiędzy kolorem skorupek jaj a wielkością inwestycji samca. Również u blisko spokrewnionej muchołówki białoszyjej *Ficedula albicollis* nie znaleziono zależności pomiędzy pigmentacją skorupek jaj a inwestycją samca (Krist & Grim, 2007), podobnie jak u głuptaka niebieskonogiego *Sula nebouxii* (Morales *et al.*, 2010). Z kolei prace na szpaku jednobarwnym *Sturnus unicolor* przyniosły niejednoznaczną odpowiedź. Korelacyjne badania pokazały, że samce mniej inwestowały w lęgi o intensywniej wybarwionych skorupkach (López-Rull *et al.*, 2007), natomiast w badaniach eksperymentalnych, gdzie oryginalne jaja zamieniano na sztuczne modele o bladym lub intensywnym kolorze, samce inwestowały więcej w lęgi z bardziej kolorowymi modelami jaj (Soler *et al.*, 2008).

U gatunków składających jaja o skorupkach wybarwionych protoporfiryną, pigmentacja skorupek jaj rzeczywiście często odzwierciedlała kondycję samicy, choć kierunek tej zależności był różny w zależności od badanego gatunku lub sposobu pomiaru kondycji samicy. Wielokrotnie zależność była odwrotna niż w przypadku biliwerdyny. Samice bogatki składające bardziej pigmentowane jaja były lżejsze (Stoddard *et al.*, 2012) i miały wyższy poziom limfocytów we krwi, co mogło świadczyć o aktualnie przechodzonych infekcjach (Hargitai *et al.*, 2016b), a samice modraszki *Cyanistes caeruleus* charakteryzowały się niższym wskaźnikiem masy ciała i wyższym poziomem białek szoku termicznego (Martínez-de la Puente *et al.*, 2007) oraz mniejszą masą ciała i wyższym poziomem pasożytów we krwi (Badás *et al.*, 2017). Z drugiej strony samice dymówki *Hirundo rustica* o ciemniejszych piórach na piersi (a więc wyższym poziomie melanin) składały jaja o większym pokryciu przez plamki

(Corti *et al.*, 2018), a samice łyski *Fulica atra z* większą liczbą plamek na jajach miały wyższy wskaźnik masy ciała, bardziej rozwinięte ornamenty płciowe i niższy współczynnik heterofilów do limfocytów, a więc niższy stres fizjologiczny (Minias *et al.*, 2020).

Ponadto w wielu badaniach znaleziono zależność pomiędzy intensywnością pigmentacji a wielkością inwestycji samic w jaja, choć i ta zależność nie była jednoznaczna. U wielu gatunków bardziej pigmentowane jaja miały większą objętość (Poláček *et al.*, 2017; Minias *et al.*, 2020) oraz masę (Martínez-Padilla *et al.*, 2010), wyższą masę żółtka i aktywność lizozymu (Krištofík *et al.*, 2013) oraz więcej przeciwciał (Holveck *et al.*, 2012). U innych gatunków bardziej pigmentowane jaja miały mniejszą masę (Walters & Getty, 2010), zawierały mniej przeciwciał (Hargitai *et al.*, 2016a) oraz mniej karotenoidów w żółtku (Hargitai *et al.*, 2016b). U dwóch gatunków, mewy delawarskiej *Larus delawarensis* i mazurka *Passer montanus,* znaleziono również pozytywną zależność pomiędzy pigmentacją skorupek jaj a masą wyklutych z nich piskląt (Hanley & Doucet, 2009; Poláček *et al.*, 2017), a u modraszki z lęgów o bardziej pigmentowanych skorupkach wykluwało się więcej piskląt (Sanz & García-Navas, 2009). Natomiast żadne z czterech badań, które szukały zależności pomiędzy pigmentacją skorupek jaj protoporfiryną a sukcesem wylotu piskląt z gniazda nie znalazły takiej zależności (Sanz & García-Navas, 2009; Martínez-Padilla *et al.*, 2010; Hargitai *et al.*, 2018; Hodges *et al.*, 2020).

Również odpowiedź samca na sygnał w postaci pigmentacji skorupek jaj była niejednoznaczna. U wielu gatunków nie znaleziono żadnego związku pomiędzy pigmentacją a inwestycją samca (np. Hanley & Doucet, 2009; Sanz & García-Navas, 2009; Stoddard *et al.*, 2012; Corti *et al.*, 2018). Natomiast dwie prace, które znalazły taką zależność, przyniosły przeciwstawne rezultaty: u mazurka znaleziono odpowiedź samca zgodną z hipotezą SSEC (większa inwestycja w bardziej pigmentowanych lęgach, Poláček *et al.*, 2017), podczas gdy u strzyżyka śpiewnego *Troglodytes aedon* zależność była odwrotna (Walters *et al.*, 2014). Dodatkowo większość badań testujących inwestycję samca prowadzono na gatunkach ptaków gniazdujących w dziuplach (najczęściej na populacjach korzystających ze skrzynek lęgowych), a to, czy ptaki są w stanie dostatecznie dobrze ocenić wygląd skorupek jaj w tak ciemnych warunkach wciąż nie zostało jednoznacznie stwierdzone (Reynolds *et al.*, 2009; Holveck *et al.*, 2010; Chaib *et al.*, 2023). Co istotne, samce z gatunków socjalnie monogamicznych, takich jak bogatki, odwiedzają gniazda sąsiednich par, a więc mają możliwość porównania pigmentacji skorupek jaj we własnych lęgach z pigmentacją skorupek innych lęgów (Holveck *et al.*, 2010). Ci sami autorzy wykorzystali modele wizualne, aby stwierdzić, czy bogatki miały możliwość dostrzeżenia różnic w kolorach lub jasności jaj w ciemnych warunkach doświadczanych

w skrzynce lęgowej. Ich wyniki nie dały jednoznacznej odpowiedzi na to pytanie – wyniki modelowania zależały wyłącznie od założeń przyjętych w danym modelu, choć różnice w jasności były z dużym prawdopodobieństwem bardziej widoczne dla ptaków, niż różnice w kolorze. Ciemne wnętrza skrzynek lęgowych niekoniecznie odpowiadają warunkom doświadczanym przez ptaki w naturalnych dziuplach, jednak badania dziupli wykorzystywanych przez bogatki, sikory ubogie *Poecile palustris* oraz muchołówki białoszyje pokazały, że naturalne dziuple są równie ciemne i zdolność do rozróżniania kolorów może być w takim środowisku ograniczona (Wesołowski & Maziarz, 2012; Maziarz & Wesołowski, 2014). Z drugiej strony inne badania pokazują, że oczy niektórych ptaków są w stanie – przynajmniej częściowo – adaptować się do ciemności w stosunkowo krótkim czasie, co pozwala ptakom na rozróżnianie obiektów o różnej jasności (Chaib *et al.*, 2023).

Potencjalne trudności z dostrzeżeniem przez samca sygnału w postaci kolorowej skorupki wewnątrz ciemnej dziupli lub skrzynki lęgowej wskazują na konieczność przeprowadzenia badań na gatunkach, które gniazdują w jaśniejszych miejscach, gdzie brak światła nie ogranicza możliwości odpowiedzi samca na ten sygnał. Co więcej, niezbędne jest wzięcie pod uwagę różnic w sposobie postrzegania świata przez różne gatunki zwierząt (Caves *et al.*, 2019) i zmierzenie wyglądu skorupek jaj z perspektywy odpowiedniego odbiorcy sygnału, którym, w przypadku hipotezy SSEC, jest samiec badanego gatunku ptaka. Znajomość liczby typów fotoreceptorów oraz ich proporcji w siatkówce oka i czułości na światło pozwana na wykorzystanie modeli wizualnych, dzięki którym można stwierdzić, czy odbiorca jest w stanie dostrzec różnicę (kontrast) koloru lub jasności dwóch bodźców świetlnych (Vorobyev & Osorio, 1998). Z drugiej strony hipoteza SSEC zakłada, że zawartość pigmentów w skorupce koreluje z kondycją samicy. Skoro odbiorcą sygnału jest samiec, dla którego jedyna dostępna informacja to zewnętrzny wygląd jaja, to aby hipoteza SSEC mogła funkcjonować, zewnętrzny wygląd jaja powinien rzetelnie odzwierciedlać zawartość pigmentu w skorupce. W kolejnym podrozdziale przedstawiam przegląd metod wykorzystywanych do pomiaru pigmentacji skorupek jaj.

Pomiary pigmentacji skorupek jaj

Hipoteza SSEC zakłada, że zewnętrzny wygląd jaja odzwierciedla zawartość pigmentu w skorupce, który jest z kolei skorelowany z kondycją samicy (Moreno *et al.*, 2006a). Wysokociśnieniowa chromatografia cieczowa (ang. *high performance liquid chromatography*, HPLC) umożliwia dokładny pomiar zawartości pigmentu w skorupce, dzięki czemu możliwe jest następnie sprawdzenie, czy zawartość pigmentu jest związana z zewnętrznym wyglądem

jaja. Pomiar przy użyciu HPLC wymaga zniszczenia skorupki wraz z rozwijającym się zarodkiem, co w przypadku większości badań na dzikich ptakach jest niemożliwe. Z tego powodu do pomiarów z użyciem HPLC stosuje się często próbkę jaj pochodzącą z porzuconych lęgów lub pojedyncze niewyklute jaja np. (Poláček *et al.*, 2017; Gómez *et al.*, 2019). W zależności od badanego gatunku, różne zmienne są skorelowane w różnym stopniu z zawartością pigmentu i nie zawsze zewnętrzny wygląd jaja jest dobrym wskaźnikiem zawartości barwnika w skorupce (Wegmann *et al.*, 2015; Butler & Waite, 2016). Przykładowo, u bogatki koncentracja protoporfiryny jest najmocniej powiązana z kolorem plam (Wegmann *et al.*, 2015), u przepiórki japońskiej z ciemnością tła (Duval *et al.*, 2013), a u sieweczki morskiej z wymiarem fraktalnym plam (matematyczną miarą samopodobieństwa, Gómez *et al.*, 2019).

Ptaki różnią się od ludzi (a także pomiędzy sobą) sposobem postrzegania świata (Martin, 2017). Wiele gatunków ptaków – choć nie wszystkie (Höglund *et al.*, 2019) – posiada w siatkówce cztery fotoreceptory (czopki) odpowiedzialne za postrzeganie kolorów, pręciki odpowiedzialne za widzenie przy niskim natężeniu światła oraz specjalny rodzaj fotoreceptorów (podwójne czopki), które odpowiadają za postrzeganie jasności oraz konturów (Martin, 2017). Oczy wielu gatunków ptaków są czułe na szeroki zakres długości fal i oprócz światła widzialnego dla ludzi (400-700 nm) widzą też światło ultrafioletowe o krótszych długościach fal (300-400 nm), które jest niewidoczne dla naszych oczu (Ödeen & Håstad, 2013; Cronin & Bok, 2016). Dodatkowo gatunki znacznie różnią się między sobą ostrością widzenia (Caves *et al.*, 2018). To oznacza, że ten sam lęg oglądany w tym samym świetle i z tej samej odległości przez trichromatycznego człowieka i przez tetrachromatycznego ptaka będzie wyglądać zupełnie inaczej, co zwraca uwagę na konieczność badania sygnałów wizualnych z perspektywy właściwego odbiorcy sygnału (Caves *et al.*, 2019).

Coraz częściej w badaniach nad pigmentacją skorupek jaj wykorzystuje się fotografię cyfrową. Fotografia posiada wiele zalet, na przykład pozwala na stosunkowo szybki pomiar wyglądu jaj, który uwzględnia całą powierzchnię skorupki, co umożliwia analizę przestrzenną wzoru plamkowania (Stevens *et al.*, 2007). Do wykorzystania tej metody wystarczy zwykły aparat cyfrowy oraz niedrogie standardy szarości (Johnsen, 2016). Nieco bardziej skomplikowane jest uwzględnienie w pomiarach światła ultrafioletowego, co wymaga usunięcia filtra UV pokrywającego matrycę światłoczułą aparatu i upewnienia się, że obiektyw przepuszcza światło UV. Konieczne jest również wykorzystanie dużo droższych standardów szarości, które odbijają równą ilość światła również w zakresie fal UV. Ze względu na niższą wrażliwość matrycy światłoczułej na światło UV, niezbędne jest wykonanie dwóch fotografii

z wykorzystaniem różnych filtrów: jednego przepuszczającego wyłącznie światło widzialne i drugiego przepuszczającego wyłącznie światło UV. Fotografie wykonuje się sekwencyjnie, zmieniając filtr i dostosowując czas naświetlania (Troscianko & Stevens, 2015a). To wydłuża czas trwania pomiaru, choć pojawiają się nowe rozwiązania, które przyspieszają ten proces (Vasas *et al.*, 2024).

Fotografia umożliwia zarówno wykonanie obiektywnych pomiarów, jak i wykorzystanie modeli wizualnych modelujących sposób widzenia sygnału wizualnego przez wybranego odbiorcę. W każdym wypadku konieczne jest pamiętanie o kilku zasadach, aby wykonany pomiar był prawidłowy. Po pierwsze, oprogramowanie aparatów wykorzystuje algorytmy przekształcające fotografie w obrazy jak najbardziej atrakcyjne wizualnie dla ludzkiego odbiorcy. Z tego względu należy zapisywać fotografie w formacie RAW, czyli w postaci bezpośredniej odpowiedzi matrycy światłoczułej na światło (Stevens *et al.*, 2007). Jeżeli jest to niemożliwe i zdjęcia są zapisywane w popularnie stosowanym formacie JPEG, niezbędne jest wykonanie dodatkowego kroku w postaci linearyzacji. Linearyzacja polega na wykonaniu fotografii zestawu standardów szarości o całej gamie jasności. Znając wartości ich reflektancji, możliwe jest odwrócenie algorytmów wykorzystywanych przez oprogramowanie aparatu i odzyskanie oryginalnej informacji z matrycy światłoczułej. Po drugie, aby pomiary były porównywalne, ustawienia przysłony i ISO powinny być takie same dla wszystkich zdjęć. Aby zapewnić właściwą ekspozycję, należy manipulować jedynie długością czasu naświetlania (Troscianko & Stevens, 2015a). Po trzecie, obok obiektu, który będzie mierzony (np. ptasie jaja) należy umieścić pasek ze skalą oraz standardy szarości. Standardy szarości stanowią punkt referencyjny do pomiaru jasności i kolorów i pozwalają na wprowadzenie poprawki ze względu na zmienne oświetlenie. Zakres jasności użytych standardów powinien być dopasowany do najjaśniejszego i najciemniejszego fragmentu obiektu, który będzie mierzony (Johnsen, 2016). Standardy szarości powinny być położone w tej samej odległości od obiektywu, co obiekt, który będzie mierzony, a także powinny być tak samo zorientowane względem światła (Troscianko & Stevens, 2015a; Johnsen, 2016). Nie jest to trudne, gdy mierzony obiekt jest stosunkowo płaski (np. skrzydła motyla, płatki kwiatów), ale praktycznie niemożliwe w przypadku jaj. Charakterystyczny kształt jaja sprawia, że z miejsc położonych przy widocznej na zdjęciu „krawędzi" jaja odbija się mniej światła trafiającego następnie do obiektywu aparatu, przez co fragmenty w centrum jaja sprawiają wrażenie jaśniejszych niż brzegi (Gómez & Liñán-Cembrano, 2017). Zastosowanie blendy albo otoczenie fotografowanego lęgu arkuszem politetrafluoroetylenu (teflonu) rozprasza światło, co pomaga

częściowo uporać się z tym problemem oraz uniknąć odblasków na skorupkach jaj (Troscianko & Stevens, 2015a).

Kolejnym utrudnieniem w wykorzystaniu metody fotograficznej jest zmienne oświetlenie. Jak pokazują badania, kalibracja z użyciem standardów szarości nie jest w stanie całkowicie zrekompensować efektu zmiennego naturalnego oświetlenia w przypadku, gdy pomiary są wykonywane przy zmiennej pogodzie (braku lub obecności chmur) oraz o różnych porach dnia (Bergman & Beehner, 2008; DeLacey *et al.*, 2022; Szala *et al.*, 2023). W literaturze istnieją dwa sposoby rozwiązania tego problemu: (1) ograniczenie wykonywania pomiarów do jednolitej pogody oraz wybranych godzin (Troscianko & Stevens, 2015a; Troscianko *et al.*, 2016) lub (2) wykorzystanie przenośnych ciemni (np. drewnianego lub plastikowego pudła), do którego wkładane są jaja i w którym zdjęcie jest wykonywane z użyciem sztucznego oświetlenia (Ornés *et al.*, 2014; Poláček *et al.*, 2017).

Cele projektu i streszczenie wyników pracy

Celem mojego projektu było zbadanie roli ubarwienia skorupek jaj protoporfiryną w pokopulacyjnym doborze płciowym. Jako gatunek modelowy wybrałam gąsiorka *Lanius collurio* (Rycina 2), ptaka krajobrazu rolniczego, który jest socjalnie monogamiczny, a potomstwem opiekują się oboje rodzice (Cramp & Perrins, 1993). Gąsiorek buduje otwarte gniazda w formie miseczki (Cramp & Perrins, 1993), do których zwykle dociera dość dużo światła słonecznego – zarówno bezpośredniego, rozproszonego przez niebo (lub chmury), jak i przefiltrowanego przez liście drzew i krzewów. W przeciwieństwie do ptaków gniazdujących w dziuplach, takie warunki dają możliwość oceny koloru i plamkowania przez samca bez żadnych przeszkód. Wyłącznie samica inkubuje jaja (Cramp & Perrins, 1993) i nie przykrywa ich materiałem gniazdowym ani na etapie składania jaj, ani w trakcie przerw w inkubacji. To oznacza, że samiec gąsiorka ma możliwość zobaczenia jaj na etapie ich składania przez samicę, a także podczas karmienia wysiadującej samicy. Gąsiorek składa jaja o dużej międzyosobniczej zmienności w kolorze tła i plam oraz w wielkości, kształcie i rozmieszczeniu plam (Rycina 3, Surmacki *et al.*, 2006; Stoddard & Stevens, 2011), co znajduje swoje odzwierciedlenie w dużej zmienności koncentracji protoporfiryny w skorupkach jaj tego gatunku (Mikšík *et al.*, 1994, 1996).

Rycina 2. Gniazdo gąsiorka *Lanius collurio* z jajami (po lewej) oraz dorosłe gąsiorki karmiące dziewięciodniowe pisklęta (po prawej). Samica jest ubarwiona na brązowo, a samiec posiada szarą głowę, rdzawobrązowy grzbiet i charakterystyczną czarną maskę na oczach. Fot. Klaudia Szala.

Używając gąsiorka jako gatunku modelowego w moich badaniach, sprawdziłam po pierwsze, czy pigmentacja skorupek jaj jest związana z kondycją samicy (Moreno & Osorno, 2003) lub z kondycją piskląt (Moreno *et al.*, 2005) oraz czy jaja pochodzące z tego samego lęgu mają podobny wygląd (Moreno *et al.*, 2004) i czy ma to związek z kondycją samicy (Reynolds

et al., 2009). Po drugie, korzystając z modeli wizualnych sprawdziłam, czy samce są w stanie dostrzec zmienność w kolorze lub jasności skorupek jaj w populacji (Holveck *et al.*, 2010). Po trzecie, zbadałam, czy samce dostosowują wielkość swojej inwestycji w potomstwo do intensywności pigmentacji skorupek jaj składanych przez ich partnerki (Moreno & Osorno, 2003).

Rycina 3. Zmienność ubarwienia jaj gąsiorka *Lanius collurio*. Każde jajo pochodzi z innego lęgu. Jaja różnią się pomiędzy sobą (kolumny od lewej do prawej): jasnością i kolorem tła, jasnością i kolorem plam, wielkością plam, ich rozmieszczeniem na jaju oraz stopniem pokrycia skorupki przez plamy. Ponadto jaja różnią się kształtem. Skala wielkości nie została zachowana. Zdjęcia skalibrowano w odniesieniu do standardów szarości w programie MICA Toolbox. Fot. Klaudia Szala.

Pomiarów pigmentacji skorupek jaj dokonałam z wykorzystaniem skalibrowanej fotografii cyfrowej oraz modeli wizualnych używając programu Multispectral Image Calibration and Analysis (MICA) Toolbox (Troscianko & Stevens, 2015b). W celu pomiaru dodatkowych aspektów wyglądu jaj (m.in. średnia wielkość plam, procent powierzchni skorupki pokrytej przez plamy oraz kolor i jasność zmierzone osobno dla plamek i osobno dla tła jaja) opracowałam własne skrypty dla programu MICA Toolbox. Na podstawie pomiarów biometrycznych samic i piskląt obliczyłam wyskalowany indeks masy ciała (ang. *scaled mass index*), który zastosowałam jako miarę ich kondycji (Peig & Green, 2009). W przypadku samic wykorzystałam dodatkowo drugą miarę kondycji w postaci szerokości dziennych prążków wzrostu na sterówkach (Grubb, 1989). Inwestycję samca określałam jako liczbę wizyt z pokarmem przypadających na jednostkę czasu i na jedno pisklę. Liczbę wizyt mierzyłam przy

użyciu kamer GoPro instalowanych przy gnieździe dwukrotnie w trakcie wzrostu piskląt: czwartego lub piątego oraz dziewiątego lub dziesiątego dnia życia piskląt. Za każdym razem kamery nagrywały zachowanie ptaków przez około półtorej godziny.

Wyniki modeli wizualnych wskazały, że samce były w stanie dostrzec różnice w kolorze i jasności pomiędzy różnymi lęgami z badanej populacji. Samice składały jaja o stosunkowo powtarzalnym wyglądzie wewnątrz lęgu, a samice składające jaja o bardziej czerwonych plamach były w gorszej kondycji fizycznej. W przypadku piskląt zależność była odwrotna: pisklęta z lęgów o bardziej czerwonych plamach na skorupce były w lepszej kondycji. Natomiast inwestycja samca nie była związana z kolorem ani plamkowaniem skorupek jaj w lęgu. To oznacza, że choć wiele założeń hipotezy SSEC zostało spełnionych, to wygląd skorupek jaj nie służy u gąsiorka jako sygnał wpływający na zachowanie samca. Szczegółową metodykę oraz wyniki tej części badań przedstawiam w Rozdziale 1, który został wysłany do czasopisma *Behavioral Ecology* (numer identyfikacyjny: BEHECO-2024-0174) i znajduje się obecnie w recenzji.

Hipoteza SSEC zakłada, że kondycja samicy jest związana z zawartością pigmentu w skorupce, ale z drugiej strony odbiorcą tego sygnału jest samiec, który jest w stanie dostrzec tylko warstwy pigmentu obecne bliżej powierzchni skorupki. Zatem aby pigmentacja mogła stanowić rzetelny sygnał kondycji samicy i aby samiec miał możliwość odpowiedzi na ten sygnał, zewnętrzny wygląd jaja powinien odpowiadać zawartości pigmentu w skorupce (Moreno *et al.*, 2006a). Dlatego celem drugiej części badań było sprawdzenie, na ile zmienne dotyczące koloru, jasności i plamkowania jaj zmierzone przy wykorzystaniu skalibrowanej fotografii cyfrowej odzwierciedlają koncentrację protoporfiryny w skorupkach zmierzoną przy użyciu wysokociśnieniowej chromatografii cieczowej (HPLC).

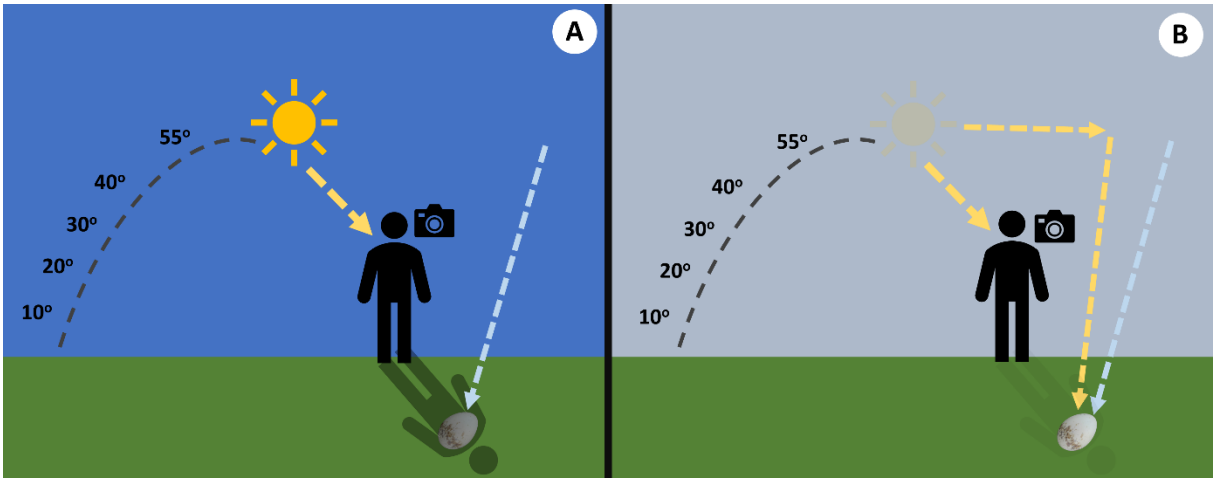
Do badań użyłam skorupek jaj gąsiorka pochodzących z porzuconych lęgów lub pojedynczych niewyklutych jaj. Jaja zostały sfotografowane w wystandaryzowanych warunkach świetlnych i z wykorzystaniem standardów szarości. Zdjęcia były analizowane przy użyciu programu MICA Toolbox oraz autorskich skryptów. Łącznie zmierzyłam 20 różnych cech wyglądu skorupek jaj, m.in. jasność, kolor, procent pokrycia powierzchni jaja przez plamy, kontrast pomiędzy plamami a tłem jaja oraz średnią wielkość plam. Następnie skorupki jaj zostały użyte do pomiaru zawartości protoporfiryny przy użyciu HPLC.

Zbadane skorupki jaj charakteryzowały się dużą zmiennością koncentracji protoporfiryny. Koncentracja protoporfiryny była związana z kolorem jaj, jednak ilość wyjaśnianej zmienności była niewielka. To oznacza, że zewnętrzny wygląd skorupek jaj

gąsiorka jest słabym wskaźnikiem zawartości protoporfiryny. Szczegółową metodykę oraz wyniki tej części badań przedstawiam w Rozdziale 2, który został wysłany do czasopisma *Biological Journal of the Linnean Society* (numer identyfikacyjny: BJLS-7727).

Fotografia cyfrowa jest użytecznym narzędziem, które przyczyniło się do rozwoju badań nad kolorami roślin i zwierząt i często jest stosowana w warunkach terenowych. Wykorzystanie standardów szarości pozwala do pewnego stopnia kontrolować zmienne naturalne warunki oświetleniowe. Jednakże wiele badań wykorzystujących fotografię cyfrową jest prowadzonych przy zróżnicowanym naturalnym oświetleniu, na przykład o różnych porach dnia oraz przy różnej pogodzie. Celem trzeciej części badań było sprawdzenie, jak zmienne naturalne warunki oświetleniowe wpływają na powtarzalność pomiarów różnych aspektów wyglądu jaj.

W tym celu wykorzystałam zestaw 36 skorupek jaj przepiórki japońskiej, które opróżniłam z zawartości przy użyciu strzykawki. Tę samą „stronę" wszystkich jaj sfotografowałam razem ze standardami szarości w zmiennym naturalnym oświetleniu: przy pięciu różnych wysokościach słońca nad horyzontem zarówno w słoneczny dzień, jak i przy jednolitej pokrywie chmur (Rycina 4). To pozwoliło mi sprawdzić, jak wiele zmienności wprowadza w pomiary zmienne oświetlenie oraz które aspekty wyglądu jaj są najbardziej narażone na ten efekt.



Rycina 4. Schemat przedstawiający metodykę badań dotyczących wpływu zmiennego naturalnego oświetlenia na pomiary skorupek jaj ptaków z wykorzystaniem skalibrowanej fotografii cyfrowej. Aby uniknąć odblasków i prześwietleń na skorupkach jaj, zdjęcia wykonywano we własnym cieniu przy (A) bezchmurnym niebie oraz (B) jednolicie zachmurzonym niebie. Szara przerywana linia przedstawia trasę pozornej wędrówki słońca po niebie, a liczby określają wysokość słońca nad horyzontem, przy której wykonywano pomiary. Żółta strzałka reprezentuje bezpośrednie światło słoneczne, a niebieska strzałka światło rozproszone przez niebo. Obecność chmur dodatkowo rozprasza światło (złamana żółta strzałka).

Wyniki pokazały, że zmienne oświetlenie wprowadzało dużo zmienności w pomiary jasności skorupek jaj oraz pomiary kontrastu pomiędzy plamkami a tłem, podczas gdy pomiary koloru i wielkości plam były dużo bardziej powtarzalne. Ponadto zmienność wynikająca z różnej wysokości słońca nad horyzontem była dużo mniejsza w przypadku obecności chmur, które rozpraszały światło. Wyniki tych badań pozwoliły wypracować szereg zaleceń metodycznych dotyczących wykorzystania fotografii cyfrowej do pomiarów wykonywanych w zmiennych warunkach oświetleniowych, jakie są doświadczane w pracy terenowej. Wyniki tej części badań przedstawiam w Rozdziale 3, który został opublikowany w czasopiśmie *Ecology and Evolution*.

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Rozdział 1 | Chapter 1

Do eggshell coloration or patterning affect provisioning effort of males in a cup-nesting passerine?

Do eggshell coloration or patterning affect provisioning effort of males in a cup-nesting passerine?

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Lay summary

Multiple ideas have been proposed to explain why avian eggshells are pigmented. One of them is that eggshell pigmentation functions as a signal of female condition or her investment into eggs that elicits higher investment of her partner. Here, we tested this hypothesis using a cup-nesting passerine and found that while there was a link between eggshell color and condition of both females and chicks, it was not related to males' provisioning effort.

Title: Do eggshell coloration or patterning affect provisioning effort of males in a cup-nesting passerine?

Running header: Appearance of eggshells and investment of males

Abstract

A striking diversity of avian eggshell coloration across species has led to the appearance of many hypotheses trying to explain the evolution of pigmented eggshells. According to the sexually selected eggshell coloration hypothesis, pigments can serve as a signal of a female's condition or of her maternal investment into eggs that elicits a higher investment from her partner. Here, we tested multiple assumptions and predictions of the hypothesis using a cup-nesting passerine with protoporphyrin-pigmented eggshells, red-backed shrike *Lanius collurio*, as our model species. We used calibrated digital photography and visual modeling to assess eggshells appearance from a point of view of a relevant signal receiver. Further, we estimated condition of chicks and females using Scaled Mass Index, and in case of females we additionally measured width of growth bars on tail feathers. We quantified investment of males in terms of provisioning effort in two stages (younger and older chicks). Our results indicate that individual females lay clutches that are consistent in appearance and that males are likely to perceive difference in color and luminance among different clutches in the studied population. We also found a link between color of spots and condition of both chicks and females. However, provisioning effort of males was not related to eggshell coloration or patterning. Thus, while many assumptions of the sexually selected eggshell coloration hypothesis are met, we did not find support that eggshell pigmentation serves as a signal affecting parental effort of males in the red-backed shrike.

Key words: eggshell pigmentation, parental investment, post-mating sexual selection, protoporphyrin IX, quantum catch

Introduction

Throughout years, many hypotheses have been proposed to explain the striking diversity of avian eggshells coloration (Cassey et al. 2010; Hamchand et al. 2020). It was demonstrated that pigments can serve as a structural reinforcement of eggshells (Gosler et al. 2005), affect developing embryo and protect it from detrimental ultraviolet (UV) light (Maurer et al. 2011), contribute to camouflage (Troscianko et al. 2016) and thermoregulation (Wisocki et al. 2020) of eggs, and protect birds from brood parasites (Samaš et al. 2021).

In 2003, in an attempt to explain the presence of conspicuous eggs in some species, Moreno and Osorno proposed that eggshell pigmentation can act as an honest signal of female condition inducing higher parental effort of the partner (Moreno and Osorno 2003). Vibrant blue-green color is produced by biliverdin, a derivative of heme (Sparks 2011). Biliverdin has antioxidant properties and therefore depositing it in the eggshell may be costly for a laying female. Moreno and Osorno (2003) argued that this is an honest signal that demonstrates the female's ability to control free radicals in the light of the handicap hypothesis (Zahavi 1975, but see: Penn and Számadó 2020). Twenty years after the original publication, the sexually selected eggshell coloration hypothesis (the SSEC hypothesis hereafter) was tested multiple times on many avian species and it gained some support in the case of biliverdin-pigmented eggshells (e.g., Moreno et al. 2004; Hanley et al. 2008; Moreno et al. 2008; Soler et al. 2008; Morales et al. 2012; but see: Krist and Grim 2007; Johnsen et al. 2011).

Another pigment present in eggshells of many avian species is protoporphyrin IX giving rise to reddish-brown hues (Kennedy and Vevers 1976). It is a direct precursor of heme, hence from one side depositing protoporphyrin in the shell can be perceived as a handicap, as it could have been used for heme synthesis instead (McGraw 2006). Further, owing to its pro-oxidant properties, protoporphyrin can induce oxidative stress leading to a higher activity of antioxidant enzymes (Afonso et al. 1999; Moreno and Osorno 2003). Alternatively, it may simply indicate high pro-oxidant level in a female's body and therefore, her worse condition (Sanz and García-Navas 2009). Thus, the predicted direction of relationship between female condition and protoporphyrin-based pigmentation is equivocal. Indeed, many studies found a negative relationship between the two: females in better condition were laying less pigmented eggs (Martínez-de la Puente et al. 2007; Stoddard et al. 2012; Hargitai, Nagy, et al. 2016; Hargitai, Boross, et al. 2016). Reynolds et al. (2009) noted that it is potentially not the high amount of pigmentation per se that signals a female's quality, but rather a consistence of pigmentation of eggshells in her clutch. Furthermore, Moreno et al. (2005) proposed that females, by means of

eggshell pigmentation, can signal the quality of the eggs and therefore, through maternal effects, a better conditions of chicks originating from such eggs. There is some support for a positive relationship between eggshell pigmentation and chicks' mass (Hanley and Doucet 2009; Poláček et al. 2017; but see: Hodges et al. 2020), or hematocrit (Hargitai et al. 2018). However, there is not much evidence that males respond to this signal: results for male parental effort are mixed. A study on the tree sparrow *Passer montanus* showed that feeding frequencies of males were higher in clutches with darker eggs (Poláček et al. 2017), while a study on the house wren *Troglodytes aedon* found an inverse relationship (Walters et al. 2014). Further, many studies did not find any relationship between eggshell appearance and male's investment both in open nesting species (Hanley and Doucet 2009; Bulla et al. 2012; Corti et al. 2018) and in cavity nesting species (e.g., Sanz and García-Navas 2009; Walters and Getty 2010; Stoddard et al. 2012; Hodges et al. 2020). Cavities are often dark environments (Wesołowski and Maziarz 2012) and there is an ongoing debate on whether birds are able to estimate color of eggs in such conditions (Reynolds et al. 2009; Holveck et al. 2010; but see: Chaib et al. 2023).

Since the SSEC hypothesis refers to the signaling function of the eggshell appearance, it is quite surprising that almost no study focusing on protoporphyrin used visual modeling to measure colors from birds' eyes perspective. As it was stressed multiple times in the literature, signals should preferably be measured from the ecologically relevant receiver's point of view (e.g., Stoddard and Stevens 2010; Caves et al. 2019). Meanwhile, only two studies followed this approach. Holveck et al. (2010) employed receptor noise-limited (RNL) model (Vorobyev and Osorio 1998) to investigate whether blue tit *Cyanistes caeruleus* males are able to perceive variation in eggshell coloration among clutches, one of the essential assumptions of the SSEC hypothesis. They concluded that whether birds can see differences in color or luminance (perceived brightness) inside of a nest-box depends entirely on assumptions of visual limitations in a model, but achromatic contrasts were more likely to be perceived than chromatic contrasts. Unfortunately, the authors did not compare results of visual modeling with males' provisioning rates. On the other hand, Stoddard et al. (2012) used visual modeling to measure speckling pattern from birds' perspective. They did not find any relationship between male provisioning rate and four explored eggshell pattern features in the great tit *Parus major*.

In this study, we aimed to test the SSEC hypothesis on a species laying protoporphyrin-pigmented eggs and nesting in cup nests where, unlike in cavities, light level does not limit birds' ability to assess eggshell color, luminance or patterning. As a model species, we selected red-backed shrike *Lanius collurio*, a monogamous Passerine with biparental care that nests in a farmland. This species exhibits a great variability in the appearance of eggshells: clutches

highly differ in terms of background coloration, color and size of spots, area covered by spots and dispersion of patterning (Surmacki et al. 2006; Stoddard and Stevens 2011). This diversity of appearance is well reflected in the high variability of concentration of protoporphyrin in eggshells (Miksik et al. 1994). Finally, red-backed shrikes build cup-nests (Cramp and Perrins 1993) and females do not cover eggs with nest material at any stage and thus, males can easily examinate coloration and patterning of clutches.

We tested the following assumptions of the SSEC hypothesis: (1) individual females lay eggs that are consistent in appearance, a premise of signaling (Moreno et al. 2004); (2) males can perceive variation in eggshell coloration or luminance among clutches in a population (Holveck et al. 2010); (3) eggshell pigmentation is related to female's and/or chicks' condition (Moreno and Osorno 2003; Moreno et al. 2005); (4) females in better condition lay more consistently pigmented clutches (Reynolds et al. 2009). Finally, (5) we investigated whether males adjust their parental investment with respect to eggshell pigmentation (Moreno and Osorno, 2003).

Methods

Fieldwork

The fieldwork was conducted between May and August in three consecutive years, 2020-2022, in a farmland in Western Poland (52°4′N, 16°18′E). The research area (around 2.5 km^2) is a mosaic of extensively used meadows, sparse fields with grain crops, abundant hedges of shrubs and trees, and drainage ditches. The area is abundant in red-backed shrikes, ranging between 7.2 and 10.9 pairs per km^2 (Bialas et al. 2024).

From the beginning of May the research area was surveyed at least twice a week in order to detect the onset of mating. When the mating began, all shrubs in the research area were searched for nests with a special attention to the shrubs within territories of red-backed shrike pairs. The majority of detected nests were up to two meters above the ground level. Nests located higher (4 out of 165) were excluded from the study due to potential difficulties with reaching eggs and chicks, or quick installation of cameras (see below). Females from this species lay one egg per day and usually 5 or 6 eggs in a clutch (Cramp and Perrins 1993). In case a nest was detected before the clutch completion, first egg laying date (FED hereafter) was calculated backward. When nest was detected at the incubation stage, FED was established later based on the date of hatching and assuming that the incubation lasts 14 days and starts when the penultimate egg is laid (Cramp and Perrins 1993). Since in this species there is a one-day

hatching asynchrony, when the hatching time approached, nests were controlled every two days to detect the day of hatching.

Eggs measurements

Photographs of clutches were taken in the second week of incubation. Eggs were placed on a custom-made holder with shallow pits for eggs. The holder was prepared using uniformly black thermoplastic mass (Fimo soft, Staedtler, Germany) and fixed to a small tripod (Malinowska et al. 2023). It was placed on the ground and aligned horizontally with the help of a bull's eye level. X-Rite ColorChecker (X-Rite, USA) including six gray standards and a scale bar was placed next to eggs, within the frame of every image. A digital camera (Sony Alpha 7 II with Sony FE 50 mm prime lens) was fixed to a tripod and levelled. Self-timer was used to avoid any camera movements. The camera was not converted to full spectrum and therefore only information in the human visible light (i.e., wavelengths between 400-700 nm) was registered.

Camera settings were kept constant throughout the entire study: aperture f/8, 50 mm focal length and ISO 320. A series of pictures with different exposition (bracketing mode) was taken and all pictures were saved in RAW format. We photographed in uniform weather conditions, only under clear skies, and avoided early morning and late evening hours when the sun is low (<10 degrees) above the horizon (Troscianko et al. 2016; Szala et al. 2023). In order to diffuse light and avoid overexposure, we took pictures of eggs in our own shade (Troscianko et al. 2016; Wilson-Aggarwal et al. 2016). We photographed all eggs from two sides with a 180° rotation around the long axis between photographs. To ensure a precise rotation, we marked a short and thin line on the side of every eggshell using a nontoxic pen. In total 470 eggs from 89 clutches were photographed.

Image analyses

Images were processed in Multispectral Image Calibration and Analysis (MICA) Toolbox version 2.1 (Troscianko and Stevens 2015a) a plug-in to an open-source ImageJ software version 1.53 (Schneider et al. 2012). The brightest and not overexposed photograph was selected from every bracketing series. Then, using six X-Rite's gray standards (reflectance: 1.99%, 8.52%, 19.03%, 37.35%, 64.51%, 92.96%), images were calibrated (Stevens et al. 2007; Troscianko and Stevens 2015a) and a 10 mm scale bar was set. Next, we selected eggs using multipoint selection tool and automatic fitting of the shape of egg (Troscianko 2014). We then searched again for overexposed pixels (values > 100) in every channel using a custom-written

function for MICA Toolbox. Because overexposure results in incorrect pixel values, we excluded an egg from further analyses if more than 0.1% of its surface was overexposed. We also excluded a clutch if more than half of its eggs was overexposed. Using Scale Bar Calculator Tool we found the smallest scale bar in our set of pictures, rounded it down to 20 px/mm and resized all images according to this factor to make comparisons of pattern among pictures possible (Troscianko and Stevens 2015b).

Using MICA Toolbox, we converted normalized images of eggs to quantum catches of birds. Cones sensitivities of the red-backed shrike are unknown (Renoult et al. 2010), hence following Stoddard and Stevens (2011) we repeated all subsequent analyses using visual models of two species: the blue tit *Cyanistes caeruleus* and the Indian peafowl *Pavo cristatus* (both modeled as trichromats – without UV light). Since results for both visual models are very similar, we provide only results for the blue tit visual model in the main text. A complete set of results for the peafowl visual model can be found in the Appendix (Tables A3-A5, Figures A3-A6). Using custom written scripts for MICA Toolbox, we segmented images of every egg into eggshell background and spots. To do this, we used a built-in ImageJ local thresholding function (Phansalkar et al. 2011). Additionally, to compensate for the effect of darker edges of eggs and improve detection of spots, we employed a difference of Gaussian blur following Gómez et al. (2018). We used double cones channel for spots detection (Stoddard and Stevens 2010) and following settings: 50 px radius for thresholding, 181 px Gaussian blur, 20 px/mm scaling factor. We also decreased selection around every egg by 5% of an egg's width to avoid measuring a black background that eggs were lying upon when photographed. We visually compared segmented images with original pictures of eggs and excluded 16 eggs with poorly detected spots. Excluding overexposed eggs (see above) and eggs with poorly detected spots resulted in the number of 410 eggs from 79 clutches that were used in further analyses. The segmented images of eggs allowed us to calculate percent of eggshell surface covered by spots (percent of spots hereafter), average spots size, and dispersion of patterning (measured as a coefficient of variation of percent of spots in six horizontal bands along the long axis of the egg; dispersion hereafter). Furthermore, using the segmented images as masks, we could measure luminance and color of spots and background separately. We measured luminance as quantum catch for double cones (Stoddard and Stevens 2011). To measure color, we used logarithms of quantum catches for long-, medium- and short-wavelength sensitive single cones (henceforth, LW, MW, and SW cones respectively) and plotted them into a perceptually calibrated color space (Kelber et al. 2003; Renoult et al. 2017; van den Berg et al. 2019). The localization of a point in the color space represents a relative stimulation of cones by a light stimulus. We did not include

cones sensitive to very short wavelengths in the calculations, and therefore, the resulting color space can be described by two opponent axes. X axis represents relative excitation of LW and MW cones, while Y axis represents relative excitation of the summed output of LW and MW cones versus SW cones. In a human perceiver, X axis corresponds to the red-green opponency channel (with higher values representing redder colors) and Y axis to the blue-yellow opponency channel (with higher values representing bluer colors). Although we do recognize that terms such as "red" and "blue" are anthropocentric and may not correspond to subjective sensations experienced by birds, for simplicity we will refer to X axis as "redness" of spots and background coloration from now on. Additionally, we calculated saturation which is a distance between the achromatic center of the color space and a point representing an object (Renoult et al. 2017). Finally, we calculated chromatic (Vorobyev and Osorio 1998) and achromatic contrasts (Siddiqi et al. 2004) between eggshell background and spots as a measure of spots distinctiveness. It has been shown that chromatic contrast between eggshell background and spots can be an important cue for birds that, for example, affects rejection probability of a foreign egg in American robin *Turdus migratorius* (Dainson et al. 2017). We assumed that light intensity in a cup nest does not limit visual discriminability and thus, we calculated quantum catches using only neural noise and selected D65 standard daylight illumination. We used Weber fraction of 0.05 for the most abundant cone type and cone ratios: 1.92:2.68:2.7 for the blue tit (Hart et al. 2000) and 1.9:2.2:2.1 for the peafowl (Hart 2002). Contrasts are expressed in units of Just Noticeable Differences (JNDs) and values <1 JNDs indicate that two stimuli are indistinguishable to a receiver, two stimuli of contrast equal to 1 JNDs are distinguishable under ideal viewing conditions, and values >1 JNDs are becoming increasingly distinguishable. All custom written scripts for MICA Toolbox are available from the GitHub repository (https://github.com/KlaudiaSzala/eggshell-spots) or by contacting directly with the corresponding author (KS).

To investigate how consistent eggs color and luminance is within clutches, we used quantum catches to calculate chromatic and achromatic contrast between pairs of eggs from the same clutches, separately for spots and eggshell background (893 pairs; hereafter contrasts within clutches) in *pavo* package for R (Maia et al. 2019). Similarly, to verify whether males can perceive a difference in coloration or luminance among eggs from different clutches in the focal population, we calculated contrasts between pairs of eggs from different clutches (82952 pairs; hereafter contrasts between clutches), separately for background and spots.

Finally, we used Batch Multispectral Image Analysis in MICA Toolbox for automatic measurements of patterning of eggs. We selected double cones channel for granularity pattern

analysis (Stoddard and Stevens 2010) and used following settings: 20 px/mm scaling factor, start size: 2 px, end size: 64 px, step size: √2, and step size was multiplied, resulting in 11 spatial scales. The four variables describing pattern spectra are as follows: maxFreq is the filter size for the dominating spot size, maxPower is the contrast for the dominating spot size, sumPower is the overall contrast of the pattern, and propPower is the degree of dominance of the spot size with the highest energy in the overall pattern (Troscianko and Stevens 2015b).

Similarly as for color and luminance, we explored how consistent egg patterning is comparing average pattern difference within and between clutches. We calculated a difference between pattern spectra for pairs of eggs (i.e., surface between two spectra) derived from granularity pattern analysis in MICA Toolbox. To obtain the sum of difference for all scales, we first calculated absolute difference in energy for every spatial scale and then added the differences over all spatial scales for a given pair of eggs. Difference between pattern spectra for pairs of eggs was calculated using a custom written script in R.

All measured continuous pigmentation traits were highly repeatable for two images of every egg (two "sides") and thus, we used averaged values in analyses. Repeatability was calculated as inter-correlation coefficient using the Linear Mixed Models-basing approach for Gaussian data in *rptR* package (Stoffel et al. 2017). Detailed repeatability values are presented in Tables A1 and A2 in the Appendix. Instead, since maxFreq is a discrete variable, we randomly sampled the value for one side of every egg. Similarly, for comparing pattern spectra among eggs we randomly sampled the spectrum of one "side" of every egg (i.e., we did not average two pattern spectra for the same egg).

Measuring condition of birds

Adult birds were captured using mist nets (16 x 16 mm, Ecotone, Poland) set in the proximity to a nest at the nestling stage. Each individual was banded and standard biometric measurements were taken following the methodology described in Svensson (1992): the length of the right wing (with a metal ruler to the nearest 0.5 mm), the length of the head with beak and the length of the left tarsus (both with a plastic caliper to the nearest 0.1 mm), and mass (with a table balance to the nearest 0.1 g). All measurements were made by one person (KS) to avoid observer-based variability (Goodenough et al. 2010). Since the precision of the measurement strongly depends on the experience of the observer, two measurements of tarsus and head with beak were made and the results were averaged (Yezerinac et al. 1992). Left innermost tail feather (or the closest, when left innermost feather was lacking) was collected

from every female for growth bars measurements. Feathers were individually stored in plastic string bags in the darkness, in the room temperature.

Nestlings were measured at 9^{th} or 10^{th} day of life (hatching day = 1) in the same way as adults i.e., mass, wing, tarsus and head with beak. Using venipuncture, a blood sample (approx. 10-20 µl) was taken from the brachial vein of every nestling. The capillaries with blood were individually placed in 1.5 ml Eppendorf tubes with 96% ethanol and stored in a refrigerator for further sex analysis. Molecular sexing of chicks was done using a standard protocol for birds (e.g., Podkowa and Surmacki 2022). Briefly, DNA was isolated from blood samples using DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). Every sample of isolated DNA was diluted 20 times and 1 µl of DNA matrix was mixed with 5 µl of PCR mix (containing 2.5 µl of polymerase, 0.1 µl of P2bs-6FAM starter, 0.1 µl of P8bs starter and 2.3 µl of H_2O MiliQ). Sex was determined based on the amplification of CHD1W and CHD1Z genes (Griffiths et al. 1998). Sex ratio was calculated as the number of females divided by the total number of chicks in a given brood.

Condition of individuals was expressed as Scaled Mass Index (SMI, Peig and Green 2009). As L_0, we used the mean length of head with beak in the studied population, separately for females and chicks (37.588 and 26.154 mm respectively). Scaling exponent (b_{SMA}) was calculated by dividing the slope from an ordinary least squares regression of mass versus head with beak length in logarithmic scale by Pearson's correlation coefficient between these two traits (b_{SMA} = 2.81 for females and 2.26 for nestlings).

Growth bars at tail feathers of females were measured manually following the protocol described in Grubb (2006). Briefly, we put a feather on a piece of paperboard covered with a piece of black paper and pinned the feather (and underlying paperboard) at the top and bottom of the feather and at the distal edge of every dark bar that was visible. Using a ruler, we measured the feather's length to the nearest 0.5 mm and marked a line of 2/3 of the feather's length starting from the calamus. Then, we selected a bar the closest to the line, marked three bars upwards and three bars downwards, whenever possible, and measured their length using the same ruler. Finally, we divided the length by the number of bars to obtain average width of growth bars. Since the red-backed shrike moults during wintering period in Africa, we assumed that average width of growth bars was a proxy of nutritional condition during the pre-breeding stage (Saino et al. 2012). All growth bars measurements (N = 44 feathers) were done by one person (KS) and to make sure the measurements were consistent, a random subsample of 22 feathers was remeasured and repeatability of measurements was calculated (R = 0.791, 95% CI:

0.548-0.899). To control for different size of females, we divided average width of growth bars by tarsus length (Grubb 2006).

In total, we measured 44 females and 217 chicks from 44 broods. Fieldwork was carried out in accordance with the current Polish law: fieldwork permit of the Regional Directorate of Environment Protection in Poznań numbers: WPN-II.6401.345.2019.KL (2020), WPN-II.6401.77.2021.MT (2021) and WST.6401.92.2022.MT.2 (2022), and permit of the Local Ethics Commission in Poznań number 13/2021. All birds were banded under the license provided by the Polish Ornithological Station in Gdańsk.

Male investment

The provisioning effort of males was measured at two stages: 4^{th} or 5^{th} and 9^{th} or 10^{th} day of chicks' life (henceforth, stage 1 and 2 respectively; hatching day = day 1). The behavior of birds was recorded with GoPro Hero 7 Black cameras. A camera was attached with multi-use plastic clamps to a branch in a close vicinity to a nest and wrapped in a piece of fabric with a camouflage pattern in a way that only the lens was visible. The camera was left turned off for around one hour to let birds to habituate. After that time, the camera was turned on remotely using GoPro Smart Remote from a distance of several dozen meters and it recorded continuously for around 1.5 hour, until the battery discharged. Then, it was removed from the shrub.

We collected recordings from 63 nests at stage 1 and from 44 nests at stage 2. Lower sample size for stage 2 is due to breeding failures as a consequence of predation, which is high in this area (Bialas et al. 2024). Recordings were analyzed in Boris software (Friard and Gamba 2016). The red-backed shrike brings only one prey item to the nest at once (Carlson 1985). Consequently, provisioning effort of males was quantified as a number of provisioning visits per one chick per one hour. Provisioning effort of females was calculated in the same fashion and was used as a covariate in models for male's investment. All recordings were analyzed by one person (KS).

Statistical analyses

First, we investigated if median chromatic and achromatic contrasts within clutches were lower than median contrasts between clutches. Since the distributions of contrasts were not normal, we used one-sided Wilcoxon rank sum test. We also checked if median contrasts between clutches were >1 JNDs using Wilcoxon signed-rank test. Following Holveck et al. (2010), we calculated contrasts separately for spots and background coloration. Furthermore,

we checked if median difference between pattern spectra was lower for pairs of eggs from the same clutches than for pairs of eggs from different clutches and due to non-normal distribution, here we also applied one-sided Wilcoxon rank sum test. In all cases, effect size r was calculated as Z-statistic divided by the square root of the sample size and the interpretation is: small effect for $r \leq 0.3$, moderate effect for r between 0.3 and 0.5 and large effect for $r \geq 0.5$ (Kassambara 2021).

Second, we explored if females in better condition (i.e., with higher SMI or wider growth bars) laid clutches that were more consistent in appearance. We quantified the consistence of appearance by means of calculating median chromatic and achromatic contrasts within clutches (separately for spots and background) and median pattern difference for pair-wise comparisons between eggs within clutches. Since the contrasts and pattern differences were not normally distributed, we used Spearman's rank correlation coefficient to assess the relationship between condition of females and consistence of their clutches appearances.

Third, to investigate relationships between eggshell pigmentation traits and (1) condition of chicks, (2) condition of females, and (3) male provisioning effort, we used linear regression models (in each case response variable was normally distributed). Since all eggshell characteristics were significantly repeatable within clutches (R from 0.962 for redness of spots to 0.548 for pattern dispersion, all $p < 0.001$, see Appendix Tables A1 and A2 for details) models were fitted using mean values for broods (i.e., mean values for pigmentation traits or for chicks condition). Prior to modeling, we checked correlation among eggshell pigmentation variables selecting variables that were not highly correlated with one another (Pearson's correlation coefficients below 0.5 or above -0.5, full correlation matrix can be found in the Appendix, Figure A1 and A2) and conveying different aspects of egg appearance. The selected variables were: percent of spots, average spot size, dispersion, propPower, redness of spots, luminance of background, and chromatic and achromatic contrast between background and spots (distinctiveness of spots). Additionally, to control for potential collinearity among explanatory variables, we calculated Variance Inflate Factors (VIFs) using *car* package (Fox and Weisberg 2019) and due to high VIF value (> 3) for achromatic contrast between background and spots, average size of spots and dispersion, we excluded these three variables from models. Prior to modeling, we centered and scaled all numeric continuous explanatory variables.

We prepared a set of five full models. In the first model we investigated the relationship between SMI of chicks and the selected eggshell pigmentation traits, while controlling for egg volume, sex-ratio of chicks and clutch size. In the second and the third model we explored the

relationship between female condition (SMI and average width of growth bars respectively) and the selected eggshell pigmentation traits, while controlling for egg volume, SMI and tarsus length of chicks, sex-ratio of chicks and clutch size. Finally, in the fourth and the fifth model we explored the relationship between eggshell pigmentation traits and male provisioning effort (for stage 1 and 2 respectively), while controlling for egg volume, chicks sex-ratio, SMI of females and provisioning effort of females. In all models we additionally controlled for possible differences among seasons by introducing a year of study as a factor. To ensure that residuals of every full model were normally distributed we employed the Shapiro-Wilk test and additionally inspected the residuals visually. We used full models to generate sets of candidate models with different combinations of predictors. We ranked candidate models with increasing AICc values and considered models with ΔAICc ≤ 2 compared to the model with the lowest AICc as equally informative (henceforth, final models). We averaged estimates of model parameters across final models and assumed estimate to be zero if an explanatory variable was not included in a given final model (i.e., we calculated full average, Bartoń 2022). This approach insulates from inflating estimates of model parameters for variables that appear only in a few number of models that are being averaged (that is, for explanatory variables that are weakly related to the dependent variable). We additionally calculated sum of weights of final models that included a given explanatory variable as our measure of importance of predictors. Model averaging has been shown to perform better at making inference than model selection based on stepwise selection procedure, especially in case when the number of variables used in a model is close to the sample size (Lukacs et al. 2010). VIFs for all final models were lower than 2 and residuals were normally distributed. Sample size differed depending on the model and was: 42 nests for condition of chicks, 34 nests for condition of females, and 33 nests for the provisioning effort of males (for both stages).

All statistical analyses were performed in RStudio version 4.3.2 (R Core Team 2023), model selection was done using *MuMIn* package (Bartoń 2022), Wilcoxon tests in *rstatix* package (Kassambara 2021) and plots were prepared in *ggplot2* (Wickham 2016).

Results

Within clutch consistence

Median chromatic contrast within clutches was significantly lower than median chromatic contrast between clutches both for spots ($W = 66196481$, $p < 0.001$, $r = 0.140$) and background ($W = 65107305$, $p < 0.001$, $r = 0.135$, Figure 1A). Likewise, median achromatic contrast within

clutches was significantly lower than median achromatic contrast between clutches both for spots ($W = 55794340$, $p < 0.001$, $r = 0.090$) and background ($W = 52518244$, $p < 0.001$, $r = 0.074$, Figure 1B). Detailed values are presented in Table 1.

Median difference between pattern spectra was 0.010 (interquartile range = 0.008) within clutches and 0.020 (interquartile range = 0.016) between clutches. Median difference in pattern spectra was lower within than between clutches, $W = 57503762$, $p < 0.001$, $r = 0.098$ (Figure 2).

Variability between clutches

Median chromatic contrast between clutches was significantly higher than 1 JNDs for spots ($W = 3196426083$, $p < 0.001$, $r = 0.743$) and for background ($W = 3118021418$, $p < 0.001$, $r = 0.704$, Figure 1A) and the same was true for achromatic contrast for spots ($W = 3218723525$, $p < 0.001$, $r = 0.754$) and background ($W = 3074962651$, $p < 0.001$, $r = 0.682$, Figure 1B).

Condition of chicks and appearance of eggshells

Redness of spots was the only significant term in the averaged model predicting SMI of chicks and it was positively related to condition of chicks (Figure 3A, Table 2). Other variables included in the model did not reach statistical significance and their sums of weights were lower than for redness of spots (Figure 4A). Final models for SMI of chicks explained between 14.26 and 20.04% of variance (adjusted R^2).

Condition of females and appearance of eggshells

SMI of females was positively related to clutch size, length of chicks' tarsus and chromatic contrast between spots and background (distinctiveness of spots) and negatively related to redness of spots (Figure 3B). Additionally, there were differences in SMI of females among years (Table 2). All significant variables had the same sum of weights (Figure 4B). Final models for SMI of females explained between 53.49 and 57.41% of variance.

Width of growth bars of females was negatively related to redness of spots (Table 2). The remaining explanatory variables in the averaged model were not statistically significant and their sums of weights were lower than for redness of spots (Figure 4C). Final models for width of growth bars of females explained between 45.05 and 48.85% of variance.

Consistence of eggshells' appearance within clutches and female condition

Females with wider growth bars laid eggs that had on average lower chromatic contrast of spots among eggs within a clutch ($r = -0.38$, $p = 0.017$). However, for all but one clutch these contrasts were < 1 JNDs. (Figure 3C). For the remaining traits, correlation between condition of females and consistence of eggshells' appearance within clutches was not significant (r between -0.27 and 0.27, $p > 0.1$).

Appearance of eggshells and male provisioning effort

Year of study was the only significant explanatory variable in the averaged model explaining male provisioning effort at stage 1 (Table 2). The sums of weights of two further variables included in the model (redness of spots and SMI of females) were smaller (Figure 4D). In the averaged model predicting male investment at stage 2, only sex ratio of chicks reached statistical significance – males provisioned more to clutches with more females among chicks. Percent of spots was the second most important explanatory variable in terms of sum of weights (Figure 4E), but it did not reach statistical significance, similarly as other explanatory variables included in the averaged model (provisioning effort of females, SMI of females and volume of eggs, Table 2). Final models for male provisioning effort explained between 13.04 and 13.87% of variance at stage 1 and between 12.75 and 27.35% of variance at stage 2.

Detailed information about the final models that were included in model averaging are provided in the Appendix (Table A6).

Discussion

The results of this study provided a mixed support for the SSEC hypothesis in the red-backed shrike. We showed that individual females laid eggs of consistent appearance within clutches and that males were likely to perceive differences in color and luminance of clutches of different females. Further, we found a relationship between both females' and nestlings' condition and different aspects of eggshell pigmentation, however, the direction of relationship differed for either of them. Finally, the appearance of eggs had no effect on the provisioning effort of males.

Our results indicate that pairs of eggs from the same clutches had on average smaller chromatic and achromatic contrasts (both for spots and background) than pairs of eggs from different clutches. Especially, in the majority of cases chromatic contrast within clutches was below the threshold of 1 JNDs, which means color of spots and background of many eggs from the same clutches were likely indistinguishable to a male. This is in line with other studies

showing that many eggshell pigmentation characteristics are repeatable within clutches (e.g., Gosler et al. 2000; Hanley and Doucet 2009; Bulla et al. 2012; Corti et al. 2018; Gómez et al. 2021), and notably with the results of Holveck et al. (2010) who also measured contrasts using RNL models. Lower average pattern difference within than between clutches also indicates some degree of consistence in patterning of eggs laid by individual females. Thus, high consistence of appearance of eggs within clutches suggests a good potential for the signaling function (Moreno et al. 2004).

Furthermore, we demonstrated that on average eggs from different clutches had contrast values above the threshold of 1 JNDs, which means differences in color and luminance were likely discriminable to birds' eyes in many cases. However, we did not include UV light in our measurements which is a simplification, because single cones sensitive to very short wavelengths take part in color vision of birds (Vorobyev and Osorio 1998). Modeling their color vision as trichromatic may have missed some of the variability of colors that birds can perceive. Instead, the perception of luminance and patterns in avian visual systems is the function of double cones that are not sensitive to light below 400 nm due to oil droplets filtering out shorter wavelengths (Hart 2001; Osorio and Vorobyev 2005). In consequence, our measurements of luminance and pattern should not be affected by the lack of UV light. Similarly as for contrasts, the average difference of pattern spectra was higher between than within clutches. However, while being significant, the effect size of the difference between groups was small. Furthermore, for pattern difference, unlike for contrasts, there is no established perceptual discrimination threshold that would allow us to state whether males could see the difference of such a magnitude.

Chicks with higher SMI, and hence in better condition, originated from clutches with redder spots, which supports the idea that eggshell pigmentation can signal maternal investment in eggs (Moreno et al. 2005). Similarly, Hanley and Doucet (2009) and Poláček et al. (2017) found a positive relationship between body mass of chicks and red chroma of eggshells in the ring-billed gull *Larus delawarensis* and darkness of eggshells in the tree sparrow respectively (but see Hodges et al. 2020 for an opposite relationship in the house wren). Higher intensity of spotting was also positively related with hematocrit of nestlings in the great tit (Hargitai et al. 2018) and with hatching success in the barn swallow *Hirundo rustica* (Corti et al. 2018). A number of studies explored a more direct relationship between pigmentation and maternal investment in eggs measuring different components of egg content, for example the amount of lysozyme (Krištofík et al. 2013) and concentration of antibodies (Holveck et al. 2012). We did not measure any aspects of egg composition, but using egg volume as a proxy of female

investment we did not find any relationship between the volume and appearance of eggshells, or between the volume and condition of chicks (Appendix Table A7 and Figure A7). Further, condition of chicks and eggshell pigmentation can be simply correlated without any signaling function (Stoddard et al. 2012) and due to the correlative nature of this study we cannot rule out such a possibility. Pigmentation can also serve some proximate functions during embryo development, such as thermoregulation (Wisocki et al. 2020) or UV protection (Shafey et al. 2004).

Instead, in case of females, we found a negative relationship between their condition and eggshell pigmentation. Specifically, females laying eggs with redder spots had lower SMI and narrower daily growth bars. This is in accordance with some of the previous studies on species with protoporphyrin-pigmented eggs. For example, higher pigmentation was associated with lower mass (Stoddard et al. 2012) and higher level of leukocytes and less colorful feathers (Hargitai, Nagy, et al. 2016) in the great tit. In the canary *Serinus canaria* females with lower Body Mass Index laid eggs with darker spots (Hargitai, Boross, et al. 2016). Similarly, blue tit females that were lighter and more infested by the blood parasite *Leucocytozoon* laid eggs more covered in spots (Badás et al. 2017). Further, higher redness of eggs was related to higher concentration of Heat Schock Proteins in the same species (Martínez-de la Puente et al. 2007). On the other hand, some studies provided evidence that females in better body condition produce more pigmented eggs. For instance, females with more pigmented eggs had longer tarsi (Sanz and García-Navas 2009) and females laying eggs with higher percent of spots exhibited more saturated breast feathers in the blue tit (Badás et al. 2017) and darker breast feathers in the barn swallow (Corti et al. 2018). Further, females with lower heterophils to lymphocytes ratio (less stressed) laid eggs with higher number of spots in the Eurasian coot *Fulica atra* (Minias et al. 2020). This apparent discrepancy may result from different measures of body condition (such as mass, condition indices, physiological indicators, color of feathers) and eggshell coloration in different studies. Nevertheless, the negative sign of relationship between condition of females and eggshell pigmentation does not exclude its signaling function. Considering pro-oxidative properties of protoporphyrin (Afonso et al. 1999), intensive pigmentation of eggshells may reflect females' capacity to remove the potentially harmful substance from their bodies (Moreno and Osorno 2003). Analogous mechanism has been described to explain a negative correlation between pheomelanin-based coloration and juvenile body condition in Eurasian nuthatches *Sitta europaea* (Galván 2017). Further, Gosler et al. (2005) showed that protoporphyrin can serve as a structural reinforcement of the shell and in

this light, females laying more pigmented eggs may be the ones, who suffer from higher calcium deficiency.

Further, we showed that females with wider growth bars laid eggs that were more similar to one another in terms of lower chromatic contrast of spots among eggs within a clutch. This supports the idea of Reynolds et al. (2009) that females in superior condition lay more consistently pigmented eggs. Similarly, it was demonstrated that females in better condition laid clutches of lower variation of color for biliverdin-based pigmentation of the great reed warbler *Acrocephalus arundinaceus* (Polačiková et al. 2009), however, in the case of protoporphyrin-based pigmentation an opposite relationship was found in the great tit (De Coster et al. 2013). Yet, since the vast majority of contrasts within clutches in our dataset did not exceed 1 JNDs (Figure 3C), it is unlikely that the consistence of appearance of eggs within a clutch could serve as a signal of female condition to males. Taken together, our results show that the red-backed shrike females in better condition produced less pigmented clutches that were more uniform in appearance. It contradicts the function of protoporphyrin as a valuable resource that a female sacrifices to advertise her good condition (sensu Zahavi 1975), but does not exclude a potential signaling function of the appearance of eggs.

Provisioning effort of males was not affected by the eggshell pigmentation. The effect of the year was the only significant term in the model predicting the provisioning effort of males towards younger chicks. For older chicks, percent of spots was a predictor with a relatively high importance (as measured by means of the sum of weights), but it did not reach statistical significance. The only significant predictor in the model was the sex ratio of chicks – males invested more into broods with higher proportion of females. This is consistent with the results of a study on a closely related species, the southern gray shrike *Lanius meridionalis* suggesting that female chicks are more costly to raise than their male siblings (Moreno-Rueda et al. 2014). For both stages, predictive power of the models, in terms of the proportion of explained variance, was low.

Lack of the response of males to eggshell pigmentation was not due to their inability to perceive variation in luminance or coloration among clutches (see above). Instead, it may suggest that males modulate their parental investment depending on other cues. As Stoddard et al. (2012) pointed out, apart from eggshell pigmentation, males can increase their parental effort in response to female's or nestlings' quality. Due to the correlative nature of our study, we could not separate these effects. However, we controlled for the condition of females, as we included it as one of the explanatory variables in the models. It did not affect the provisioning effort of males. Some studies used cross-fostering experiments to break the relationship between

condition of females and eggshell pigmentation. However, none of the four cross-fostering experiments performed on species with protoporphyrin-based pigmentation found any relationship between investment of males and pigmentation of cross-fostered eggs (Hanley and Doucet 2009; Walters and Getty 2010; Stoddard et al. 2012; Hodges et al. 2020). Finally, the SSEC hypothesis assumes that eggshell pigmentation, as estimated visually by a male, is a good predictor of pigment concentration in the shell. However, this is often not the case (e.g., Wegmann et al. 2015; Poláček et al. 2017), likely because layers of pigment can be deposited deeper in the shell (Harrison 1966).

The SSEC hypothesis assumes that it is possible to males to observe clutches of their conspecifics. This is likely to be the case in colonial breeding birds, in polygynous species, or in species exhibiting some levels of extra-pair copulation (Moreno and Osorno 2003), whereas most studies to date (including this one) were carried out on socially monogamous species with established territories (Riehl 2011, but see: Holveck et al. 2010). Furthermore, Cherry and Gosler (2010) pointed out that reducing investment into the present reproductive attempt in response to a lower than expected quality of the mate is of questionable value to the fitness in species with low probability of surviving until the next breeding season. Birds with high mortality rates should maximize reproduction in the current breeding season (Williams 1966). In this context, Cherry and Gosler (2010) suggested that the SSEC hypothesis is more likely to work in long-living species, such as seabirds, where partners breed together for several years and where adjusting relative investment of both mates could potentially reduce parental conflict and increase reproductive success of either part. Alternatively, Mock et al. (2005) and Lyu et al. (2017) provided theoretical models indicating that costly post-mating signals advertised by females may evolve in response to males experiencing a trade-off between investing into parental care and seeking opportunities for extra-pair copulations during the same breeding season. The models predict that in case a male is paired with a female exhibiting higher expression of costly traits, he should allocate more effort to the current breeding attempt instead of searching for other opportunities. Empirical support for the models have been provided (Mock et al. 2005). This creates an intriguing, yet largely unexplored (but see: Badás et al. 2017) line of research: can eggshell pigmentation influence a male's decision to invest into the current breeding attempt rather than seeking for other opportunities?

Our study is a rare example of a study in which multiple assumptions of the SSEC hypothesis were tested at once. Moreover, we quantified many aspects of eggshell appearance and used methods which directly refer to vision of birds. We found that many, but not all, tested assumptions of the SSEC hypothesis are met in the red-backed shrike. Namely, eggs from the

same clutches have similar appearance and further, visual models suggest that males can perceive differences in color and luminance between clutches. Also, there was a link between eggshell color and female and chicks condition. However, males did not adjust the amount of their provisioning effort to eggshell pigmentation. Therefore, we did not find support for the idea that eggshell pigmentation acts as a signal affecting investment of males in the red-backed shrike. These results highlight the importance of a complex approach when testing the SSEC hypothesis.

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Data Availability Statement

Data and R code are currently provided in the supplementary material to facilitate the review process. They will be uploaded to Dryad repository upon acceptance of the manuscript. Custom-written scripts for MICA Toolbox are available from the GitHub repository: https://github.com/KlaudiaSzala/eggshell-spots

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. A) Comparison of average chromatic contrast for pairs of eggs from the same clutches (blue boxplots) and from different clutches (red boxplots). **B)** Comparison of average achromatic contrast for pairs of eggs from the same clutches (blue boxplots) and from different clutches (red boxplots). Contrasts for background and spots were calculated separately. Units for y-axes are Just Noticeable Differences (JNDs). Horizontal dashed lines indicate JNDs = 1, a threshold above which the contrast is considered to be perceived by a receiver. Numbers above plots indicate percent of pairs of eggs in a given group for which contrast values were >1 JNDs. In both cases, the visual model of the blue tit *Cyanistes caeruleus* was used.

Figure 2. Difference between pattern energy spectra of eggs from different clutches (red boxplot) and the same clutches (blue boxplot) for the visual model of the blue tit *Cyanistes caeruleus*.

Figure 3. A) The relationship between average condition (Scaled Mass Index) of chicks in a brood and redness of eggshell spots; **B)** The relationship between female condition (Scaled Mass Index) and redness of eggshell spots; **C)** The relationship between median chromatic contrast of spots between pairs of eggs from the same clutches and average width of female growth bars. Units for the y-axis are Just Noticeable Differences (JNDs). Horizontal dashed line indicates JNDs = 1, a threshold above which the contrast is considered to be perceived by a receiver. In all three cases, red dashed line shows linear regression and shaded area depicts 95% confidence interval. Visual model of the blue tit *Cyanistes caeruleus* was used.

Figure 4. The importance of predictors in the models. Headers indicate the response variables. Heights of the bars show sum of weight for predictors and numbers above bars indicate number of models in which predictors were present. Total number of models with ΔAICc ≤ 2 (that were later averaged) were: 5 for Scale Mass Index of chicks, 2 for Scale Mass Index of females, 8 for width of growth bars of females, 3 for male effort at stage 1 (chicks between 4^{th} and 5^{th} day of life) and 8 for male effort at stage 2 (chicks between 9^{th} and 10^{th} day of life). Visual model of the blue tit *Cyanistes caeruleus* was used.

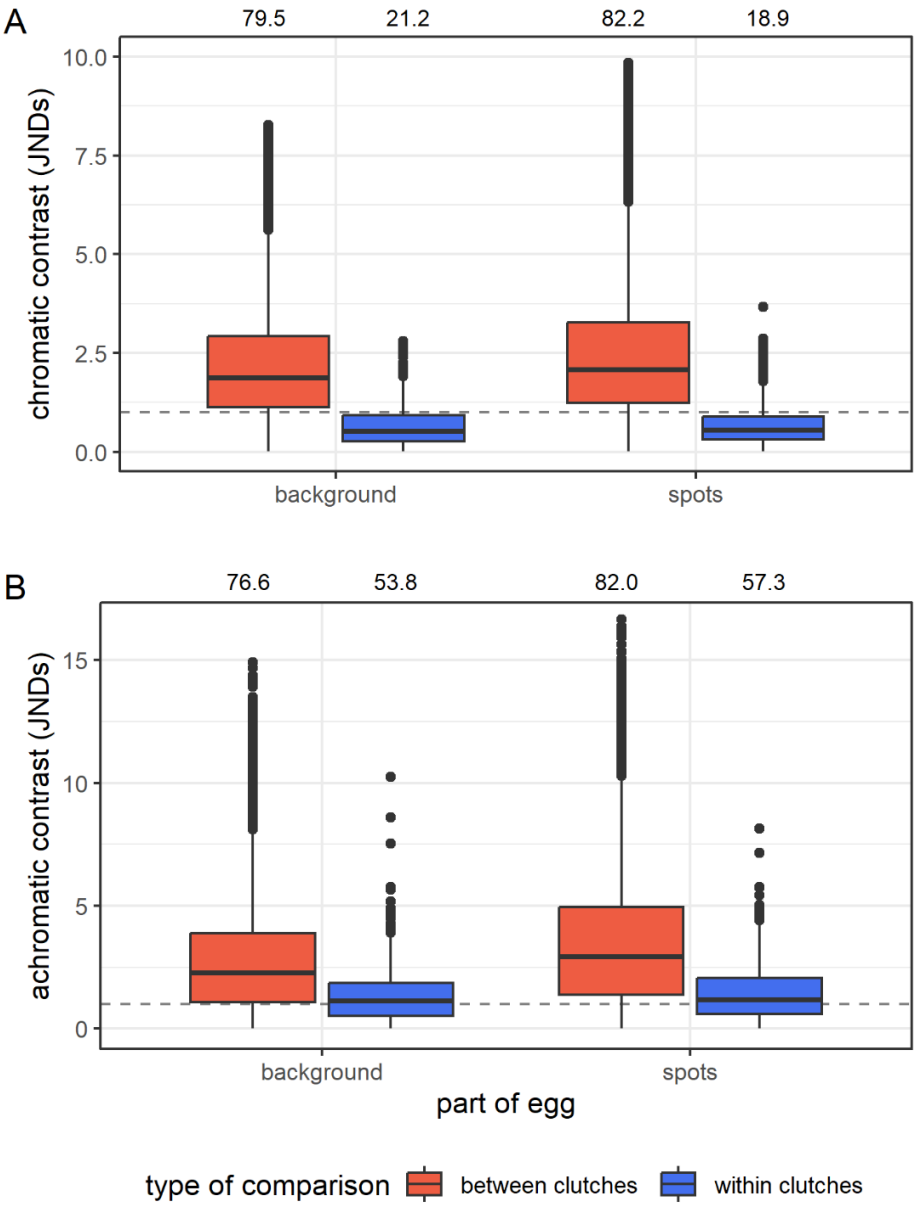
Tables and Table Legends

Table 1. Median and interquartile range (IQR) of chromatic and achromatic contrasts for pairwise comparisons between eggs of the red-backed shrike *Lanius collurio*. Pairwise comparisons were calculated for pairs of eggs from the same clutches (within clutches) and pairs of eggs from different clutches (between clutches), separately for eggshell background and for spots. Visual model of the blue tit *Cyanistes caeruleus* was used.

Part of egg	Type of comparison	Chromatic contrast		Achromatic contrast	
		median	IQR	median	IQR
background	between clutches	1.872	1.800	2.265	2.813
background	within clutches	0.508	0.658	1.133	1.354
spots	between clutches	2.083	2.031	2.922	3.556
spots	within clutches	0.540	0.580	1.189	1.469

Table 2. Results of averaged models (full average). SE stands for standard error and SMI for Scaled Mass Index, sex-ratio is sex-ratio among chicks (calculated as number of females / number of all chicks), dS is chromatic contrast between spots and eggshell background (distinctiveness of spots), spots_X is redness of spots, bg_lum is luminance of background, and propPower is proportion of energy (how much dominating spot size dominates in overall patterning). Suffix "_F" indicates measurements of females and "_PULL" measurements of chicks. Visual model of the blue tit *Cyanistes caeruleus* was used.

Scaled Mass Index of chicks					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	21.1369	0.2235	0.2303	91.7905	<0.001
percent_spots	-0.3071	0.2248	0.2293	1.3390	0.1806
spots_X	0.5334	0.2043	0.2106	2.5323	0.0113
propPower	-0.0954	0.1858	0.1884	0.5065	0.6125
year2021	0.0929	0.2786	0.2823	0.3290	0.7422
year2022	-0.0263	0.1995	0.2056	0.1280	0.8982
dS	-0.0305	0.1121	0.1143	0.2670	0.7895
Scaled Mass Index of females					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	24.6407	1.7069	1.7881	13.7801	<0.001
clutchSize	1.0754	0.3114	0.3263	3.2961	0.001
dS	0.7708	0.2300	0.2406	3.2035	0.001
SMI_PULL	0.2284	0.2576	0.2626	0.8696	0.385
spots_X	-0.6499	0.2494	0.2598	2.5016	0.012
tarsus_PULL	0.7494	0.2214	0.2316	3.2361	0.001
year2021	-1.2081	0.4820	0.5047	2.3935	0.017
year2022	-2.1053	0.5254	0.5506	3.8239	<0.001
Width of growth bars of females					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	0.1187	0.0051	0.0052	22.6214	<0.001
propPower	-0.0011	0.0012	0.0012	0.9160	0.3596
spots_X	-0.0035	0.0010	0.0011	3.2171	0.0013
dS	-0.0016	0.0014	0.0014	1.1211	0.2623
bg_lum	0.0003	0.0008	0.0008	0.4319	0.6658
clutchSize	0.0003	0.0009	0.0009	0.3631	0.7165
Male effort - stage 1					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	1.125	0.3300	0.3438	3.2724	0.0011
year2021	0.5074	0.4646	0.4839	1.0486	0.2944
year2022	1.3998	0.5171	0.5389	2.5974	0.0094
SMI_F	0.0502	0.1379	0.1414	0.3550	0.7226
spots_X	-0.0355	0.1159	0.1192	0.298	0.7657
Male effort - stage 2					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	3.0632	0.2730	0.2848	10.7539	<0.001
percent_spots	-0.5876	0.3179	0.3278	1.7923	0.0731
sex_ratio	0.7046	0.2835	0.2955	2.3849	0.0171
Ffeeds_rate	-0.1623	0.2671	0.2720	0.5965	0.5508
SMI_F	0.1436	0.2586	0.2635	0.5449	0.5858
volume	0.1178	0.2336	0.2384	0.4940	0.6213



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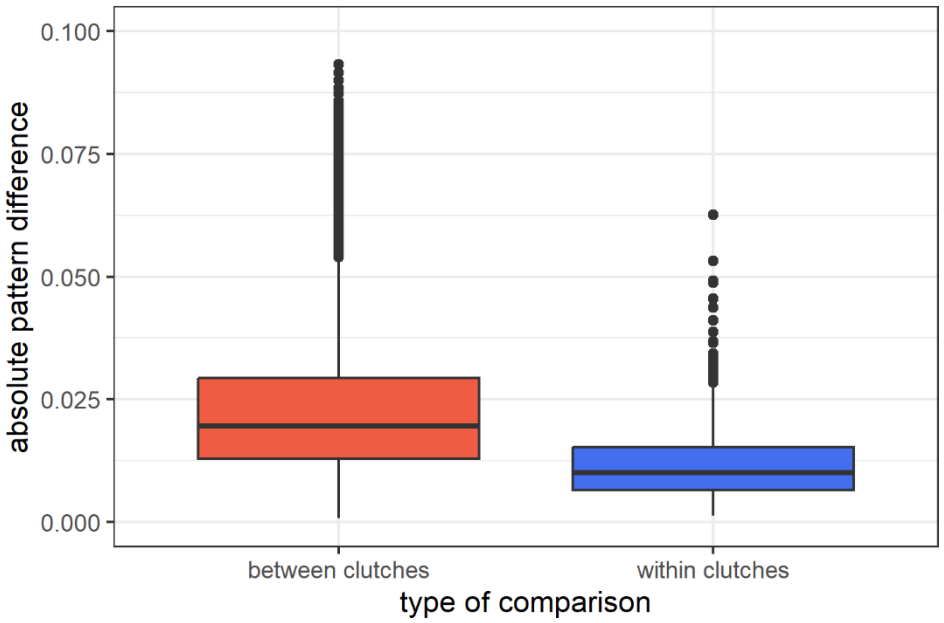

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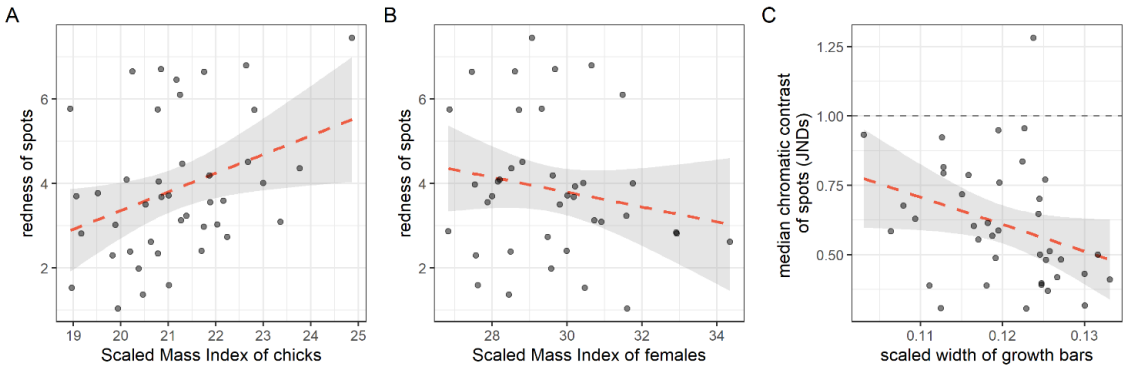
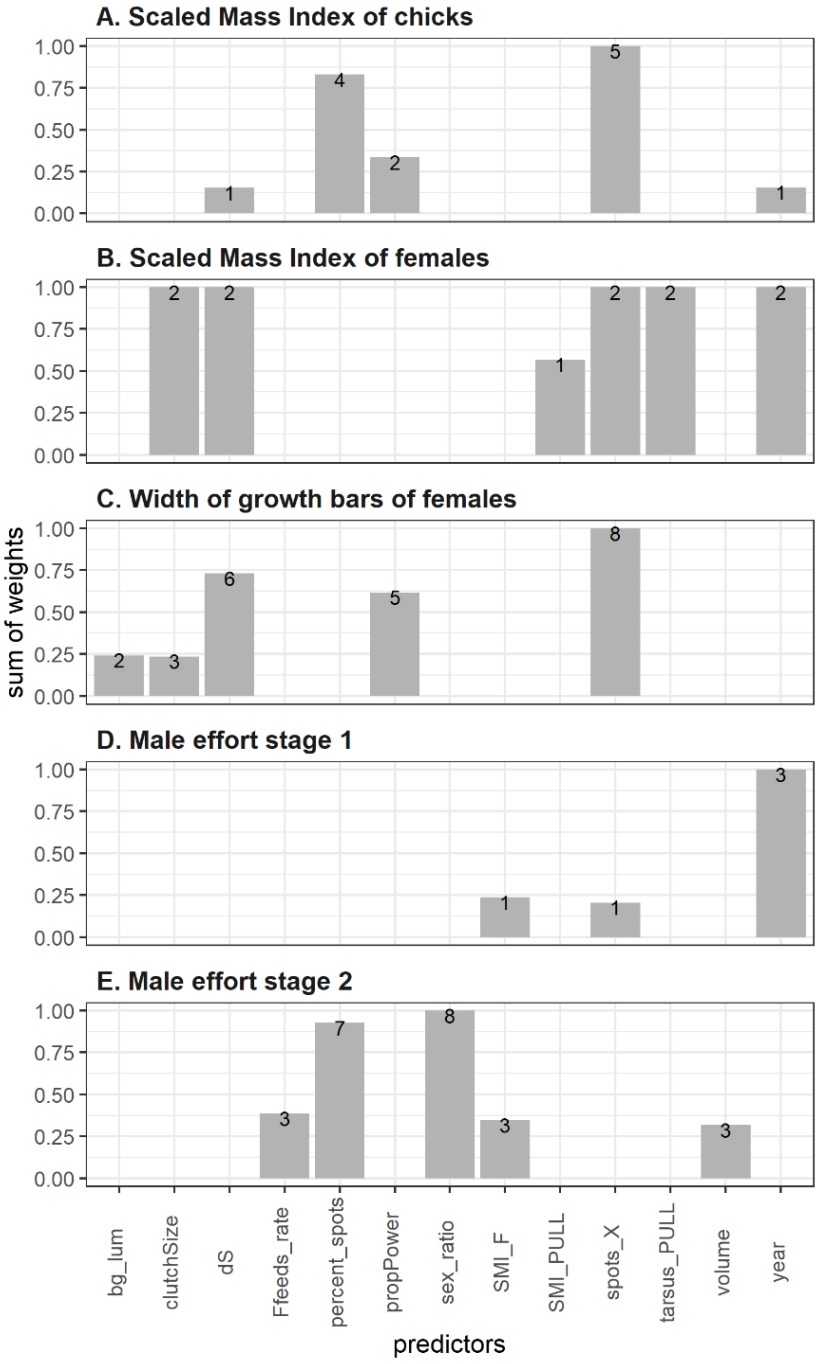


Figure 3. A) The relationship between average condition (Scaled Mass Index) of chicks in a brood and redness of eggshell spots; **B)** The relationship between female condition (Scaled Mass Index) and redness of eggshell spots; **C)** The relationship between median chromatic contrast of spots between pairs of eggs from the same clutches and scaled width of female growth bars. Units for the y-axis are Just Noticeable Differences (JNDs). Horizontal grey dashed line indicates JNDs = 1, a threshold above which the contrast is considered to be perceived by a receiver. In all three cases, red dashed line shows linear regression and shaded area depicts 95% confidence interval. Visual model of the blue tit *Cyanistes caeruleus* was used.



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Appendix

Szala K., Tobolka M., Surmacki A., Do eggshell coloration or patterning affect provisioning effort of males in a cup-nesting passerine?

Correlation between pigmentation traits and within-egg and within-clutch repeatability

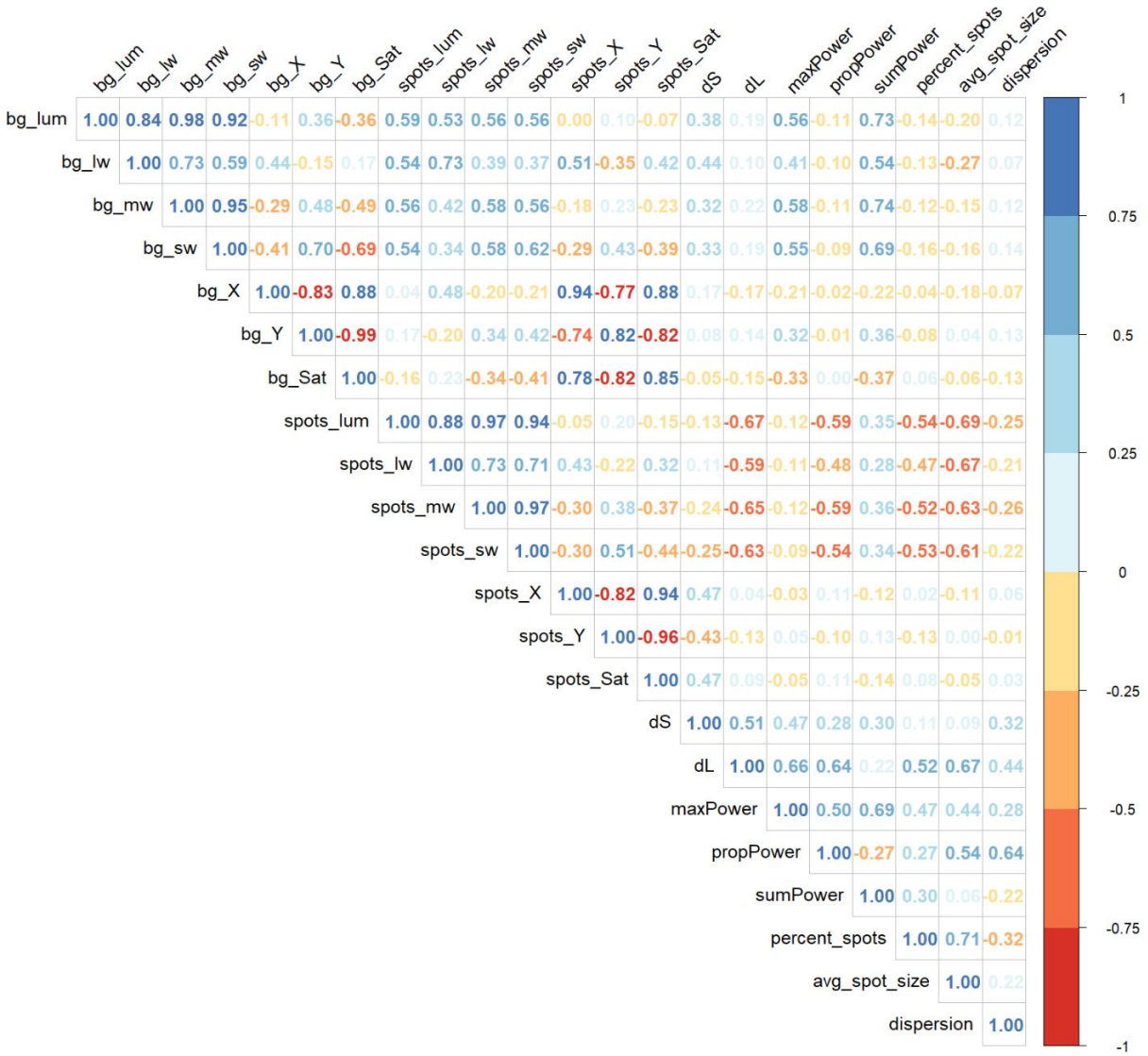

Figure A1. Correlation matrix (Pearson correlation coefficient) between measured pigmentation traits. Lw, mw and sw refer to quantum catch of long-, medium- and short-wavelength sensitive single cones respectively. X and Y refer to two axes in the color space, higher values for X axis indicate higher relative stimulation of lw cones ("redder colors") and higher values for Y axis indicate higher relative stimulation of sw cones ("bluer" colors). Sat is saturation (distance from the achromatic center in the color space) and dS and dL are chromatic and achromatic contrast between spots and eggshell background respectively. Visual model of the blue tit *Cyanistes caeruleus* was used. N = 79 clutches of the red-backed shrike *Lanius collurio*.

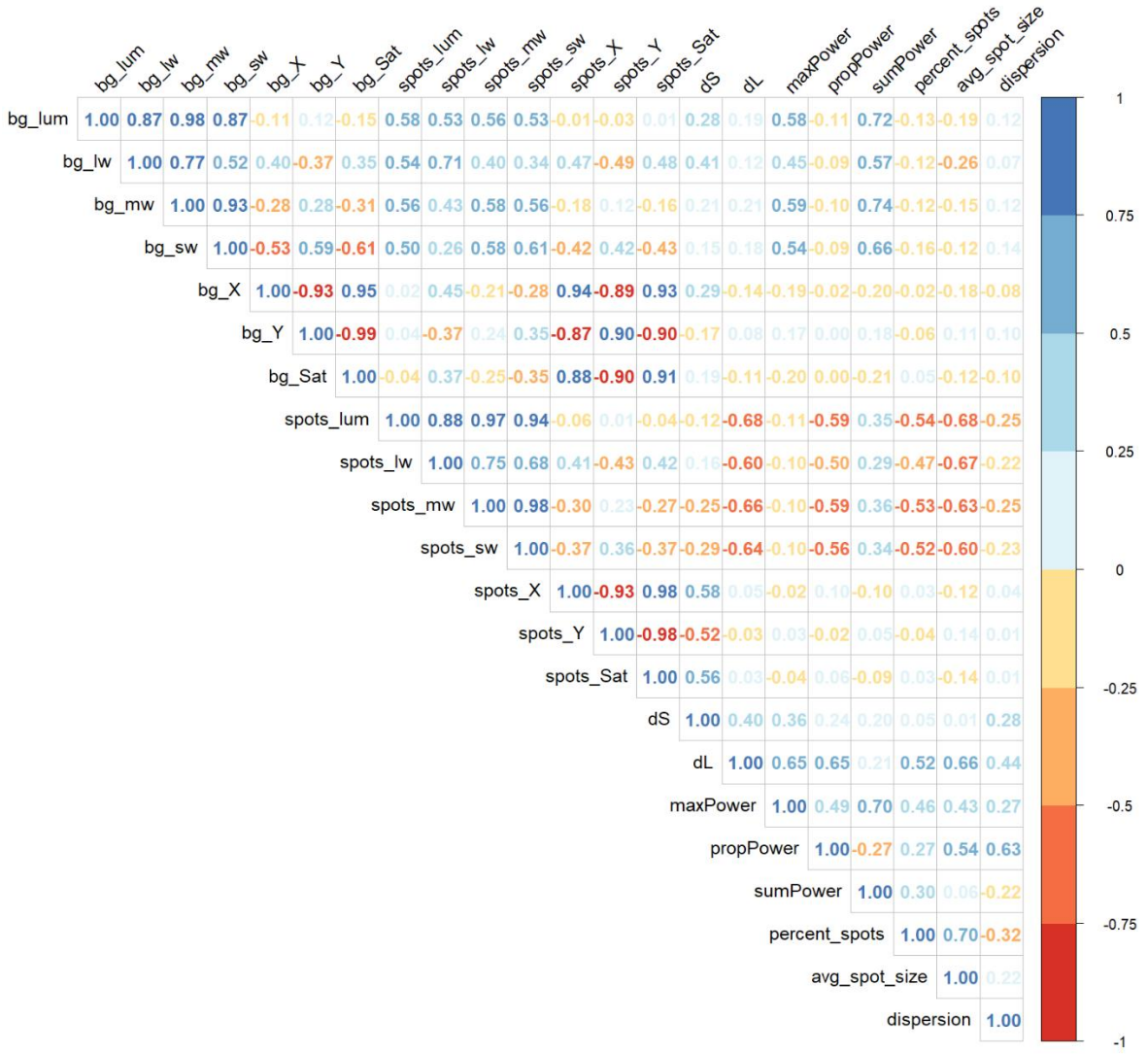

Figure A2. Correlation matrix (Pearson correlation coefficient) between measured pigmentation traits. Lw, mw and sw refer to quantum catch of long-, medium- and short-wavelength sensitive single cones respectively. X and Y refer to two axes in the color space, higher values for X axis indicate higher relative stimulation of lw cones ("redder colors") and higher values for Y axis indicate higher relative stimulation of sw cones ("bluer" colors). Sat is saturation (distance from the achromatic center in the color space) and dS and dL are chromatic and achromatic contrast between spots and eggshell background respectively. Visual model of the peafowl *Pavo cristatus* was used. N = 79 clutches of the red-backed shrike *Lanius collurio*.

Table A1. Within egg and within clutch repeatability of measurements of red-backed shrike *Lanius collurio* eggs. Repeatability was calculated as inter-correlation coefficient using the Linear Mixed Models-basing approach for Gaussian data (note that not all variables met the assumption of normal distribution). Lw, mw and sw refer to quantum catch of long-, medium- and short-wavelength sensitive single cones respectively and CI stands for confidence intervals. X and Y refer to two axes in the color space, higher values for X axis indicate higher relative stimulation of lw cones ("redder colors") and higher values for Y axis indicate higher relative stimulation of sw cones ("bluer" colors). Visual model of the blue tit *Cyanistes caeruleus* was used and color and luminance were calculated separately for spots and eggshell background. N = 392 eggs from 79 clutches.

	Within egg					Within clutch				
Trait	R	Lower 95% CI	Upper 95% CI	Standard error	Normal distribution	R	Lower 95% CI	Upper 95% CI	Standard error	Normal distribution
luminance										
spots_lum	0.899	0.878	0.917	0.010	no	0.801	0.737	0.846	0.028	yes
bg_lum	0.911	0.893	0.926	0.009	yes	0.729	0.656	0.786	0.033	yes
color										
spots_lw	0.916	0.899	0.928	0.008	no	0.847	0.795	0.888	0.023	yes
spots_mw	0.901	0.880	0.917	0.010	no	0.800	0.737	0.849	0.029	no
spots_sw	0.920	0.904	0.933	0.008	no	0.795	0.735	0.843	0.027	no
spots_X	0.989	0.987	0.991	0.001	no	0.962	0.947	0.971	0.006	yes
spots_Y	0.926	0.912	0.940	0.008	yes	0.797	0.741	0.844	0.030	yes
spots_saturation	0.956	0.947	0.964	0.004	yes	0.871	0.825	0.901	0.020	yes
bg_lw	0.920	0.902	0.934	0.008	yes	0.812	0.758	0.857	0.026	yes
bg_mw	0.909	0.892	0.925	0.009	yes	0.719	0.631	0.779	0.036	yes
bg_sw	0.952	0.942	0.961	0.005	yes	0.751	0.682	0.806	0.033	yes
bg_X	0.995	0.994	0.996	0.001	no	0.948	0.929	0.962	0.008	yes
bg_Y	0.980	0.975	0.984	0.002	no	0.853	0.797	0.885	0.023	yes
bg_saturation	0.983	0.980	0.986	0.002	no	0.866	0.810	0.900	0.023	yes
spots achromatic contrast	0.886	0.863	0.905	0.011	yes	0.738	0.665	0.797	0.036	yes
spots chromatic contrast	0.902	0.882	0.919	0.010	no	0.764	0.696	0.815	0.032	yes
pattern										
maxPower	0.832	0.803	0.860	0.015	no	0.621	0.523	0.689	0.044	yes
propPower	0.829	0.799	0.858	0.015	no	0.674	0.599	0.743	0.037	no
sumPower	0.902	0.882	0.920	0.009	yes	0.707	0.618	0.771	0.037	yes
percent_spots	0.852	0.822	0.875	0.014	yes	0.746	0.665	0.798	0.034	yes
avg_spot_size	0.773	0.729	0.813	0.021	no	0.663	0.570	0.727	0.039	no
dispersion	0.827	0.790	0.853	0.016	yes	0.548	0.455	0.623	0.046	yes
dimensions										
length	0.992	0.990	0.993	0.001	no	0.721	0.635	0.778	0.035	yes
width	0.979	0.975	0.983	0.002	yes	0.667	0.585	0.733	0.038	yes
volume	0.984	0.980	0.987	0.002	yes	0.704	0.622	0.763	0.037	yes

Table A2. Within egg and within clutch repeatability of measurements of red-backed shrike *Lanius collurio* eggs. Repeatability was calculated as inter-correlation coefficient using the Linear Mixed Models-basing approach for Gaussian data (note that not all variables met the assumption of normal distribution). Lw, mw and sw refer to quantum catch of long-, medium- and short-wavelength sensitive single cones respectively and CI stands for confidence intervals. X and Y refer to two axes in the color space, higher values for X axis indicate higher relative stimulation of lw cones ("redder colors") and higher values for Y axis indicate higher relative stimulation of sw cones ("bluer" colors). Visual model of the peafowl *Pavo cristatus* was used and color and luminance were calculated separately for spots and eggshell background. N = 392 eggs from 79 clutches.

	Within egg					Within clutch				
Trait	R	Lower 95% CI	Upper 95% CI	Standard error	Normal distribution	R	Lower 95% CI	Upper 95% CI	Standard error	Normal distribution
luminance										
spots_lum	0.897	0.877	0.915	0.010	no	0.799	0.735	0.844	0.028	no
bg_lum	0.908	0.888	0.923	0.009	yes	0.725	0.647	0.790	0.037	yes
colour										
spots_lw	0.912	0.893	0.926	0.009	no	0.841	0.786	0.880	0.025	no
spots_mw	0.901	0.881	0.918	0.010	no	0.801	0.737	0.846	0.028	no
spots_sw	0.917	0.903	0.932	0.008	no	0.802	0.735	0.846	0.030	no
spots_redGreenOpponency	0.990	0.987	0.991	0.001	no	0.963	0.947	0.973	0.007	no
spots_blueYellowOpponency	0.952	0.942	0.960	0.005	yes	0.878	0.831	0.908	0.020	yes
spots_saturation	0.973	0.967	0.978	0.003	no	0.921	0.891	0.941	0.013	no
bg_lw	0.914	0.897	0.929	0.008	yes	0.796	0.726	0.840	0.029	yes
bg_mw	0.909	0.888	0.923	0.009	yes	0.719	0.645	0.778	0.035	yes
bg_sw	0.953	0.943	0.962	0.005	yes	0.755	0.678	0.812	0.034	yes
bg_redGreenOpponency	0.995	0.994	0.996	0.001	no	0.950	0.930	0.963	0.008	no
bg_blueYellowOpponency	0.980	0.976	0.983	0.002	yes	0.905	0.869	0.929	0.016	yes
bg_saturation	0.984	0.980	0.987	0.002	no	0.911	0.879	0.933	0.014	no
spots achromatic contrast	0.885	0.859	0.906	0.012	yes	0.738	0.664	0.793	0.034	yes
spots chromatic contrast	0.923	0.907	0.935	0.007	no	0.808	0.745	0.855	0.029	no
pattern										
maxPower	0.829	0.796	0.856	0.016	no	0.613	0.510	0.690	0.046	no
propPower	0.829	0.796	0.859	0.016	no	0.673	0.582	0.741	0.040	no
sumPower	0.898	0.877	0.915	0.010	yes	0.708	0.627	0.770	0.039	yes
percent_spots	0.851	0.825	0.875	0.013	yes	0.745	0.662	0.800	0.035	yes
avg_spot_size	0.776	0.734	0.811	0.020	no	0.663	0.573	0.734	0.042	no
dispersion	0.829	0.795	0.856	0.016	yes	0.548	0.462	0.635	0.044	yes
dimensions										
length	0.992	0.991	0.994	0.001	no	0.721	0.643	0.786	0.036	no
width	0.979	0.975	0.983	0.002	yes	0.667	0.579	0.737	0.040	yes
volume	0.984	0.981	0.987	0.002	yes	0.704	0.623	0.767	0.036	yes

Results for the peafowl visual model

Median chromatic contrast within clutches was significantly lower than median chromatic contrast between clutches both for spots ($W = 66462313$, $p < 0.001$, $r = 0.141$) and background ($W = 65349429$, $p < 0.001$, $r = 0.136$, Figure A3A). Likewise, median achromatic contrast within clutches was significantly lower than median achromatic contrast between clutches both for spots ($W = 55756514$, $p < 0.001$, $r = 0.090$) and background ($W = 52385131$, $p < 0.001$, $r = 0.074$, Figure A3B). Detailed values are presented in Table A3.

Median difference between pattern spectra was 0.010 (interquartile range = 0.009) within clutches and 0.020 (interquartile range = 0.017) between clutches. Median difference in pattern spectra was lower within clutches than between clutches, $W = 57507938$, $p < 0.001$, $r = 0.098$ (Figure A4).

Further, median chromatic contrast between clutches was significantly higher than 1 JNDs for spots ($W = 3069307955$, $p < 0.001$, $r = 0.679$) and for background ($W = 2990226851$, $p < 0.001$, $r = 0.639$) and the same was true for achromatic contrast for spots ($W = 3222446984$, $p <0.001$, $r = 0.756$) and background ($W = 3072198074$, $p < 0.001$, $r = 0.681$).

Results of averaged models are presented in Table A4, selected relationships are plotted in Figure A5, and importance of predictors is shown in Figure A6. Details on selected models that were subject to model averaging are included in Table A5.

Table A3. Mean, median and standard deviation (SD) of chromatic and achromatic contrasts for pairwise comparisons between eggs of the red-backed shrike *Lanius collurio*. Pairwise comparisons were calculated for pairs of eggs from the same clutches (within clutches) and pairs of eggs from different clutches (between clutches), separately for eggshell background and for spots. Visual model of the peafowl *Pavo cristatus* was used.

Part of egg	Type of comparison	Chromatic contrast		Achromatic contrast	
		median	IQR	median	IQR
background	between clutches	1.789	1.942	2.256	2.806
background	within clutches	0.434	0.532	1.135	1.370
spots	between clutches	1.978	2.200	2.949	3.588
spots	within clutches	0.434	0.452	1.206	1.494

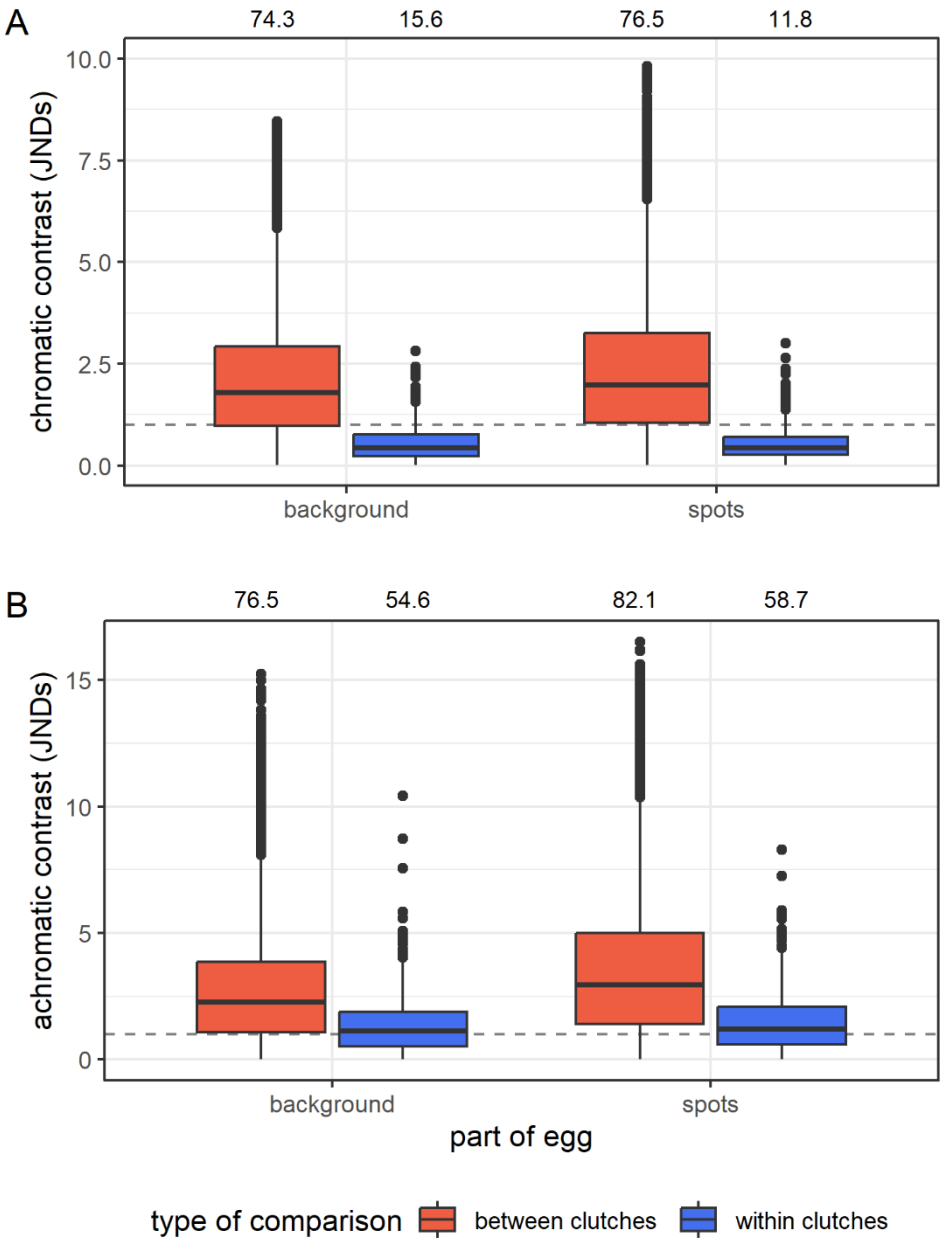

Figure A3. A) Comparison of average chromatic contrast for pairs of eggs from the same clutches (blue boxplot) and from different clutches (red boxplot). **B)** Comparison of average achromatic contrast for pairs of eggs from the same clutches (blue boxplot) and from different clutches (red boxplot). Contrasts for background and spots were calculated separately. Units for y-axes are Just Noticeable Differences (JNDs). Horizontal dashed lines indicate JNDs = 1, a threshold above which the contrast is considered to be perceived by a receiver. Numbers above plots indicate percent of pairs of eggs in a given group for which contrast values were >1 JNDs. In both cases, the visual model of the peafowl *Pavo cristatus* was used.

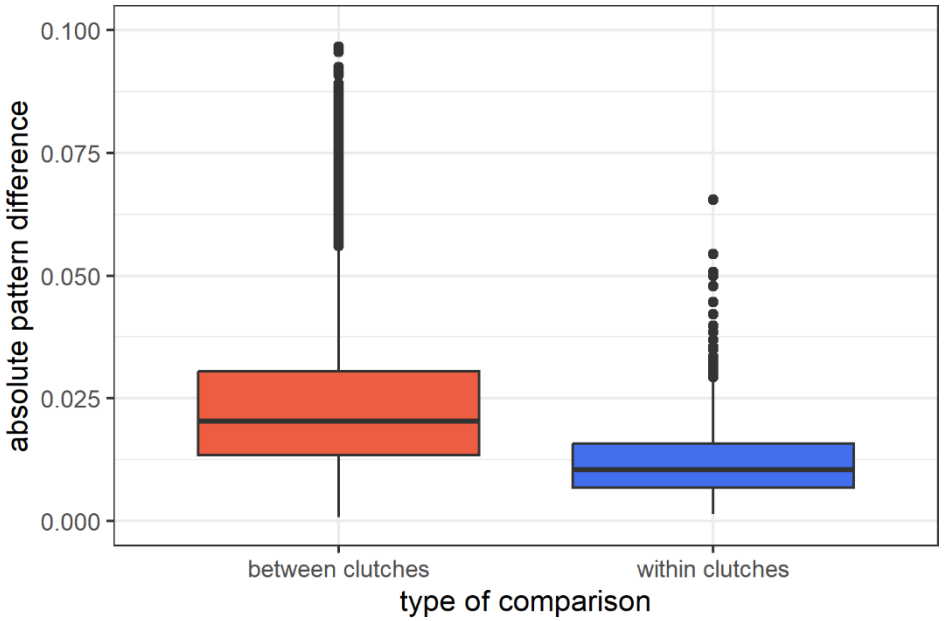

Figure A4 Difference between pattern energy spectra of eggs from different clutches (red boxplot) and the same clutches (blue boxplot) for the visual model of the peafowl *Pavo cristatus.*

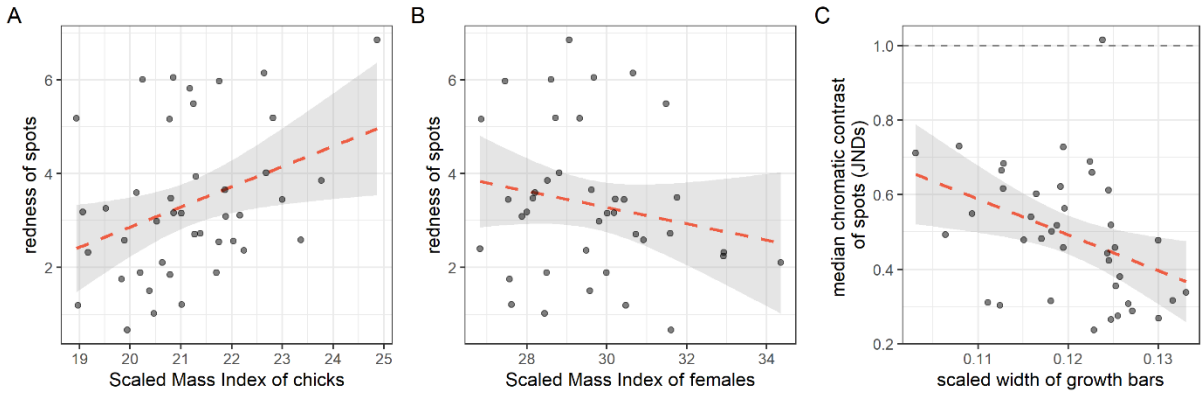

Figure A5. A) The relationship between average condition (Scaled Mass Index) of chicks in a brood and redness of eggshell spots; **B)** The relationship between female condition (Scaled Mass Index) and redness of eggshell spots; **C)** The relationship between median chromatic contrast of spots between pairs of eggs from the same clutches and average width of female growth bars. Units for the y-axis are Just Noticeable Differences (JNDs). Horizontal dashed line indicates JNDs = 1, a threshold above which the contrast is considered to be perceived by a receiver. In all three cases, red dashed line shows linear regression and shaded area depicts 95% confidence interval. Visual model of the peafowl *Pavo cristatus* was used.

Table A4. Results of averaged models (full average). SE stands for standard error and SMI for Scaled Mass Index, sex-ratio is sex-ratio among chicks (calculated as number of females / number of all chicks), dS is chromatic contrast between spots and eggshell background, spots_X is redness of spots, bg_lum is luminance of background, and propPower is proportion of energy (how much dominating spot size dominates in overall patterning). Suffix "_F" indicates measurements of females and "_PULL" measurements of chicks. Presented results are for the visual model of the peafowl *Pavo cristatus.*

Scaled Mass Index of chicks					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	21.1409	0.219	0.2257	93.6841	<0.001
percent_spots	-0.274	0.2323	0.2361	1.1606	0.246
spots_X	0.5487	0.2132	0.2194	2.5003	0.012
dS	-0.0474	0.1427	0.1449	0.327	0.744
propPower	-0.0811	0.1741	0.1765	0.4595	0.646
year2021	0.0801	0.2605	0.2639	0.3035	0.762
year2022	-0.0232	0.1853	0.191	0.1215	0.903
Scaled Mass Index of females					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	24.1879	1.7168	1.7996	13.4405	<0.001
clutchSize	1.1586	0.3137	0.3288	3.5234	<0.001
dS	0.7873	0.2552	0.2672	2.9461	0.003
SMI_PULL	0.2876	0.2805	0.2863	1.0044	0.315
spots_X	-0.7899	0.2823	0.2941	2.6861	0.007
tarsus_PULL	0.7624	0.2262	0.2368	3.2200	0.001
year2021	-1.2006	0.4895	0.5130	2.3403	0.019
year2022	-2.1452	0.5386	0.5647	3.7987	<0.001
percent_spots	0.0695	0.1578	0.1608	0.4320	0.666
Width of growth bars of females					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	0.1194	0.0041	0.0042	28.7411	<0.001
propPower	-0.0017	0.0012	0.0012	1.4077	0.159
spots_X	-0.0036	0.0011	0.0011	3.1631	0.002
dS	-0.0008	0.0012	0.0012	0.6464	0.518
clutchSize	0.0002	0.0007	0.0007	0.2689	0.788
Male effort - stage 1					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	1.1249	0.3299	0.3437	3.2725	0.001
year2021	0.5073	0.4644	0.4837	1.0488	0.294
year2022	1.4004	0.5172	0.539	2.5981	0.009
SMI_F	0.0501	0.1378	0.1412	0.3546	0.723
spots_X	-0.0363	0.1169	0.1201	0.302	0.763
Male effort - stage 2					
	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	3.0632	0.273	0.2849	10.7533	<0.001
percent_spots	-0.5912	0.3149	0.3251	1.8188	0.069
propFemales	0.7014	0.2836	0.2956	2.373	0.018
SMI_F	0.1331	0.2512	0.2559	0.5201	0.603
Ffeeds_rate	-0.1485	0.2587	0.2634	0.5637	0.573
volume	0.1121	0.2294	0.234	0.4791	0.632
dS	-0.0164	0.0962	0.0987	0.1658	0.868

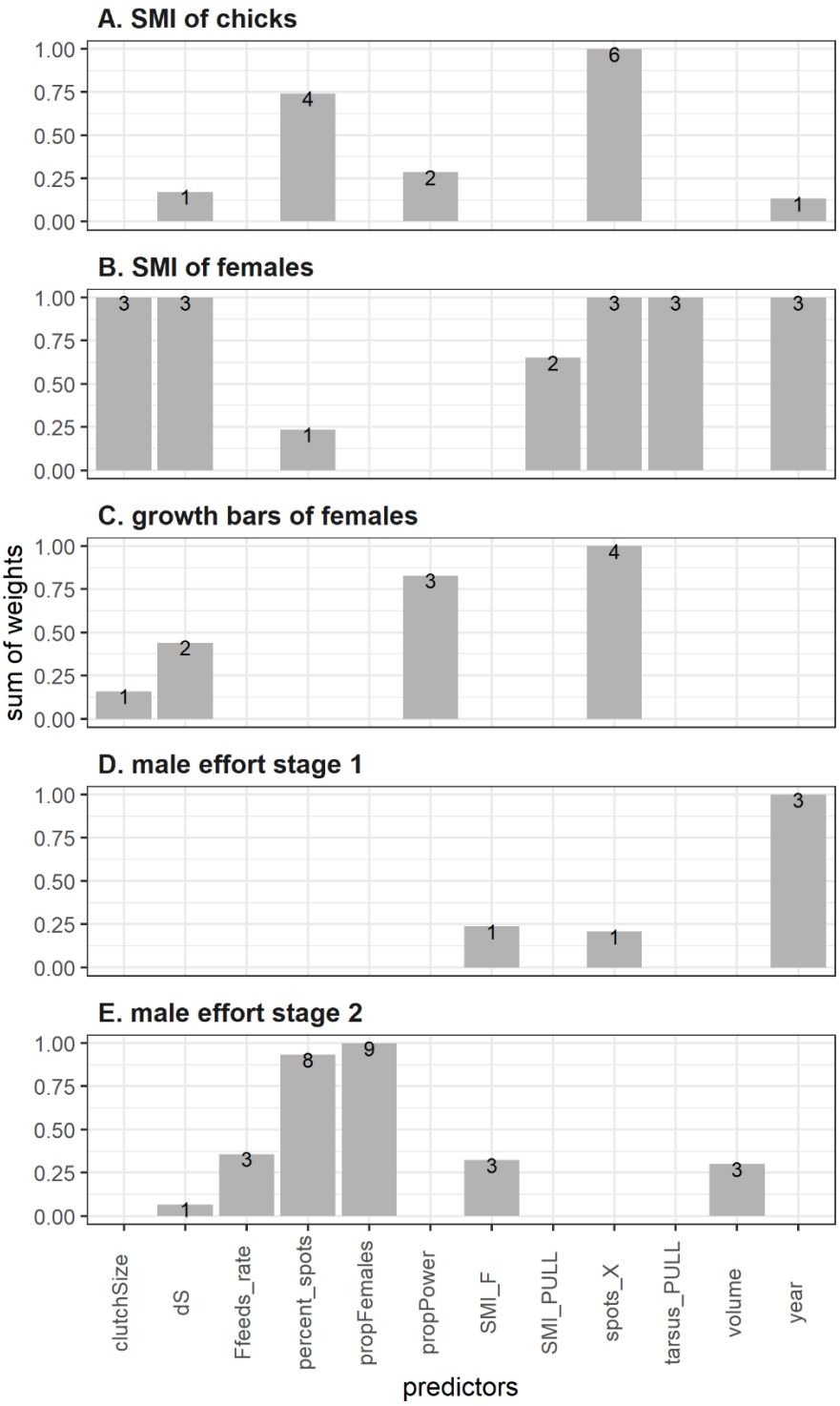

Figure A6. The importance of predictors in the models. Headers indicate the response variables. Heights of the bars show sum of weight for predictors and numbers above bars indicate number of models in which predictors were present. Total number of models with ΔAICc ≤ 2 (that were later averaged) were: 6 for Scale Mass Index of chicks, 3 for Scale Mass Index of females, 4 for width of growth bars of females, 3 for male effort at stage 1 (chicks in age between 4 and 5 days) and 9 for male effort at stage 2 (chicks in age between 9 and 10 days).

Table A5. The best models explaining Scaled Mass Index of females red-backed shrike *Lanius collurio*, growth bars of females, Scaled Mass Index of chicks, and parental effort of males in stage 1 (chicks in 4-5th day of life) and stage 2 (chicks in 9-10th day of life). Models are ranked according to their ΔAICc and only models with ΔAICc ≤ 2 are presented. Presented results are for the peafowl *Pavo cristatus* visual model.

Model	df	logLik	AICc	delta	weight	F	p	adjusted R2 [%]
Scaled Mass Index of females (N = 34 females)								
clutchSize + dS + SMI_PULL + spots_X + tarsus_PULL + year	9	-46.763	119.026	0	0.416	6.76	<0.001	55.01
clutchSize + dS + spots_X + tarsus_PULL + year	8	-48.808	119.377	0.351	0.349	6.76	<0.001	51.13
clutchSize + dS + percent_spots + SMI_PULL + spots_X + tarsus_PULL + year	10	-45.303	120.171	1.145	0.235	6.48	<0.001	57.06
Growth bars of females (N = 34 females)								
propPower + spots_X	4	132.136	-254.893	0	0.401	14.71	<0.001	45.39
dS + propPower + spots_X	5	133.121	-254.099	0.794	0.27	10.65	<0.001	46.74
dS + spots_X	4	131.283	-253.186	1.707	0.171	13.23	<0.001	42.58
clutchSize + propPower + spots_X	5	132.587	-253.032	1.861	0.158	10.02	<0.001	45.04
Scaled Mass Index of chicks (N = 42 broods)								
percent_spots + spots_X	4	-65.826	140.733	0	0.298	5.33	0.009	17.44
dS + percent_spots + spots_X	5	-65.083	141.832	1.099	0.172	4.04	0.014	18.21
propPower + spots_X	4	-66.528	142.138	1.404	0.148	4.51	0.017	14.63
percent_spots + propPower + spots_X	5	-65.294	142.254	1.521	0.139	3.88	0.016	17.39
percent_spots + spots_X + year	6	-63.973	142.346	1.613	0.133	3.62	0.014	20.32
spots_X	3	-68.039	142.709	1.976	0.111	5.84	0.020	10.56
Parental effort of male at stage 1 (N = 33 broods)								
year	4	-48.751	106.931	0	0.556	3.58	0.040	13.87
SMI_F + year	5	-48.207	108.636	1.706	0.237	2.71	0.064	13.79
spots_X + year	5	-48.339	108.899	1.969	0.208	2.61	0.071	13.10
Parental effort of male at stage 2 (N = 33 broods)								

percent_spots + propFemales	4	-60.545	130.519	0	0.174	5.35	0.010	21.39
percent_spots + propFemales + SMI_F	5	-59.294	130.809	0.29	0.15	4.48	0.011	24.62
Ffeeds_rate + percent_spots + propFemales	5	-59.306	130.835	0.316	0.148	4.47	0.011	24.56
Ffeeds_rate + percent_spots + propFemales + volume	6	-58.07	131.372	0.853	0.113	4.04	0.010	27.51
percent_spots + propFemales + volume	5	-59.61	131.442	0.923	0.109	4.22	0.014	23.16
Ffeeds_rate + percent_spots + propFemales + SMI_F	6	-58.238	131.706	1.187	0.096	3.92	0.012	26.77
percent_spots + propFemales + SMI_F + volume	6	-58.446	132.122	1.603	0.078	3.79	0.014	25.84
propFemales	3	-62.808	132.444	1.925	0.066	5.67	0.024	12.75
dS + percent_spots + propFemales	5	-60.123	132.467	1.948	0.066	3.79	0.021	20.74

Table A6. The best models explaining Scaled Mass Index of females red-backed shrike *Lanius collurio*, growth bars of females, Scaled Mass Index of chicks, and parental effort of males in stage 1 (chicks in 4-5th day of life) and stage 2 (chicks in 9-10th day of life). Models are ranked according to their ΔAICc and only models with ΔAICc ≤ 2 are presented. Presented results are for blue tit *Cyanistes caeruleus* visual model.

Model	df	logLik	AICc	ΔAICc	weight	F	p	adjusted R^2 [%]
Scaled Mass Index of females (N = 34 females)								
clutchSize + dS + SMI_PULL + spots_X + tarsus_PULL + year	9	-45.83	117.16	0	0.567	7.36	<0.001	57.41
clutchSize + dS + spots_X + tarsus_PULL + year	8	-47.97	117.69	0.54	0.433	7.33	<0.001	53.49
Growth bars of females (N = 34 females)								
propPower + spots_X	4	132.21	-255.03	0	0.194	14.84	<0.001	45.61
dS + propPower + spots_X	5	133.43	-254.71	0.32	0.165	11.03	<0.001	47.69
dS + spots_X	4	132.03	-254.68	0.35	0.163	14.53	<0.001	45.05
bg_lum + dS + spots_X	5	133.29	-254.44	0.60	0.144	10.86	<0.001	47.27
bg_lum + dS + propPower + spots_X	6	134.38	-253.66	1.38	0.098	8.88	<0.001	48.85
clutchSize + dS + propPower + spots_X	6	134.25	-253.38	1.65	0.085	8.75	<0.001	48.44
clutchSize + dS + spots_X	5	132.64	-253.14	1.89	0.076	10.08	<0.001	45.22
clutchSize + propPower + spots_X	5	132.62	-253.10	1.93	0.074	10.06	<0.001	45.16
Scaled Mass Index of chicks (N = 42 broods)								
percent_spots + spots_X	4	-65.87	140.83	0	0.356	5.27	0.009	17.25
propPower + spots_X	4	-66.62	142.32	1.49	0.169	4.41	0.019	14.26
percent_spots + propPower + spots_X	5	-65.33	142.34	1.51	0.168	3.84	0.017	17.22
percent_spots + spots_X + year	6	-64.05	142.50	1.67	0.155	3.57	0.015	20.04
dS + percent_spots + spots_X	5	-65.43	142.52	1.69	0.153	3.77	0.018	16.87
Parental effort of male at stage 1 (N = 33 broods)								
year	4	-48.75	106.93	0	0.557	3.58	0.040	13.87

SMI_F + year	5	-48.21	108.63	1.71	0.237	2.71	0.064	13.79
spots_X + year	5	-48.35	108.92	1.99	0.206	2.60	0.071	13.04
Parental effort of male at stage 2 (N = 33 broods)								
percent_spots + propFemales	4	-60.59	130.62	0	0.184	5.30	0.011	21.16
Ffeeds_rate + percent_spots + propFemales	5	-59.33	130.89	0.27	0.160	4.45	0.011	24.45
percent_spots + propFemales + SMI_F	5	-59.33	130.89	0.27	0.160	4.45	0.011	24.44
Ffeeds_rate + percent_spots + propFemales + volume	6	-58.11	131.45	0.83	0.121	4.01	0.011	27.35
percent_spots + propFemales + volume	5	-59.67	131.57	0.95	0.114	4.16	0.014	22.87
Ffeeds_rate + percent_spots + propFemales + SMI_F	6	-58.25	131.73	1.12	0.105	3.92	0.012	26.71
percent_spots + propFemales + SMI_F + volume	6	-58.50	132.23	1.61	0.082	3.75	0.014	25.60
propFemales	3	-62.81	132.44	1.83	0.074	5.67	0.024	12.75

Egg volume

We found no relationship between average egg volume and average Scaled Mass Index of chicks of the red-backed shrike (r_P = -0.04, p = 0.81, 95% CI: -0.34-0.27, Figure A7).

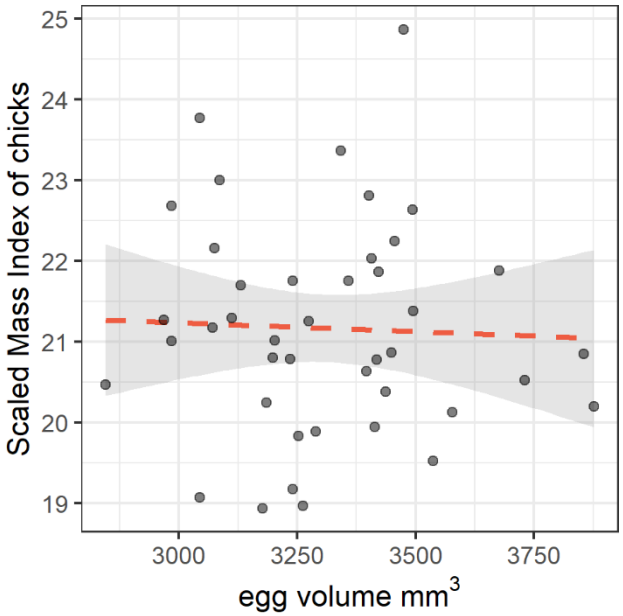

Figure A7. Relationship between average condition (Scaled Mass Index) of chicks in a brood and average volume of eggs in a clutch. Red dashed line shows linear regression and shaded area depicts 95% confidence interval.

Further, we fitted a full linear regression model with volume as a response variable and five pigmentation traits as explanatory variables while controlling for the year of study (factor). Pigmentation traits used in the model were the same five features that were used for other models (see the manuscript): redness of spots, luminance of background, percent of egg surface covered by spots, chromatic contrast between spots and eggshell background and propPower. Three of pigmentation traits entered to the final averaged model, but only year was statistically significant (Table A7). For the details about the model selection and model averaging procedure, please, see the manuscript.

Table A7. Results of averaged models (full average) predicting average egg volume. SE stands for standard error, spots_X is redness of spots, propPower is proportion of energy (how much dominating spot size dominates in overall patterning).

	beta	SE	adjusted SE	Z-value	p-value
(Intercept)	0.4156	0.1948	0.198	2.0993	0.0358
year2021	-0.6176	0.2606	0.2649	2.3318	0.0197
year2022	-0.5939	0.2843	0.289	2.0552	0.0399
spots_X	0.0194	0.0631	0.0637	0.3039	0.7612
percent_spots	0.0176	0.0606	0.0613	0.2877	0.7736
propPower	-0.0125	0.0534	0.0541	0.2311	0.8172

Rozdział 2 | Chapter 2

Eggshell coloration and patterning traits derived from calibrated photographs are poor predictors of protoporphyrin IX concentration in eggshells of the red-backed shrike Lanius collurio

Eggshell coloration and patterning traits derived from calibrated photographs are poor predictors of protoporphyrin IX concentration in eggshells of the red-backed shrike *Lanius collurio*

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Running title: Eggshell appearance and protoporphyrin IX quantity

Abstract

Multiple hypotheses have been proposed to explain adaptive roles of pigmentation of avian eggshells and its variability across species. Testing hypotheses focusing on mechanical or structural functions of the pigmentation requires an accurate measurement of the quantity of pigment(s) in the eggshells. High-performance liquid chromatography (HPLC) is best suited for this task but is invasive and simply impossible in many kinds of research. Hence, measurements of the external appearance of eggs are often used as a proxy for the concentration of pigments(s), yet, literature shows appearance features best predicting the quantity of pigment(s) differ among species and usually their predictive power is low. Here, we explored whether external appearance of eggshells of the red-backed shrike *Lanius collurio* is a reliable predictor of protoporphyrin IX concentration. We collected 43 eggs and photographed them in standardised light conditions to objectively measure 20 different metrics of coloration and patterning and used HPLC to measure the exact concentration of the pigment. Our analysis revealed, green chroma was the best predictor of protoporphyrin IX concentration. However, the amount of explained variance was low which is consistent with the results from the literature. We discuss possible reasons for such a weak relationship.

Keywords: calibrated digital photography; chroma; full spectrum; granularity; grey standards; high-performance liquid chromatography; pigmentation; ultraviolet

Introduction

Five pigments have been identified in avian eggshells so far: blue-green biliverdin, yellow-brown bilirubin, red-brown protoporphyrin IX, red-orange uroerythrin, and purple mesobiliviolin (Kennedy & Vevers, 1976; Hamchand *et al.*, 2020). They are all tetrapyrrole compounds that are related to the metabolic pathway of haem (Sparks, 2011; Hamchand *et al.*, 2020). The pigments can be evenly deposited in the calcium carbonate matrix producing a uniform ground coloration of the eggshell (Sparks, 2011) and they can be mixed in different proportions resulting in new colours (Hanley *et al.*, 2015; Hamchand *et al.*, 2020). Additionally, protoporphyrin can occur as maculation patterns of higher pigment concentration: speckles, spots, or streaks differing in size, shape, and distribution (Kennedy & Vevers, 1976; Pike, 2015).

This diversity of eggshell appearances that can be observed across avian species gave rise to multiple hypotheses seeking to explain the evolution of pigmented eggshells and their advantages over white and plain eggshells. Extensive reviews of potential adaptive functions of pigmentation are provided elsewhere (e.g. Underwood & Sealy, 2002; Kilner, 2006; Cherry & Gosler, 2010; Maurer *et al.*, 2011; Brulez *et al.*, 2015). Briefly, pigmentation can serve as structural reinforcement of the eggshell (Gosler *et al.*, 2005), aid thermoregulation (Wisocki *et al.*, 2020), protect an embryo from detrimental ultraviolet (UV) radiation (Maurer *et al.*, 2015), and provide antimicrobial protection (Ishikawa *et al.*, 2010). Furthermore, coloration and patterning can serve as a signature of identity helping to recognise one's own eggs in crowded breeding colonies (Birkhead, 1978; Quach *et al.*, 2021) or in clutches parasitised by brood parasites (Caves *et al.*, 2015). Finally, eggshell coloration can provide camouflage against visually-oriented predators (Troscianko *et al.*, 2016), or serve as a signal of female condition that elicits higher parental investment of a male (Moreno & Osorno, 2003).

Patterning present in some eggshells consists of protoporphyrin (Kennedy & Vevers, 1976) and layers of this pigment can occur at different depths of the calcium carbonate matrix (Harrison, 1966; Mikhailov, 1997). While for some of the above-mentioned functions only the external pigmentation is of interest (e.g. for camouflage or providing signatures of identity), a proper test of the other hypotheses requires measuring the exact quantity of the pigment(s) included not only at the surface, but in the entire cross-section of the eggshell. The most accurate method is to measure concentration of pigment(s) using high-performance liquid chromatography (HPLC); however, this requires destroying of the eggshell and therefore, the embryo developing inside. To avoid such an invasive approach, an array of other methods has been developed, inevitably at the expense of accuracy. Most of them consist of quantifying of

the external brightness, coloration, or patterning using spectrometry, photography, or visual scoring (Stevens, 2011; Brulez *et al.*, 2014).

Pigments absorb light and the amount of absorbed light (as the function of wavelength) depends on their concentration. Hence, brightness, hue, or saturation of colour should be a good proxy for the concentration of pigments (Andersson & Prager, 2006; Johnsen, 2012). However, while usually some metrics of eggshell colour measured at the surface correlate with concentration of protoporphyrin or biliverdin, their predictive power is often limited (Wegmann *et al.*, 2015; Butler & Waite, 2016). For instance, in the case of protoporphyrin-based pigmentation, contrast of spots combined with pattern coverage and fractal dimension of spottiness were better predictors of the pigment concentration than coloration for eggshells of great tits *Parus major* and Kentish plovers *Charadrius alexandrinus* respectively (Wegmann *et al.*, 2015; Gómez *et al.*, 2019). This highlights the importance of measuring many different aspects of eggshell appearance and assessing how well they predict the actual concentration of pigment(s) measured with HPLC. Such a calibration should preferably be done for the focal species and not inferred from studies on other species, as the exact relationship between concentration of pigments and external appearance can differ from one species to another (Butler *et al.*, 2011).

Here we employed calibrated digital photography to quantify multiple aspects of eggshell appearance and explored how well they predict the actual concentration of protoporphyrin IX measured using HPLC in eggshells of red-backed shrikes *Lanius collurio*. This species exhibits a large variability of eggshell pigmentation (Figure 1A), both in terms of the amount of protoporphyrin (Mikšík *et al.*, 1994, 1996) and appearance of eggshells (Surmacki *et al.*, 2006; Stoddard & Stevens, 2010).

Material and methods

Fieldwork

We collected unhatched eggs and abandoned clutches of the red-backed shrike between June and July during three field seasons (2019-2021) in farmland close to Terespol (52°4′ N, 16°18′ E) in Western Poland. We searched for and controlled nests of red-backed shrikes for the purpose of another study. We collected 10 eggs from 7 clutches in 2019, 14 from 8 clutches in 2020, and 9 from 8 clutches in 2021. After collection, the eggs were stored in a fridge unblown until photographing and then were prepared for pigment extraction (see below). Additionally, we used 10 eggs from another population of the red-backed shrike close to

Odolanów (51°34′ N, 17°40′ E) in Western Poland. Three of them (all from the same clutch) were collected in 2020 and processed as the rest of the eggs. The remaining seven eggs were collected between 2009 and 2011, blown, and stored at room temperature and in darkness since then. We do not possess information about clutch ID for these older eggs. In total, we used 43 eggs.

Fieldwork was conducted in accordance with the current Polish law and in line with the permission from the Directorate of Environment Protection, numbers: DZP-WG.6401.03.111.2019.ks (2019), WPN-II.6401.345.2019.KL (2020), WPN-II.6401.77.2021.MT (2021).

Measuring eggshell appearance

Eggshells were put sideways on a custom-made black plastic holder with shallow pits for eggs. Photographs were taken in a darkroom and the only source of illumination was an Iwasaki eyeColor arc lamp MT70D E27 6500K (Iwasaki Electric Co., Ltd., Tokyo, Japan) with its UV filter removed following Troscianko & Stevens (2015a). The light was diffused using a sheet of PTFE. We used a Nikon D90 camera converted to full spectrum and fitted with a Nikkor EL 80 mm lens. We photographed sequentially through a Baader UV pass filter and a Baader IR/UV cut filter (Baader Planetarium, Mammendorf, Germany), therefore, in the UV light (300-400 nm) and in the human-visible light (VIS, 400-700 nm) respectively. The filters were attached to a slider mounted in front of the lens enabling fast and smooth change of the filters without moving of the camera. The camera was fixed to a tripod with a lens axis aligned perpendicularly to the ground. To additionally minimise movements of the camera, we used a remote shutter release. The distance between the eggs and the lens was constant (1 m), and a scale bar was present in every photograph. As a reference, we used 20% and 99% Spectralon grey standards (Labsphere, Congleton, UK). We set the same aperture (f/8) and ISO (400) for all pictures, and to ensure good exposure, we used bracketing mode with a ±1 EV step. All images were saved as RAW files.

We processed pictures in ImageJ version 1.53 (Schneider *et al.*, 2012) with Multispectral Image Calibration and Analysis (MICA) Toolbox version 2.2.2 (Troscianko & Stevens, 2015b). First, we calibrated the pictures with respect to the grey standards and aligned the same images in VIS and UV light using automatic alignment with the following settings: offset = 16, scaling loops = 8, and scale step size = 0.005. As a result, we obtained multispectral images with five channels: red (R), green (G), blue (B), UVB and UVR, where

pixel values are expressed on a reflectance scale (between 0 and 100%). Next, we set a scale bar and selected eggs using automatic fitting of the egg shape (Troscianko, 2014). Using a custom-written script, we measured the number of overexposed pixels (values >100) in every channel and excluded an egg if more than 0.5% of its surface was overexposed in any channel (7 eggs in total, see below). This is important because the true information for overexposed pixels is lost and measuring such areas would increase measurement error (Troscianko & Stevens, 2015a). To make pattern measurements comparable across pictures, we calculated the minimum px/mm factor in our set of photographs (15.96 px/mm), scaled it down to 15.5 px/mm and used this value for scaling (Troscianko & Stevens, 2015a). Since the aim of this study was to measure eggshell colour and pattern features objectively, we did not assume any particular viewer, and therefore, we did not use visual models.

To measure the pattern, as well as the overall brightness and coloration of eggshells, we employed the Batch Multispectral Image Analysis tool in the MICA Toolbox. Following Spottiswoode & Stevens (2010), we selected the green channel for granularity pattern analysis and used the following settings: start size = 2 px, end size = 64 px, and step = $\sqrt{2}$ (steps were multiplied), resulting in 11 spatial scales. We used standard metrics summarising pattern spectra (Troscianko & Stevens, 2015a): maxPower (contrast for the dominating spot size), sumPower (overall contrast), and propPower (maxPower divided by sumPower; it indicates how much the dominating spot size dominates).

Since spots are likely to contain more protoporphyrin than the eggshell background, apart from measuring the overall brightness and coloration, we measured the brightness and colour of spots and background separately as well. For this purpose, we used a custom-written script employing local thresholding with the Phansalkar method (Phansalkar *et al.*, 2011) and a 50 px radius value to segment an egg image into spots and eggshell background (Figure 1B). Because of their shape, different regions of eggs reflect different amounts of light to a camera sensor, resulting in an apparent darkening of the edges of eggs (Gómez & Liñán-Cembrano, 2017). Thus, to avoid detecting shades as spots, we used a difference of 128 px Gaussian blur (Gómez *et al.*, 2018) and additionally decreased the selection around every egg by 10% of the egg's width. Similarly as for the granularity analysis, we used the green channel to detect spots. In all cases, we calculated brightness as the sum of reflectance values for all channels (brightness = R + G + B + UVB + UVR; it could range between 0 and 500), red chroma as R / brightness, green chroma as G / brightness, blue chroma as B / brightness and UV chroma as (UVB + UVR) / brightness (all four chroma metrics could range between 0 and 1). Finally,

using segmented images, we calculated pattern coverage (as area covered by spots × 100% / total area) and average size of spots.

Measuring pigment concentration

After taking pictures, eggs were cut in halves using a razor blade, emptied, and rinsed with distilled water. After drying, eggshells were ground to a fine powder in a mortar and weighed to the nearest 0.01 mg. They were transferred directly to glass vials and mixed with 450 µl of 3M HCl. Shells were left in the solution of acid overnight in darkness at 4°C. The next morning, the content of the vials was mixed with 375 µl of acetonitrile and 250 µl of water. The whole mixture was vortexed for 5 minutes and centrifuged at 14000 g for 5 minutes. The supernatant was collected and transferred to new vials for subsequent HPLC analysis.

The extracts were analysed using a mass spectrometry-coupled reversed-phase HPLC apparatus (Agilent 1260 Infinity II with the Agilent 6470 B mass detector, Agilent Technologies, Santa Clara, CA, USA). Initial runs were performed on samples of protoporphyrin IX diluted in the same medium as the actual samples to establish a calibration curve. Moreover, mass spectra and fragmentation patterns of known protoporphyrin IX samples were used to validate the identity of the pigment peak observed in samples extracted from the eggshells. Solvent A was 0.05% trifluoroacetic acid (TFA), and solvent B was acetonitrile. Elution was started at 90% A and 10% B and carried on in a linear gradient of concentration to 0% A and 100% B. Detection was done at 405 nm, protoporphyrin IX eluted at ~26.9 minutes. We measured the pigment content (in µg) and concentration (in µg of protoporphyrin IX per mg of eggshell). However, due to the variable mass of individual eggshell samples (mean = 125.8 mg, SD = 28.5, minimum = 58.8, maximum = 177.5), we focused here on the concentration of protoporphyrin rather than on its absolute content.

Statistical analyses

First, we calculated mean concentration of protoporphyrin, standard deviation (SD), minimum, and maximum values using all 43 sampled eggs. A number of eggs in our dataset originated from the same clutches, and therefore, were not independent from one another. Since we did not possess information on clutch ID for seven older eggs collected in Odolanów, we excluded them from further analyses. We also excluded two outliers with exceptionally high concentration of protoporphyrin. As mentioned above, we analysed overall brightness and coloration and repeated these analyses separately for eggshell backgrounds and spots. For the analyses based on whole eggs and on their backgrounds, we excluded images of six

overexposed eggs. Overexposed pixels appeared only in the background area, and thus, we did not exclude these eggs from analyses focusing solely on the brightness and coloration of spots. Finally, we excluded one egg with incorrectly detected spots from the analyses for background and for spots as both require an accurate detection of spots to properly segment the egg image. Prior to modelling, we centred and scaled all numeric continuous variables. The final sample size was 28 eggs from 22 clutches for analyses based on whole eggs, 27 eggs from 22 clutches for background and 33 eggs from 24 clutches for spots.

To initially explore the relationship between the concentration of protoporphyrin and colour and pattern traits measured from calibrated digital images, we calculated correlation coefficients. We checked distribution of variables using the Shapiro-Wilk test and when the distribution of the variable was normal, we calculated Pearson correlation coefficient, otherwise, we calculated Spearman rank correlation coefficient. The distribution of the concentration of protoporphyrin was normal in all cases.

In further analyses, we focused on eggshell appearance traits that correlated with the concentration of protoporphyrin. We fitted linear mixed models using the concentration of protoporphyrin as the dependent variable and, sequentially, each of the selected variables as the explanatory predictor. To control for non-independence of data points we introduced clutch ID as a random factor in all models. Protoporphyrin-based pigmentation is known to fade over time, especially during the first days after oviposition (Hanley *et al.*, 2016; Cembrano *et al.*, 2021; but see: Cassey *et al.*, 2012b). Although it was impossible to standardise the number of days eggs were exposed to daylight in the nests until their collection, we introduced the year of collection as a covariate in the models to control for a potentially higher degree of fading for older eggs. However, it is important to note here that our objective was not to approximate the original amount of protoporphyrin in the eggshell of a freshly laid egg but to investigate whether external eggshell appearance is a good predictor of the amount of the pigment. We also controlled for potential differences between the two populations, introducing population ID as another covariate in the models. We calculated variance inflation factors (VIFs) to explore collinearity between explanatory variables. In all cases, VIFs were below 2; thus, explanatory variables were not collinear. Residuals and random effects were normally distributed, with no outliers or overly influential observations. We calculated R^2 as a measure of the predictive power of the model. Since we had both fixed (selected eggshell appearance trait, year of collection, and population ID) and random (clutch ID) variables, we focused on marginal R^2 that measures the amount of explained variance attributed to fixed effects. We report results of

the models as regression coefficients $\beta \pm$ standard error and provide 95% confidence intervals ($CI_{0.95}$) for the main effect (the selected eggshell appearance trait) alongside the *p*-value for the main effect and covariates.

All analyses were performed in RStudio version 4.3.2 (R Core Team, 2023). Linear mixed models were fitted using *lme4* (Bates *et al.*, 2015) and *lmerTest* packages (Kuznetsova *et al.*, 2017). The performance of the models was assessed using *performance* package (Lüdecke *et al.*, 2021), VIFs calculated in *car* package (Fox & Weisberg, 2019) and marginal R^2 using *MuMIn* package version 1.46.0 (Bartoń, 2022). Correlations were calculated using *rstatix* package version 0.7.0 (Kassambara, 2021). Plots were prepared in *ggplot2* package (Wickham, 2016). R code and database with a complete set of measurements are provided in the Supporting Information.

Results

Mean concentration of protoporphyrin in eggshells of the red-backed shrike was 0.070 μg/mg of eggshell (SD = 0.049, $N = 43$, Table 1, Figure 2A). Correlation coefficients between protoporphyrin concentration and eggshell appearance traits measured from calibrated photographs are presented in Table 2. Most of the relationships were weak, with absolute correlation coefficients below 0.3. Only overall green chroma correlated significantly with protoporphyrin concentration ($r = –0.38$, $p = 0.047$, Figure 2B) and the correlation between protoporphyrin concentration and spots green chroma was marginally significant ($r = –0.32$, $p = 0.066$, Figure 2C).

We fitted two linear mixed models using these two variables as predictors. There was a negative relationship between the concentration of protoporphyrin and overall green chroma ($\beta = –0.022 \pm 0.008$, $F_{1,13.2} = 7.870$, $p = 0.015$, $CI_{0.95} = –0.035$ to $–0.008$) when we controlled for the year of collection (year2020: $\beta = 0.010 \pm 0.018$, $p = 0.575$; year2021: $\beta = 0.023 \pm 0.017$, $p = 0.211$; $F_{2,14.2} = 0.858$, $p = 0.445$) and population ID ($\beta = –0.064 \pm 0.032$, $F_{1,8.0} = 4.061$, $p = 0.079$). Fixed effects explained 29.9% of the variance. Similarly, there was a negative relationship between the concentration of protoporphyrin and spots green chroma ($\beta = –0.019 \pm 0.007$, $F_{1,17.1} = 7.205$, $p = 0.016$, $CI_{0.95} = –0.032$ to $–0.006$) when we controlled for the year of collection (year2020: $\beta = 0.007 \pm 0.016$, $p = 0.676$; year2021: $\beta = 0.015 \pm 0.017$, $p = 0.387$; $F_{2,14.2} = 0.394$, $p = 0.682$) and population ID ($\beta = –0.065 \pm 0.032$, $F_{1,8.0} = 4.154$, $p = 0.077$). Fixed effects explained 24.8% of the variance. Detailed results of models fitted for all measured eggshell appearance metrics can be found in Table S1 in the Supporting Information.

Discussion

Our results indicate that the concentration of protoporphyrin in the eggshells of the red-backed shrike was variable with minimum and maximum values differing by two orders of magnitude. However, the external appearance of eggshells measured utilising calibrated digital photography was a poor predictor of the pigment concentration.

Minimum protoporphyrin concentration in the investigated eggshells was similar to minimum values from two other studies conducted on the same species in the Czech Republic (Mikšík *et al.*, 1994, 1996; Table 1). Furthermore, our mean concentration was intermediate between mean concentration values provided by the studies from the Czech Republic and the maximum value was slightly lower than the maximum concentration reported by Mikšík *et al.* (1996). Overall, our study supports the conclusions of Mikšík *et al.* (1994, 1996) that protoporphyrin concentration is highly variable in eggshells of the red-backed shrike.

Among 20 measured eggshell coloration and patterning traits, most of them did not significantly correlate with protoporphyrin concentration. Unlike other studies (e.g. Cassey *et al.*, 2012b; Wegmann *et al.*, 2015; Gómez *et al.*, 2019), we found no relationship between the pigment concentration and pattern features. On the other hand, our results are in line with the studies on the blue tit *Cyanistes caeruleus* and the house wren *Troglodytes aedon* that demonstrated pattern metrics based on visual scoring and pattern coverage based on photography were poor predictors of the concentration of protoporphyrin respectively (Brulez *et al.*, 2014; Thompson *et al.*, 2022). We also found no significant relationship between protoporphyrin concentration and brightness of the eggshell that was found in other studies focusing on this pigment (e.g. Cassey *et al.*, 2012b; Wegmann *et al.*, 2015; Poláček *et al.*, 2017). Finally, unlike many studies (e.g. Wegmann *et al.*, 2015; Hargitai *et al.*, 2016a,b, 2018; Thompson *et al.*, 2022), we found no relationship between the concentration of protoporphyrin and red chroma. In fact, the only eggshell appearance metric that significantly correlated with the pigment concentration in our study was overall green chroma. This is consistent with the results of Cassey *et al.* (2012a) who found a negative relationship between blue-green chroma and protoporphyrin concentration in the eggshells of the blackbird *Turdus merula*. This species possesses blue-green eggshells mottled with fine brownish protoporphyrin-based patterning often difficult to differentiate from the background coloration (e.g. see spectra for blackbird in Cassey *et al.*, 2008).

Many pigments, including biliverdin and protoporphyrin, selectively absorb light of some wavelengths to a higher extent than light of other wavelengths. As a pigment

concentration increases, the amount of reflected light decreases to a higher degree in the region of maximum absorbance of the pigment than in other regions of the spectrum (Andersson & Prager, 2006; Johnsen, 2012). In other words, colour of an object with high concentration of pigments seems more saturated not because it reflects more light in the region of peak reflectance, but because it reflects less light outside of this region (Węgrzyn *et al.*, 2011; see also spectra in Walters & Getty, 2010). A more direct relationship between the concentration of pigments and the amount of absorbed light can be the reason we found a relationship between the concentration of protoporphyrin and green chroma but did not find such a relationship for red chroma. Protoporphyrin possesses five absorption peaks in the visible light: at 402, 503, 537, 574, and 626 nm (Lozovaya *et al.*, 1990) and three of them (503, 537 and 574 nm) coincided with the range of high sensitivity of the green channel of our camera.

Despite correlations being significant at $\alpha = 0.05$, in many studies (including this one), the strength of the relationship between eggshell appearance and concentration of pigment(s) was often moderate or even weak (Table S2 in the Supporting Information). Furthermore, the studies that provided the proportion of variance explained by models were consistent in that, in most cases, colour or pattern metrics were poor predictors of pigment(s) concentration, rarely exceeding 50% of explained variance (Wegmann *et al.*, 2015; Butler & Waite, 2016; Poláček *et al.*, 2017; but see: Cassey *et al.*, 2012b). What can be the reason for such a weak relationship? It has been shown that the presence of the cuticle (Fecheyr-Lippens *et al.*, 2015; Igic *et al.*, 2015) affects eggshell reflectance, especially in the ultraviolet part of the spectrum which can make the relationship between eggshell coloration and pigment concentration more complex. For example, Mari *et al.* (2024) demonstrated that varied reflectance in the ultraviolet part of the spectrum (with reflectance in the other parts of the spectrum being relatively constant) affected blue-green chroma measurements of eggshells of the pied flycatcher *Ficedula hypoleuca*. The red-backed shrike belongs to Passeriformes that usually possess a thin cuticle at the very surface of the eggshell (Mikhailov, 1997) which means, the cuticle can influence the spectrum reflected by the eggshells, irrespective of the pigment concentration. Furthermore, in some species, pigment deposition occurs in final hours before oviposition and thus, the layers of pigments are primarily present at the surface of the shell (Tamura & Fujii, 1967; Wang *et al.*, 2007; Fargallo *et al.*, 2014). Yet, in many other species from diverse taxa, layers of pigment occur at varying depths in the calcium carbonate matrix (Harrison, 1966; Baird *et al.*, 1975; Mikhailov, 1997; Jagannath *et al.*, 2008). Unfortunately, to our knowledge, no study to date has explored the distribution of protoporphyrin layers in the cross-section of the red-backed shrike's eggshells specifically, but comparative studies by Harrison (1966) and Mikhailov (1997)

demonstrated that layers of pigment occur at varying depths in Passeriformes. Moreover, comparing the appearance of the red-backed shrike eggs (Figure 1C) with Figure 1 from Jagannath *et al.* (2008) gives a clue that paler spots often present in eggshells of the red-backed shrike can indeed be layers of protoporphyrin deposited deeper in the eggshell. Consistent with these observations, Butler & Waite (2016) noted that including eggshell thickness as a covariate in models predicting concentration of biliverdin in the European starling *Sturnus vulgaris* eggshells, improved their predictive power (but it was still not excessive, maximum $R^2 = 0.53$). Finally, Kennedy & Vevers (1976) showed that even eggshells that are apparently white like, for example, in the white stork *Ciconia ciconia*, barnacle goose *Branta leucopsis*, European scops owl *Otus scops*, or wood pigeon *Columba palumbus* do contain detectable amounts of protoporphyrin.

Conclusion

Overall green chroma was the best predictor of the protoporphyrin concentration in eggshells of the red-backed shrike. However, similarly to other studies, we found that external eggshell appearance explained only a moderate proportion of variance in the concentration of protoporphyrin. This highlights the importance of investigating the relationship between eggshell appearance and concentration of pigment(s) in the focal species prior to utilising eggshell colour or pattern as a proxy of pigment quantity. Importantly, this does not apply to studies exploring signalling functions of the eggshell pigmentation, as in such cases, only the external eggshell appearance visible to a signal receiver is relevant.

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Author contributions

Klaudia Szala: Conceptualisation (equal); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (equal); software (lead); visualisation (lead); writing—original draft (lead); writing—review and editing (equal).

Szymon M. Drobniak: investigation (equal); methodology (equal); resources (equal); validation (equal); writing—review and editing (equal).

Adrian Surmacki: Conceptualisation (equal); funding acquisition (supporting); investigation (equal); methodology (equal); project administration (supporting); resources (equal); supervision (lead); validation (equal); writing—review and editing (equal).

Conflicts of interest

The authors declare no conflicts of interest.

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Data availability statement

Data and R code are currently provided in the Supporting Information to facilitate the review process. They will be uploaded to Zenodo repository upon acceptance of the manuscript. Custom-written scripts for MICA Toolbox are available from the GitHub repository: https://github.com/KlaudiaSzala/eggshell-spots

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Supporting Information

List of files contained in the Supporting Information:

1. SupportingInformation_pigmentation_Lancol.docx

   Table S1. Detailed results of linear mixed models for all 20 eggshell appearance metrics measured for the eggshells of the red-backed shrike *Lanius collurio*.

   Table S2. Relationships between the amount of biliverdin and protoporphyrin in avian eggshells and different metrics of eggshell appearance measured using visual scoring, spectrometry, or digital photography compiled from the literature.
2. Lancol_pigmentation_database_2024-08-19.csv – a database with all data necessary to reproduce the results of this study
3. Lancol_pigmentation_analysis_2024-09-03.R – code used to prepare the results and the figures
4. README_Lancol_pigmentation.txt – detailed description of data and file structure

Table 1. Comparison of the concentration of protoporphyrin IX in eggshells of the red-backed shrike Lanius collurio in this study and from the literature. Mean, minimum and maximum concentrations are provided in μg of the pigment/mg of eggshell. SD stands for standard deviation.

reference	**mean**	**minimum**	**maximum**	**SD**	***N***
this study	0.070	0.002	0.225	0.049	43
Mikšík *et al.* 1994[1]	0.029	0.003	0.070	0.020	22
Mikšík *et al.* 1996[2]	0.124	0.005	0.254	0.058	49

[1]This paper provides concentration of protoporphyrin in pmol/mg. We recalculated it to μg/mg to make the results comparable with ours. Molar mass of protoporphyrin IX = 562.658 g/mol.

[2]This paper provides only the content of protoporphyrin in nmol/whole eggshell. We recalculated it to concentration expressed in μg/mg using an average red-backed shrike's eggshell mass of 181 mg (N = 1334) derived from Kuźniak and Tryjanowski (2003) who cite Makatsch (1955).

Table 2. Correlation between the concentration of protoporphyrin IX in eggshells of the red-backed shrike *Lanius collurio* and different colour and pattern traits (indicated by the leftmost column, detailed explanations are provided in the text). Brightness and colour measurements are divided into three categories: overall, spots, and background (upper index "O", "Sp" and "Bg" respectively). We calculated Pearson correlation coefficient for normally distributed variables (upper index "P"), otherwise, we calculated Spearman rank correlation coefficient (upper index "S"). Correlation coefficients are arranged in a descending order (for absolute values). Sample size is 28 eggs for overall measurements, 27 for background and 33 for spots. A detailed explanation of the different *N* is provided in the text.

Eggshell appearance variable	***r***	***p***
green chroma^{O}	-0.38^{P}	0.047
green chroma^{Sp}	-0.32^{P}	0.066
maxPower	-0.3^{P}	0.118
green chroma^{Bg}	-0.27^{S}	0.171
sumPower	-0.23^{P}	0.232
blue chroma^{Bg}	-0.22^{S}	0.279
blue chroma^{O}	-0.2^{P}	0.303
propPower	-0.18^{P}	0.369
brightness^{O}	-0.17^{P}	0.392
blue chroma^{Sp}	-0.16^{S}	0.369
ultraviolet chroma^{Bg}	0.15^{P}	0.458
ultraviolet chroma^{Sp}	0.15^{P}	0.405
ultraviolet chroma^{O}	0.13^{P}	0.52
percent of spots	0.12^{S}	0.507
red chroma^{O}	0.11^{S}	0.572
average spot size	-0.11^{S}	0.529
brightness^{Bg}	-0.09^{P}	0.672
red chroma^{Sp}	-0.05^{S}	0.8
red chroma^{Bg}	0.02^{S}	0.931
brightness^{Sp}	0^{P}	0.994

Figure 1. (A) Variability of eggshell pigmentation in the red-backed shrike *Lanius collurio*. Eggs are arranged with increasing concentration of protoporphyrin IX, from left to right: minimum, 1st quartile, mean, 3rd quartile, and maximum concentration; **(B)** A preview of the detection of spots: original egg (on the left) and segmented image (on the right). Segmented images were used as masks to measure the brightness and colour of background and spots separately; **(C)** The high variation of brightness and colour of spots can indicate that layers of protoporphyrin IX occur at different depths within the calcium carbonate matrix. See the text for the discussion. In all cases, colour images are normalised images prepared in the MICA Toolbox and converted to RGB.

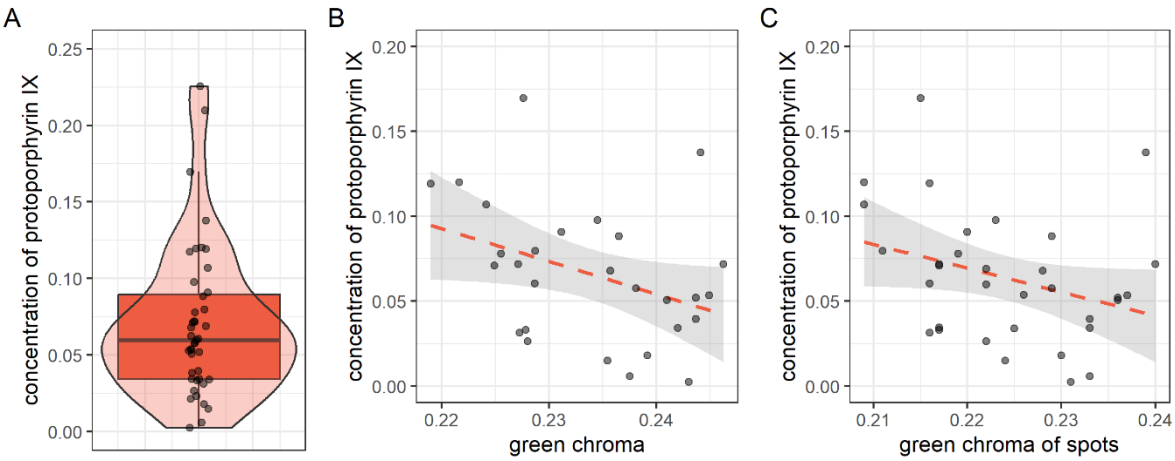

Figure 2. (A) Concentration of protoporphyrin IX (in µg/mg of eggshell) in eggshells of the red-backed shrike *Lanius collurio* (*N* = 43). Points were jittered to avoid overplotting. Note two outliers with concentration of the pigment >20 µg/mg of eggshell. The horizontal line indicates the median value, the box represents interquartile range, and tails minimum and maximum values. The pale orange shape depicts kernel density estimation. **(B)** Relationship between the concentration of protoporphyrin IX and overall green chroma (unitless, *N* = 28). **(C)** Relationship between the concentration of protoporphyrin IX and spots green chroma (unitless, *N* = 33). In both cases dashed lines depict linear regression and shaded area are 95% confidence intervals. A detailed explanation of the different *N* is provided in the text.

Supporting Information

Szala K., Drobniak S.M., Surmacki A., Eggshell coloration and patterning traits derived from calibrated photographs are poor predictors of protoporphyrin IX concentration in eggshells of the red-backed shrike *Lanius collurio*

Table S1. Detailed results of linear mixed models for all 20 eggshell appearance metrics measured for the eggshells of the red-backed shrike *Lanius collurio*. In every case, dependent variable is the concentration of protoporphyrin IX (in µg/mg of eggshell). Sample size is 28 eggs for overall measurements, 27 for background and 33 for spots. For details about methods and sample size, please, see the main text. Four models marked with asterisks (*) had a singular fit and thus, were probably overfitted.

Overall brightness (R^2 = 0.065)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0911	0.0446	12.4259	2.0415	0.063
brightness	0.0044	0.0115	22.2758	0.3814	0.7065
year2020	0.0084	0.0227	16.1558	0.3719	0.7148
year2021	0.0226	0.0276	21.1365	0.8197	0.4215
siteIDTER	-0.0393	0.0442	13.12	-0.8895	0.3898
Brightness of spots (R^2 = 0.113)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1006	0.0431	11.7564	2.333	0.0383
spots_brightness	0.0152	0.0109	23.7353	1.3859	0.1787
year2020	0.0141	0.0198	13.8884	0.7118	0.4884
year2021	0.0414	0.0279	18.7268	1.4866	0.1538
siteIDTER	-0.0555	0.0433	12.0923	-1.2836	0.2233
Brightness of background* (R^2 = 0.079)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0877	0.0363	22	2.4136	0.0246
bg_brightness	0.0054	0.0123	22	0.4367	0.6666
year2020	0.0111	0.0202	22	0.5491	0.5885
year2021	0.0263	0.0282	22	0.9345	0.3602
siteIDTER	-0.0384	0.0357	22	-1.0741	0.2944
Overall ultraviolet chroma (R^2 = 0.209)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1154	0.0408	11.7796	2.8255	0.0155
UVchroma	0.0219	0.0107	17.219	2.056	0.0553
year2020	0.0021	0.0207	15.8982	0.1019	0.9201
year2021	0.0432	0.0232	15.844	1.8576	0.0819
siteIDTER	-0.0704	0.0402	11.3847	-1.7513	0.1068
Ultraviolet chroma of spots (R^2 = 0.185)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1253	0.0372	5.5724	3.3665	0.0169
spots_UVchroma	0.0206	0.0096	15.6892	2.1326	0.0491

year2020	-0.0061	0.018	7.2726	-0.3409	0.7428
year2021	0.0349	0.0198	11.2692	1.7627	0.105
siteIDTER	-0.0712	0.0351	5.018	-2.0301	0.0979
Ultraviolet chroma of background* (R^2 = 0.207)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1101	0.0349	22	3.1545	0.0046
bg_UVchroma	0.021	0.01	22	2.0891	0.0485
year2020	0.0065	0.0186	22	0.3471	0.7318
year2021	0.0429	0.0221	22	1.9429	0.0649
siteIDTER	-0.066	0.0342	22	-1.93	0.0666
Overall blue chroma (R^2 = 0.065)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0873	0.0428	11.6364	2.041	0.0646
Bchroma	-0.0034	0.0127	20.8489	-0.2709	0.7891
year2020	0.0092	0.0226	16.0299	0.409	0.688
year2021	0.011	0.0288	20.3599	0.3838	0.7051
siteIDTER	-0.0314	0.0399	10.9854	-0.7876	0.4476
Blue chroma of spots (R^2 = 0.059)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0839	0.0426	11.1149	1.9701	0.0742
spots_Bchroma	-0.0046	0.0115	23.9284	-0.3996	0.693
year2020	0.0132	0.0205	13.9261	0.6445	0.5297
year2021	0.0076	0.0288	20.729	0.2636	0.7947
siteIDTER	-0.0275	0.0397	10.5864	-0.6915	0.5041
Blue chroma of background* (R^2 = 0.072)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0848	0.036	22	2.353	0.028
bg_Bchroma	-0.0006	0.0115	22	-0.0496	0.9609
year2020	0.0109	0.0203	22	0.5386	0.5955
year2021	0.0169	0.0271	22	0.622	0.5403
siteIDTER	-0.0321	0.033	22	-0.9737	0.3408
Overall green chroma (R^2 = 0.299)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1141	0.0338	9.0742	3.3786	0.0081
Gchroma	-0.0215	0.0077	13.1953	-2.8053	0.0147
year2020	0.0103	0.018	12.7587	0.5755	0.5749
year2021	0.0227	0.0173	14.7255	1.3077	0.211
siteIDTER	-0.064	0.0318	7.9845	-2.0152	0.0787
Green chroma of spots (R^2 = 0.248)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1188	0.0342	8.4726	3.476	0.0077
spots_Gchroma	-0.0191	0.0071	17.0496	-2.6841	0.0157
year2020	0.0069	0.016	10.4733	0.4305	0.6755
year2021	0.0153	0.0173	17.3349	0.8869	0.3873
siteIDTER	-0.0654	0.0321	7.7378	-2.0381	0.0771
Green chroma of background (R^2 = 0.249)					

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1136	0.0347	2.3499	3.2709	0.066
bg_Gchroma	-0.0202	0.0083	4.1769	-2.4218	0.0699
year2020	0.0106	0.0183	3.8076	0.5762	0.5968
year2021	0.0273	0.0187	5.4402	1.462	0.199
siteIDTER	-0.0666	0.0333	1.9067	-1.9997	0.1897

Overall red chroma (R^2 = 0.066)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0895	0.0436	12.0499	2.0513	0.0626
Rchroma	-0.0064	0.0138	22.2348	-0.4648	0.6466
year2020	0.0071	0.0231	16.2813	0.306	0.7635
year2021	0.0258	0.0302	20.8505	0.8526	0.4036
siteIDTER	-0.038	0.0421	12.127	-0.9017	0.3848

Red chroma of spots (R^2 = 0.053)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0837	0.0438	10.6511	1.9111	0.0833
spots_Rchroma	-0.001	0.0127	21.1456	-0.0778	0.9387
year2020	0.0121	0.0219	13.7752	0.5533	0.5889
year2021	0.0168	0.028	18.1276	0.6004	0.5557
siteIDTER	-0.0291	0.0416	10.3655	-0.699	0.4999

Red chroma of background* (R^2 = 0.096)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0886	0.0356	22	2.4885	0.0209
bg_Rchroma	-0.0103	0.0126	22	-0.8166	0.4229
year2020	0.0094	0.0201	22	0.4672	0.645
year2021	0.0342	0.0284	22	1.2057	0.2407
siteIDTER	-0.0411	0.0343	22	-1.2004	0.2428

maxPower (R^2 = 0.092)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0857	0.0401	10.4971	2.1357	0.0572
maxPower	-0.0099	0.0112	19.6992	-0.8813	0.3888
year2020	0.0097	0.0216	15.5111	0.4512	0.6581
year2021	0.0022	0.0269	22.9374	0.0823	0.9352
siteIDTER	-0.0265	0.0379	10.0737	-0.6991	0.5003

sumPower (R^2 = 0.065)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.087	0.042	11.1866	2.0716	0.0622
sumPower	-0.0028	0.0111	21.0902	-0.2485	0.8062
year2020	0.0086	0.0224	16.3792	0.3841	0.7059
year2021	0.0122	0.0278	22.5012	0.4407	0.6636
siteIDTER	-0.0311	0.0393	10.5152	-0.7907	0.4466

propPower (R^2 = 0.089)

term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0819	0.0425	11.8927	1.9295	0.0779
propPower	-0.0072	0.0076	21.6325	-0.9407	0.3573
year2020	0.0128	0.0226	16.4165	0.5665	0.5787

year2021	0.0174	0.0211	17.6356	0.8225	0.4218
siteIDTER	-0.029	0.0394	11.2133	-0.7358	0.477
Average size of spots (R^2 = 0.087)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.1049	0.0477	14.7454	2.1973	0.0444
avg_spot_size	-0.0093	0.009	27.9875	-1.0287	0.3124
year2020	0.0112	0.0205	14.3807	0.5443	0.5946
year2021	0.0136	0.0212	17.5511	0.641	0.5298
siteIDTER	-0.0509	0.0456	14.4466	-1.1144	0.2833
Percent of spots (R^2 = 0.056)					
term	Estimate	Std. Error	*df*	*t*	*p*
(Intercept)	0.0748	0.0491	12.9167	1.5228	0.1519
percent_spots	0.0033	0.0102	21.5259	0.3264	0.7473
year2020	0.0132	0.0205	13.8052	0.6454	0.5293
year2021	0.013	0.0225	17.3092	0.5771	0.5713
siteIDTER	-0.019	0.0484	13.043	-0.3927	0.7009

Table S2. Relationships between the amount of biliverdin (BV) and protoporphyrin (PP) in avian eggshells and different metrics of eggshell appearance measured using visual scoring, spectrometry, or digital photography compiled from the literature. Full list of references is provided below the table. For a detailed way of measuring different eggshell appearance metrics, please, consult the original papers.

species	**pigment**	**eggshell appearance metric**	**measurement method**	**effect size**	**type of effect size**	**p-value or 95% CI**	R^2	***N***	**reference**
Ficedula hypoleuca	BV	blue-green chroma	spectrometry	0.5	correlation (without details)	0.004	-	31	[1]
Sturnus unicolor	BV	PC1 (a measure of brightness)	spectrometry	-0.61	Pearson correlation coefficient	<0.01	-	80	[2]
Sturnus unicolor	BV	PC2 (reflectance in blue-green part of spectrum)	spectrometry	not provided	Pearson correlation coefficient	>0.05	-	80	[2]
Sturnus unicolor	BV	blue-green chroma	spectrometry	0.66	Pearson correlation coefficient	0.0005	-	80	[3]
Sturnus unicolor	BV	PC1 (a measure of brightness)	spectrometry	-0.69	Pearson correlation coefficient	<0.0001	-	80	[3]
Sturnus unicolor	BV	PC2 (reflectance in blue-green part of spectrum)	spectrometry	not provided	Pearson correlation coefficient	0.68	-	80	[3]
Sturnus unicolor	PP	blue-green chroma	spectrometry	not provided	Pearson correlation coefficient	0.93	-	80	[3]
Sturnus unicolor	PP	PC1 (a measure of brightness)	spectrometry	not provided	Pearson correlation coefficient	0.55	-	80	[3]
Sturnus unicolor	PP	PC2 (reflectance in blue-green part of spectrum)	spectrometry	not provided	Pearson correlation coefficient	0.33	-	80	[3]
Ficedula albicollis	BV	blue-green chroma	spectrometry	0.46	slope in generalized linear model	0.06 to 0.86	-	24	[4]
Ficedula albicollis	BV	ultraviolet chroma	spectrometry	-0.42	slope in generalized linear model	-0.83 to -0.01	-	24	[4]
Turdus merula	BV	blue-green chroma	spectrometry	not provided	slope in generalized linear model	>0.05	-	21	[5]
Turdus merula	BV	brightness	spectrometry	not provided	slope in generalized linear model	>0.05	-	21	[5]
Turdus merula	PP	blue-green chroma	spectrometry	not provided (negatively related)	slope in generalized linear model	<0.05	-	21	[5]

Turdus merula	PP	brightness	spectrometry	not provided	slope in generalized linear model	>0.05	-	21	[5]
Turdus philomelos	BV	blue-green chroma	spectrometry	not provided	slope in generalized linear model	>0.05	-	24	[5]
Turdus philomelos	BV	brightness	spectrometry	not provided	slope in generalized linear model	>0.05	-	24	[5]
Turdus philomelos	PP	blue-green chroma	spectrometry	not provided	slope in generalized linear model	>0.05	-	24	[5]
Turdus philomelos	PP	brightness	spectrometry	not provided	slope in generalized linear model	>0.05	-	24	[5]
49 avian genera	BV	L' (lightness in CIELUV)	photography	0.049	slope in phylogenetic multiple linear regression	<0.001	-	49	[6]
49 avian genera	BV	u' (red-green opponency in CIELUV)	photography	-0.008	slope in phylogenetic multiple linear regression	<0.001	-	49	[6]
49 avian genera	BV	v' (blue-yellow opponency in CIELUV)	photography	-0.003	slope in phylogenetic multiple linear regression	0.001	-	49	[6]
49 avian genera	BV	pattern coverage	photography	0.112	slope in phylogenetic multiple linear regression	0.003	0.18	49	[6]
49 avian genera	PP	L' (lightness in CIELUV)	photography	-0.207	slope in phylogenetic multiple linear regression	0.454	-	49	[6]
49 avian genera	PP	u' (red-green opponency in CIELUV)	photography	0.011	slope in phylogenetic multiple linear regression	0.01	-	49	[6]
49 avian genera	PP	v' (blue-yellow opponency in CIELUV)	photography	0.006	slope in phylogenetic multiple linear regression	0.29	-	49	[6]
49 avian genera	PP	pattern coverage	photography	0.144	slope in phylogenetic multiple linear regression	<0.001	0.51	49	[6]
Coturnix japonica	BV	blue-green chroma of spots	spectrometry	0.5	correlation (without details)	0.01	-	24	[7]
Coturnix japonica	PP	brightness of background	spectrometry	-0.5	correlation (without details)	0.01	-	24	[7]
Ficedula hypoleuca	BV	blue-green chroma	spectrometry	0.46	standardised slope in linear mixed model	<0.001	-	165?	[8]
Parus major	PP	intensity of spots	visual scoring	0.58	Pearson correlation coefficient	0.001	-	28	[9]
Parus major	PP	size of spots	visual scoring	0.47	Pearson correlation coefficient	0.01	-	28	[9]

Parus major	PP	intensity of spots on the blunt end	visual scoring	-0.61	Pearson correlation coefficient	<0.001	-	45	[9]
Parus major	PP	intensity of spots on the equator	visual scoring	0.47	Pearson correlation coefficient	0.001	-	45	[9]
Parus major	PP	pattern coverage on the blunt end	visual scoring	0.35	Pearson correlation coefficient	0.02	-	45	[9]
Parus major	PP	pattern coverage on the equator	visual scoring	0.15	Pearson correlation coefficient	0.32	-	45	[9]
Parus major	PP	pattern distribution	visual scoring	-0.19	Pearson correlation coefficient	0.33	-	45	[9]
Parus major	PP	PC1	visual scoring	-0.54	Pearson correlation coefficient	0.003	-	45	[9]
Parus major	PP	PC2	visual scoring	0.26	Pearson correlation coefficient	0.18	-	45	[9]
Cyanistes caeruleus	PP	intensity of spots	visual scoring	-0.11	Pearson correlation coefficient	0.57	-	27	[9]
Cyanistes caeruleus	PP	size of spots	visual scoring	-0.04	Pearson correlation coefficient	0.8	-	27	[9]
Cyanistes caeruleus	PP	intensity of spots on the blunt end	visual scoring	-0.3	Pearson correlation coefficient	0.13	-	27	[9]
Cyanistes caeruleus	PP	intensity of spots on the equator	visual scoring	0.04	Pearson correlation coefficient	0.84	-	27	[9]
Cyanistes caeruleus	PP	pattern coverage on the blunt end	visual scoring	0.41	Pearson correlation coefficient	0.04	-	27	[9]
Cyanistes caeruleus	PP	pattern coverage on the equator	visual scoring	0.14	Pearson correlation coefficient	0.5	-	27	[9]
Cyanistes caeruleus	PP	pattern distribution	visual scoring	0.05	Pearson correlation coefficient	0.8	-	27	[9]
Cyanistes caeruleus	PP	PC1	visual scoring	0.056	Pearson correlation coefficient	0.78	-	27	[9]
Cyanistes caeruleus	PP	PC2	visual scoring	0.1	Pearson correlation coefficient	0.62	-	27	[9]
71 species of British passerines	BV	L* (lightness in CIELAB)	photography	-0.04	slope in averaged phylogenetic generalized linear models	-0.06 to -0.03	-	71	[10]
71 species of British passerines	BV	a* (red-green opponency in CIELAB)	photography	-0.06	slope in averaged phylogenetic generalized linear models	-0.09 to -0.03	-	71	[10]
71 species of British passerines	BV	b* (blue-yellow opponency in CIELAB)	photography	-0.01	slope in averaged phylogenetic generalized linear models	-0.03 to 0.02	-	71	[10]

71 species of British passerines	BV	pattern coverage	photography	0.003	slope in averaged phylogenetic generalized linear models	-0.004 to 0.01	-	71	[10]
71 species of British passerines	PP	L* (lightness in CIELAB)	photography	-0.03	slope in averaged phylogenetic generalized linear models	-0.05 to -0.02	-	71	[10]
71 species of British passerines	PP	a* (red-green opponency in CIELAB)	photography	0.03	slope in averaged phylogenetic generalized linear models	0.0009 to 0.05	-	71	[10]
71 species of British passerines	PP	b* (blue-yellow opponency in CIELAB)	photography	0.011	slope in averaged phylogenetic generalized linear models	-0.004 to 0.03	-	71	[10]
71 species of British passerines	PP	pattern coverage	photography	0.01	slope in averaged phylogenetic generalized linear models	0.008 to 0.02	-	71	[10]
Parus major	PP	intensity of spots	visual scoring	0.42	correlation (without details)	0.004	-	45	[11]
Parus major	PP	size of spots	visual scoring	0.54	correlation (without details)	<0.001	-	45	[11]
Parus major	PP	pattern coverage	visual scoring	0.33	correlation (without details)	0.03	-	45	[11]
Parus major	PP	pattern distribution	visual scoring	0.05	correlation (without details)	0.75	-	45	[11]
Parus major	PP	red chroma	spectrometry	0.5	correlation (without details)	0.001	-	44	[11]
Serinus canaria	BV	overall blue-green chroma	spectrometry	0.31	Pearson correlation coefficient	0.12	-	26	[12]
Serinus canaria	BV	blue-green chroma of spots	spectrometry	0.36	Pearson correlation coefficient	0.075	-	26	[12]
Serinus canaria	BV	blue-green chroma of background	spectrometry	-0.03	Pearson correlation coefficient	0.88	-	27	[12]
Serinus canaria	BV	overall blue-green chroma 2	spectrometry	0.45	Pearson correlation coefficient	0.021	-	26	[12]
Serinus canaria	BV	blue-green chroma 2 of spots	spectrometry	0.39	Pearson correlation coefficient	0.046	-	26	[12]
Serinus canaria	BV	blue-green chroma 2 of background	spectrometry	0.27	Pearson correlation coefficient	0.18	-	27	[12]
Serinus canaria	BV	overall brightness	spectrometry	0.07	Pearson correlation coefficient	0.74	-	26	[12]
Serinus canaria	BV	brightness of spots	spectrometry	0.27	Pearson correlation coefficient	0.18	-	26	[12]
Serinus canaria	BV	brightness of background	spectrometry	-0.21	Pearson correlation coefficient	0.31	-	26	[12]
Serinus canaria	PP	overall red chroma	spectrometry	0.49	Pearson correlation coefficient	0.008	-	28	[12]
Serinus canaria	PP	red chroma of spots	spectrometry	0.42	Pearson correlation coefficient	0.025	-	28	[12]

Serinus canaria	PP	red chroma of background	spectrometry	0.39	Pearson correlation coefficient	0.038	-	29	[12]
Serinus canaria	PP	overall brightness	spectrometry	-0.4	Pearson correlation coefficient	0.033	-	28	[12]
Serinus canaria	PP	brightness of spots	spectrometry	-0.56	Pearson correlation coefficient	0.002	-	28	[12]
Serinus canaria	PP	brightness of background	spectrometry	-0.07	Pearson correlation coefficient	0.73	-	28	[12]
Serinus canaria	PP	intensity of spots	visual scoring	0.4	Pearson correlation coefficient	0.029	-	29	[12]
Serinus canaria	PP	size of spots	visual scoring	0.29	Pearson correlation coefficient	0.12	-	29	[12]
Serinus canaria	PP	pattern coverage	visual scoring	0.3	Pearson correlation coefficient	0.12	-	29	[12]
Serinus canaria	PP	pattern distribution	visual scoring	0.17	Pearson correlation coefficient	0.37	-	29	[12]
Sturnus vulgaris	BV	hue	photography	0.0056	slope in linear regression	<0.0001	0.476	66	[13]
Sturnus vulgaris	BV	saturatiom	photography	0.03514	slope in linear regression	<0.0001	0.271	66	[13]
Sturnus vulgaris	BV	brightness	photography	-0.00631	slope in linear regression	0.1062	0.044	66	[13]
Sturnus vulgaris	BV	B1	spectrometry	-0.00202	slope in linear regression	<0.0001	0.246	66	[13]
Sturnus vulgaris	BV	B2	spectrometry	-0.80842	slope in linear regression	<0.0001	0.246	66	[13]
Sturnus vulgaris	BV	B3	spectrometry	-0.41144	slope in linear regression	0.0463	0.062	66	[13]
Sturnus vulgaris	BV	S1R	spectrometry	-6.40491	slope in linear regression	<0.0001	0.338	66	[13]
Sturnus vulgaris	BV	S1G	spectrometry	4.49384	slope in linear regression	<0.0001	0.384	66	[13]
Sturnus vulgaris	BV	S1B	spectrometry	5.35164	slope in linear regression	<0.0001	0.455	66	[13]
Sturnus vulgaris	BV	S1U	spectrometry	5.35164	slope in linear regression	<0.0001	0.346	66	[13]
Sturnus vulgaris	BV	S1Y	spectrometry	-2.94172	slope in linear regression	0.0019	0.143	66	[13]
Sturnus vulgaris	BV	S1V	spectrometry	-2.755	slope in linear regression	<0.0001	0.354	66	[13]
Sturnus vulgaris	BV	S2	spectrometry	0.05541	slope in linear regression	0.0004	0.181	66	[13]
Sturnus vulgaris	BV	S3	spectrometry	3.2625	slope in linear regression	<0.0001	0.463	66	[13]
Sturnus vulgaris	BV	S5a	spectrometry	0.02412	slope in linear regression	<0.0001	0.414	66	[13]
Sturnus vulgaris	BV	S5b	spectrometry	0.01342	slope in linear regression	<0.0001	0.291	66	[13]
Sturnus vulgaris	BV	S5c	spectrometry	0.01197	slope in linear regression	<0.0001	0.245	66	[13]
Sturnus vulgaris	BV	S6	spectrometry	0.53085	slope in linear regression	0.0933	0.044	66	[13]
Sturnus vulgaris	BV	S7	spectrometry	0.11024	slope in linear regression	<0.0001	0.441	66	[13]
Sturnus vulgaris	BV	S8	spectrometry	0.31082	slope in linear regression	<0.0001	0.279	66	[13]

Sturnus vulgaris	BV	S9	spectrometry	0.57941	slope in linear regression	<0.0001	0.371	66	[13]
Sturnus vulgaris	BV	H1	spectrometry	0.00172	slope in linear regression	0.1687	0.03	66	[13]
Sturnus vulgaris	BV	H3	spectrometry	0.00063	slope in linear regression	<0.0001	0.454	66	[13]
Sturnus vulgaris	BV	H4a	spectrometry	0.09972	slope in linear regression	0.189	0.027	66	[13]
Sturnus vulgaris	BV	H4b	spectrometry	0.25568	slope in linear regression	0.0042	0.123	66	[13]
Sturnus vulgaris	BV	H4c	spectrometry	0.01747	slope in linear regression	0.5349	0.006	66	[13]
Sturnus vulgaris	BV	blue-green chroma	spectrometry	2.75955	slope in linear regression	<0.0001	0.454	66	[13]
Sturnus vulgaris	BV	red chroma	spectrometry	-11.53451	slope in linear regression	<0.0001	0.255	66	[13]
Sturnus vulgaris	BV	S1B	spectrometry	not provided (positively related)	slope in multiple linear regression (eggshell thickness controlled)	<0.01	0.53	66	[13]
Sturnus vulgaris	BV	blue-green chroma	spectrometry	not provided (positively related)	slope in multiple linear regression (eggshell thickness controlled)	<0.01	0.51	66	[13]
Parus major	PP	intensity of spots	visual scoring	0.38	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	size of spots	visual scoring	0.42	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	pattern distribution	visual scoring	-0.18	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	PC1	visual scoring	-0.45	Pearson correlation coefficient	<0.05	-	38	[14]
Parus major	PP	PC2	visual scoring	0.17	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - dispersion	photography	0.14	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - contrast	photography	0.32	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - coverage	photography	-0.38	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - mark size	photography	-0.05	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - PC1	photography	-0.07	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	granularity - PC2	photography	0.33	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	brightness	spectrometry	0.31	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	ultraviolet chroma	spectrometry	-0.39	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	red chroma	spectrometry	0.51	Pearson correlation coefficient	<0.05	-	38	[14]

Parus major	PP	Euclidian coordinates	photography	-0.5	Pearson correlation coefficient	<0.05	-	38	[14]
Parus major	PP	luminance	spectrometry	-0.01	Pearson correlation coefficient	>0.05	-	38	[14]
Parus major	PP	intensity of spots	visual scoring	0.06	slope in multiple linear regression	0.01	0.4	38	[14]
Parus major	PP	size of spots	visual scoring	0.15	slope in multiple linear regression	0.06	not included in final model	38	[14]
Parus major	PP	pattern distribution	visual scoring	-0.02	slope in multiple linear regression	0.62	not included in final model	38	[14]
Parus major	PP	granularity - dispersion	photography	-0.02	slope in multiple linear regression	0.27	not included in final model	38	[14]
Parus major	PP	granularity - contrast	photography	0.001	slope in multiple linear regression	<0.001	0.40 (contrast and coverage together)	38	[14]
Parus major	PP	granularity - coverage	photography	-0.01	slope in multiple linear regression	0.001	0.40 (contrast and coverage together)	38	[14]
Parus major	PP	granularity - mark size	photography	-0.001	slope in multiple linear regression	0.75	not included in final model	38	[14]
Parus major	PP	brightness	spectrometry	0.003	slope in multiple linear regression	0.03	0.27 (brightness, UV chroma and red chroma together)	38	[14]
Parus major	PP	ultraviolet chroma	spectrometry	-0.06	slope in multiple linear regression	0.01	0.27 (brightness, UV chroma and red chroma together)	38	[14]
Parus major	PP	red chroma	spectrometry	3.87	slope in multiple linear regression	0.04	0.27 (brightness, UV chroma and red chroma together)	38	[14]
Parus major	PP	Euclidian coordinates	spectrometry	-0.12	slope in multiple linear regression	<0.001	0.26	38	[14]

Parus major	PP	luminance	spectrometry	0.58	slope in multiple linear regression	0.12	not included in final model	38	[14]
Passer montanus	BV	lightness	photography	not provided (negatively related)	slope in linear mixed model	0.002	0.58	13	[15]
Passer montanus	PP	lightness	photography	not provided (negatively related)	slope in linear mixed model	0.03	0.4	13	[15]
Serinus canaria	BV	overall blue-green chroma	spectrometry	not provided	correlation (without details)	>0.05	-	21	[16]
Serinus canaria	BV	blue-green chroma of spots	spectrometry	not provided	correlation (without details)	>0.05	-	21	[16]
Serinus canaria	BV	blue-green chroma of background	spectrometry	0.44	correlation (without details)	0.049	-	21	[16]
Serinus canaria	PP	overall red chroma	spectrometry	0.54	correlation (without details)	0.011	-	21	[16]
Serinus canaria	PP	red chroma of spots	spectrometry	not provided	correlation (without details)	>0.05	-	21	[16]
Serinus canaria	PP	red chroma of background	spectrometry	not provided	correlation (without details)	>0.05	-	21	[16]
Serinus canaria	PP	intensity of spots	visual scoring	0.64	correlation (without details)	<0.001	-	22	[16]
Serinus canaria	PP	pattern distribution	visual scoring	-0.1	correlation (without details)	0.66	-	22	[16]
Charadrius alexandrinus	BV	overall red chroma	photography	-0.02	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	overall green chroma	photography	0.02	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	overall blue chroma	photography	-0.02	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	overall brightness	photography	-0.07	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	red chroma of spots	photography	0.07	Spearman rank correlation coefficient	>0.05	-	22	[17]

Charadrius alexandrinus	BV	green chroma of spots	photography	-0.09	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	blue chroma of spots	photography	-0.08	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	brightness of spots	photography	-0.09	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	red chroma of background	photography	-0.02	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	green chroma of background	photography	0.04	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	blue chroma of background	photography	0.01	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	brightness of background	photography	0.15	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	pattern coverage	photography	0.22	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	BV	fractal dimension of spottiness	photography	0.4	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	overall red chroma	photography	-0.39	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	overall green chroma	photography	-0.28	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	overall blue chroma	photography	0.35	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	overall brightness	photography	0.2	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	red chroma of spots	photography	-0.26	Spearman rank correlation coefficient	>0.05	-	22	[17]

Charadrius alexandrinus	PP	green chroma of spots	photography	-0.33	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	blue chroma of spots	photography	0.3	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	brightness of spots	photography	-0.13	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	red chroma of background	photography	-0.41	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	green chroma of background	photography	-0.31	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	blue chroma of background	photography	0.38	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	brightness of background	photography	0.46	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	pattern coverage	photography	0.35	Spearman rank correlation coefficient	>0.05	-	22	[17]
Charadrius alexandrinus	PP	fractal dimension of spottiness	photography	0.46	Spearman rank correlation coefficient	<0.05	-	22	[17]
Troglodytes aedon	PP	pixel value in red channel	photography	0.0938	slope in generalized linear mixed model	0.0014	-	46	[18]
Troglodytes aedon	PP	pixel value in green channel	photography	not provided	slope in generalized linear mixed model	0.4621	-	46	[18]
Troglodytes aedon	PP	pixel value in blue channel	photography	not provided	slope in generalized linear mixed model	0.1135	-	46	[18]
Troglodytes aedon	PP	pattern coverage	photography	not provided	slope in generalized linear mixed model	0.2735	-	46	[18]

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Rozdział 3 | Chapter 3

Presence of the cloud cover and elevation angle of the sun affect measurements of eggshell coloration and patterning obtained from calibrated digital images

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RESEARCH ARTICLE

Presence of the cloud cover and elevation angle of the sun affect measurements of eggshell coloration and patterning obtained from calibrated digital images

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Abstract

Calibrated digital photography is frequently used in studies focusing on avian eggshell appearance to measure colour and pattern features. Photographs are often taken in natural light conditions, yet little is known to what extent the normalisation process is able to control for varied light. Here, we photographed 36 blown eggs of the Japanese quail *Coturnix japonica* at five different elevation angles of the sun on both sunny and uniformly overcast days alongside grey standards. We normalised and processed the photographs in the MICA Toolbox software and checked how much noise was introduced by different natural light conditions to the colour and pattern measurements of the same set of eggs. Our results indicate that natural variation of light conditions affects eggshell colour and pattern measurements obtained by means of calibrated digital photography. Depending on a trait, the elevation angle of the sun had similar or even greater effect on the measurement than the presence of the cloud cover. Furthermore, measurements taken in cloudy conditions were more repeatable than those taken in sunny conditions. Based on the results, we propose practical guidelines regarding measuring colour and pattern of eggshells using calibrated digital photography in outdoor conditions.

KEYWORDS
granularity, light, normalised digital photography, repeatability, repeated-measures ANOVA, weather conditions

TAXONOMY CLASSIFICATION
Behavioural ecology, Sensory ecology

1 | INTRODUCTION

Over the past few decades, there has been a growing interest in the pigmentation of avian eggshells. Many functions have been proposed to explain the vast diversity of this feature. Among them, there are camouflage (Gómez et al., 2018), thermoregulatory function (Bakken et al., 1978; Wisocki et al., 2020), arms-race with brood-parasites (Davies & Brooke, 1989; Øien et al., 1995), signalling in post-reproductive sexual selection (Moreno & Osorno, 2003), structural reinforcement (Gosler et al., 2005) and protection of an embryo against UV light (Maurer et al., 2011).

Regardless of the hypothesis being tested, the first step in all studies is to accurately and objectively measure the eggshell pigmentation (Pike, 2019). Reflectance spectrometry is a golden standard

in studies on avian integuments coloration and works well in species with uniformly coloured eggshells (e.g. López-Rull et al. 2008; Moreno et al., 2006; but see Butler & Waite, 2016). Since spectrometers have its own stable light source, reflectance measurements are independent of ambient light variation. Moreover, information is provided with a very good spectral resolution, often down to 1 nm. Using spectrometry can be challenging, though, in the case of eggshells with finely mottled pattern. The most common approach in such cases is to measure the colour of spots and background separately (e.g. Duval, Cassey, Lovell, et al., 2013; Duval, Cassey, Miksík, et al., 2013; Hargitai et al., 2018) or to avoid spots at all and focus only on the background coloration (e.g. Cassey et al., 2010). However, since spectrometry consists of point measurements, it is technically very difficult to grasp the entire diversity of all aspects of complex eggshell patterning, like distribution and abundance of spots. Importantly, reconstructing patterning from point measurements is tedious, and the loss of information is high (Stevens et al., 2007). Finally, spectrometry is more suitable for laboratory studies or museum collections (Pike, 2011; Stevens et al., 2007). Nevertheless, it was successfully applied in a number of field studies (e.g. Moreno et al., 2006).

One of the most promising and convenient tools for assessing eggshell colour and pattern is digital photography. Fast development of this technique and growing accessibility of excellent cameras provide new opportunities and a good complement to spectrometry. Photography enables measuring the entire eggshell pattern and does not require excessive equipment, and measurement is relatively quick, which is of great importance in the case of studies in situ (Stevens et al., 2007). However, it comes with a cost of much lower spectral resolution than in the case of spectrometry, since information is usually provided only in three broad channels of a camera. To capture coloration within a human-visible light spectrum, one needs a camera capable of producing RAW images, with manual control of exposure settings, and a set of grey standards (Troscianko & Stevens, 2015a, 2015b). Along with the development of photographic equipment, computer software designed for processing digital images and easy measurements of different aspects of eggshell appearance emerged, for instance: ImageJ with MICA Toolbox (Troscianko & Stevens, 2015a) and QCPA (van den Berg et al., 2019), SpotEgg (Gómez & Liñán-Cembrano, 2017) and NaturePatternMatch (Stoddard et al., 2014). Such software provide a graphical user interface that facilitate linearisation and normalisation processes and output various colour and pattern descriptors (for details of normalisation and linearisation, see for example, Johnsen, 2016; Stevens et al., 2007). Additionally, programs often contain special functions dedicated to measuring eggshells' characteristics, such as fitting egg shape in MICA Toolbox (Troscianko, 2014). Another software, *pavo*, makes it possible to combine measurements from digital images and spectrometry (Maia et al., 2019).

Despite numerous advantages, it is important to bear several limitations of the photographic method in mind. While the normalisation process can cope, to some extent, with the variability of illumination, the variability of the light during the day, both in terms of intensity and the shape of spectrum, is high (Cronin et al., 2014). The presence of clouds and their thickness makes these differences even greater (Condit & Grum, 1964). Every method has its measurement error and digital photographs are no exception. It has already been outlined in the literature that corrections for a non-linear response of a camera and for inconstant light conditions have to be made using grey standards of known reflectance (Stevens, 2011; Stevens et al., 2007). Furthermore, Troscianko and Stevens (2015b and in their extensive online User Guide http://www.empiricalimaging.com/knowledge-base/, last accessed 8th May 2023) advised taking pictures under similar weather conditions whenever possible (i.e. either sunny or overcast sky, not both) and to put effort to match the angle of the measured surface to the angle of grey standard(s), especially under directional light. In some other studies carried out in outdoor conditions, authors have also restricted the timeframe for taking pictures to make sure illumination is comparable. Mostly, they avoided early morning and late evening hours when the sun is low over the horizon (Gómez et al., 2018; Stevens et al., 2017; Troscianko, Wilson-Aggarwal, Spottiswoode, & Stevens, 2016; Troscianko, Wilson-Aggarwal, Stevens, & Spottiswoode, 2016; Wilson-Aggarwal et al., 2016; but see Stoddard et al., 2016). Despite limitations and pitfalls of using digital photography to study egg coloration have been carefully discussed in the aforementioned articles, still many studies lack basic details regarding light conditions and camera settings what limits their usefulness for meta-analyses or comparative studies.

In light of the above, this is surprising that there has been virtually no study investigating how consistent are measurements taken with digital imaging under varied natural light conditions. The exception is the project devoted to the colour of skin patches in Gelada *Theropithecus gelada* (Bergman & Beehner, 2008; DeLacey et al., 2022). However, the measurements were performed on red squares from the ColorChecker chart, which substituted skin patches. Bergman and Beehner (2008) found that measurements of colour were significantly different in overcast conditions compared to control (that was the mean of all measurements in four light conditions: sun, shaded from the sun, backlit and overcast) for two analysed X-Rite ColorChecker red squares. Furthermore, for one of them ('moderate red square'), measurements differed depending on whether they were taken in the morning compared to the midday and the evening. Similarly, DeLacey et al. (2022) took photographs in four different light conditions. They also used two red patches from X-Rite ColorChecker as a substitute of the monkey's skin but converted normalised digital images into cone-catch values of the *Papio* using MICA Toolbox software. Then, based on long and medium wavelengths, they calculated red-green opponency as a measure of redness. Backlit conditions differed from the intercept (cloudy conditions) for 'light skin square', and shade and sunny conditions differed from the intercept for 'moderate red square'.

Eggs, unlike flat skin patches, are three-dimensional objects and may have complex patterning. Due to their spatial nature, edges of eggs are often darker in digital images (Gómez & Liñán-Cembrano, 2017), while taking pictures in direct light can lead to

TABLE 1 Timing of photographs series. All photographs were taken in Poznań, Poland (N 52°28′2″, E 16°55′36″). 55° is the maximum elevation angle of the sun in May at this latitude. In the case of cloudy conditions series at 10°, 20° and 30°, we took photographs in the middle of July, because rain and strong wind usually accompanied overcast sky and made it impossible to take photographs earlier.

Elevation angle of the sun (in degrees above the horizon)	Sunny conditions	Cloudy conditions
10	11.05.2021, 6:20	15.07.2021, 6:10
20	10.05.2021, 7:29	15.07.2021, 7:20
30	10.05.2021, 8:35	15.07.2021, 8:25
40	10.05.2021, 9:44	14.05.2021, 9:32 and 17.07.2021, 9:30
55	12.05.2021, 12:48	14.05.2021, 12:48

glare (Troscianko & Stevens, 2015b). Moreover, eggs with pale background and dark patterning are especially difficult to measure, as it is easy either to overexpose the background or to underexpose the spots. All this makes egg a challenging object to measure its colour and pattern properly. Furthermore, two different approaches are used, depending on a tested hypothesis. In the first approach, objective colour and pattern descriptors independent of any particular receiver are used as a proxy of eggshells' pigment content. It is applied in studies dedicated to mechanical functions of pigmentation, such as, for example, structural reinforcement (Gosler et al., 2005) or protection of an embryo against ultraviolet radiation (Maurer et al., 2011). Another approach relies on visual models of an appropriate receiver and is applied in studies investigating signalling functions of eggshell appearance. In the latter case, ecologically relevant illumination conditions should be used.

In this study, we explored the constancy of colour and pattern measurements of eggshells taken under sunny and overcast weather and during different times of the day using calibrated digital photographs. We focused on the objective approach that do not assume any signal's receiver. Our aim was to work out practical guidelines regarding optimal conditions at which photographs should be taken. Such information would be of great importance for designing fieldwork plans in studies dedicated to the appearance of eggshells. Additionally, to obtain a broader picture of light conditions that are commonly used when measuring eggshell appearance by means of digital photography, we reviewed a set of research articles.

2 | MATERIALS AND METHODS

2.1 | Preparations and measurements

We used 36 fresh eggs of the Japanese quail *Coturnix japonica* collected on 10 April 2021 at a commercial farm in Western Poland. We selected Japanese quail eggs due to their easy accessibility and complex patterning (Duval, Cassey, Miksík, et al., 2013), which was a subject of numerous studies (e.g. Coleman & McNabb, 1975; Duval et al., 2014; Gómez et al., 2016). Eggshells of Japanese quail contain biliverdin, which is responsible for greenish hues of background coloration and protoporphyrin that give rise to brownish-yellow background coloration and forms pattern of dark spots of variable sizes (Duval, Cassey, Lovell, et al., 2013). Since all eggs were collected on the same morning, we assume that each was laid by a different female and that samples in our study are independent. It is crucial because, as Duval, Cassey, Lovell, et al. (2013) have shown, eggshell traits of eggs laid by one female are repeatable (i.e. more affected by female identity than by environmental factors). Eggs were emptied with a syringe, cleaned with distilled water and kept in the dark before and between measurement sessions, as contact with direct light can lead to pigment degradation (Cassey et al., 2012).

The outdoor photographs were taken in an open area near the Faculty of Biology, Adam Mickiewicz University, Poznań, Poland (coordinates: N 52°28′2″, E 16°55′36″). We took pictures at five different elevation angles of the sun (the height of the sun above the horizon in degrees: 10°, 20°, 30°, 40° and 55°) in two different weather conditions: a clear blue sky without any cloud cover and uniformly overcast sky. The elevation angle of the sun and respective time of day were computed using an online calculator (https://darekk.com/sun/solar-position-calculator, last accessed: 15th July 2021). Direct sunlight could lead to the over-exposition of grey standards and eggs, as well as to the appearance of glare. Therefore, we photographed eggs in our own shade following other studies (e.g. Troscianko, Wilson-Aggarwal, Spottiswoode, & Stevens, 2016; Troscianko, Wilson-Aggarwal, Stevens, & Spottiswoode, 2016; Wilson-Aggarwal et al., 2016). We took photographs between May and July (Table 1). This is the time period when many avian species of the northern hemisphere's temperate zone lay eggs.

We used Nikon D90 converted to full spectrum with Nikkor EL 80 mm lens and photographed with Baader IR/UV cut filter, therefore, in the human-visible light (400–700 nm). The camera was fixed to a tripod with a lens axis aligned perpendicularly to the ground. The distance between eggshells and the lens was constant (1 m). To minimise movements of the camera, we used a remote shutter release. As grey standards, we used X-Rite ColorChecker (X-Rite, Grand Rapids) with a scale bar present next to the eggs in every photograph. We used aperture f/8 and ISO 400 and to avoid overexposure we manipulated only the shutter speed. We used exposure bracketing with ±1 step. Depending on the ambient light intensity, integration time in our set of pictures varied between 1/1250 and 1/160 s. All images were saved as RAW files. After taking every photograph, we checked the histogram for exposure. Eggshells were put sideways on a custom-made black plastic supporter with shallow pits for eggs (Figure 1). The supporter was aligned parallelly to the ground using a bubble level. The entire set was always surrounded by a brownish-grey box to prevent eggs from moving by the wind.

FIGURE 1 Set for photographing eggs: custom-made supporter on a tripod with pits for eggs, grey standards and scale bar. All set was surrounded on the sides by a box to prevent Japanese quail *Coturnix japonica* blown eggs from shaking due to wind.

Eggs were positioned according to the marks drawn on the eggshell. Thus, on each occasion, the same side of egg was photographed. Nevertheless, to make sure that eggs were set in the same position between consecutive pictures series, we took additional measurements of a subset of 18 eggs. We photographed them twice in artificial light in a darkroom. We used the Iwasaki eyeColor arc lamp MT70D E27 6500K (Iwasaki Electric Co., Ltd.), which has a broad spectrum of light similar to CIE D65 daylight illumination (Troscianko & Stevens, 2015b). We diffused the light with a 0.5 mm PTFE sheet that screened the eggs to avoid the glare and used the same camera setting as in the outdoor conditions.

2.2 | Image analyses

We processed photographs in MICA Toolbox (version 2.2.1), a plug-in for ImageJ software (Troscianko & Stevens, 2015a). Prior to colour and pattern measurements, we checked all images for exposition using the Photo Screening tool. We selected the brightest and simultaneously not over-exposed photograph from every bracketing series. Next, in every image we selected two X-Rite grey standard patches, the brightest and the darkest (92.96% and 1.99%, respectively, as measured with a spectrometer) to normalise it. Then, we set the scale (10 mm bar) and selected eggs using a multipoint tool to indicate eight anchor points along an egg's edges (Troscianko, 2014). We used the Scale Bar Calculator tool to check the minimum px/mm factor in our set of pictures (15.83 px/mm rounded down to 15.5 px/mm). This factor is essential in pattern analysis and should always be rounded down to rescale all images and make pattern measurements from different images comparable (Troscianko & Stevens, 2015b). Furthermore, we applied the Batch Multispectral Image Analysis tool for automatic measurements of colour and pattern. We rescaled our images and selected green channel for pattern analysis, as it closely matches the sensitivity of double cones in birds (Spottiswoode & Stevens, 2010). In pattern granularity analysis, we used the following settings: method—fast Fourier transform (FFT), start size—2 px, end size—256 px, step size—square root of 2, and steps were multiplied.

The software exports the results as a csv file where reflectance in every channel is measured compared to the reflectance of grey standards, therefore in the range between 0 (black) and 100 (white). Following methods used for spectrometry, we calculated brightness as the sum of reflectance in red, green and blue channels, and red chroma as reflectance in red channel divided by brightness (e.g. Butler & Waite, 2016; Hargitai et al., 2016). Further, following DeLacey et al. (2022), we converted our images into human cone-catch in CIE D65 illumination and calculated red-green opponency as (LW − MW)/(LW + MW). We also selected *sumPower* from granularity analysis as our measure of total pattern contrast, *maxPower* as a measure of contrast for dominating spot size, *propPower* as a measure of pattern diversity and *maxFreq* as a measure of dominating spot size (Troscianko & Stevens, 2015b). Granularity analysis based on fast Fourier bandpass filtering is often used to describe patterns of animals or eggshells (e.g. Stoddard et al., 2019; Šulc et al., 2019).

2.3 | Statistical analyses

All statistical analyses were performed in RStudio (version 4.0.5, R Core Team, 2021). To prepare diagnostic plots and plots with results, we used the following packages: *ggplot2* (Wickham, 2016), *ggsignif* (Ahlmann-Eltze & Patil, 2021), *ggpubr* (Kassambara, 2021).

To check if measurements of the same eggs taken in different light conditions differ significantly, we used repeated-measures ANOVA with two independent variables: weather and elevation angle of the sun, as well as their interaction. The weather was a factor with two levels ('sun' and 'cloud'), while the elevation angle of the sun was a factor with five levels (10°, 20°, 30°, 40° and 55°). We checked the assumptions and calculated repeated-measures ANOVA in *rstatix* package (Kassambara, 2021, see Appendix S1 for details). Generalised eta square (*ges*, hereafter) measures how much variation is explained by each model term and is a recommended effect-size statistic for repeated-measures designs (Bakeman, 2005), thus, we used it in our study. Pairwise comparisons were calculated using the Bonferroni correction for multiple comparisons.

Granularity's *maxFreq* is a variable corresponding to the size of dominating spot size on a logarithmic scale. Therefore, prior to analyses, we applied log2 transformation to obtain the values on a linear scale, from 1 (the smallest spot size) to 8 (the biggest spot size) in 0.5 increments. Since this is a discrete variable, we could not use repeated-measures ANOVA and used the Friedman test instead. Because it is a one-way test, we aggregated our two variables: weather and degrees into a one-factor variable, 'level', a combination of these two (cloud10, sun40 etc.). To perform Friedman test, we used *rstatix* package (Kassambara, 2021).

To estimate the repeatability of measurements taken in different light conditions, we calculated the inter-correlation coefficient using the LMM-basing approach for Gaussian data in *rptR* package (Nakagawa & Schielzeth, 2010; Stoffel et al., 2017) and following the same transformations as for ANOVA analysis (see Appendix S1). We calculated repeatability for the whole dataset ('natural'), as well as

separately for sun and cloud conditions. Additionally, we calculated the repeatability of measurements for a subset of 18 eggs photographed in constant artificial light conditions to check how much noise arises from putting the eggs on the egg-holder and selecting their edges in the software. In all cases, we used 500 bootstraps to obtain 95% confidence limits. We did not calculate repeatability for *maxFreq* variable due to its discrete character. Data and R code used for the statistical analyses are available from the Zenodo Digital Repository (Szala et al., 2023) and from the GitHub repository.

2.4 | Literature review

To investigate how other authors approached the issue of variable light in their research, we reviewed the literature as follows. We looked for all papers that cited the work of Troscianko and Stevens (2015a) in Google Scholar (last accessed on 21st October 2022). We found 334 records and filtered them by looking for the word 'egg' within the text, which limited the number of papers to 126. Among them, basing on abstracts and/or methods section, we looked for articles that were original research papers focused on avian eggs and used calibrated digital photography to measure eggshell colour and/or pattern trait(s). We found 22 such articles (Table S1). Furthermore, we extracted information about the conditions in which pictures were taken. We particularly focused on the time of the day, weather (i.e. overcast or clear sky) and light diffusion techniques (i.e. light diffuser or shade).

3 | RESULTS

Results of the repeated-measures ANOVA showed that both weather and elevation angle of the sun, as well as their interaction significantly affected all six measurements of the eggshells appearance that we focused on (Tables 2 and 3). In the case of red chroma and human red-green opponency, variability caused by the elevation angle of the sun was higher than variability caused by the weather, as *ges* indicated. For the rest of variables *ges* of both factors and their interaction had similar values. The trait that turned out to be the most resistant to variability due to different illumination was *propPower*.

Since the interaction between weather and the elevation angle of the sun was significant in all cases, we additionally calculated one-way ANOVA models where we checked the effect of the elevation angle of the sun on either level of the weather factor (Table 4). In the case of *propPower*, the effect of the elevation angle of the sun was similar for both weather conditions, whereas, for the rest of variables, the effect of the elevation angle of the sun was more pronounced in the sunny conditions. In Figure 2, we presented all pairwise comparisons that did not significantly differ from each other (except for *propPower*, where significantly different pairs were marked because there were too many non-significantly different comparisons for the plot to be clear and interpretable). Apart from *propPower*, red chroma and human red-green opponency were the traits that had the most non-significant pairs of measurements—which is also indicated by their low values of *ges* (Table 2). Also, for every trait, there were from one to three pairs of measurements taken under different weather conditions, but at the same elevation angle of the sun, that did not differ significantly (Figure 2).

The Friedman test revealed no significant effect of illumination on *maxFreq* values (Friedman $\chi^2 = 6.7013$, df = 9, $p = .6682$), and the additional test showed that the effect size was small according to Cohen's interpretation guidelines (Kendall's $W = 0.0207$). Figure 3 presents a histogram of occurrences of different spot size categories in different light conditions.

Depending on the eggshell colour or pattern feature, the repeatability of measurements highly varied (Figure 4; Table S2). It was the lowest for *sumPower* measured in sunny conditions ($R = 0.347$, 95%

TABLE 2 Results of repeated-measures ANOVA for chromatic and achromatic features for a set of 36 Japanese quail *Coturnix japonica* eggs photographed in different weather conditions and elevation angles of the sun ('degrees'). Generalised eta square (*ges*) is the amount of variability due to the within-subjects factor.

Effect	DFn	DFd	*F*-value	*p*-value	*ges*
Brightness					
Weather	1	35	160.08	<.001	0.061
Degrees	2.95	103.21	74.064	<.001	0.061
Interaction weather:degrees	2.77	96.94	99.491	<.001	0.082
Red chroma					
Weather	1	35	6.604	<.05	0.003
Degrees	2.67	93.37	44.609	<.001	0.017
Interaction weather:degrees	2.55	89.09	18.746	<.001	0.01
Human red-green opponency					
Weather	1	35	25.123	<.001	0.018
Degrees	2.64	92.3	49.894	<.001	0.042
Interaction weather:degrees	2.73	95.72	32.099	<.001	0.041

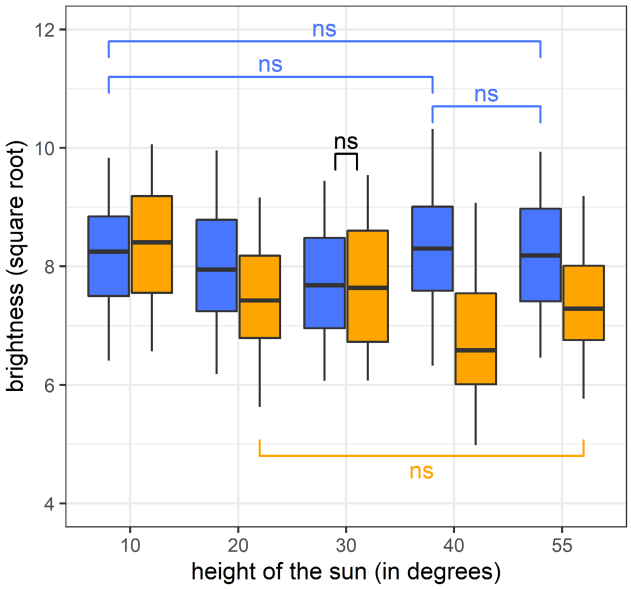
Effect	DFn	DFd	*F*-value	*p*-value	*ges*
sumPower					
Weather	1	34	259.303	<.001	0.2
Degrees	2.51	85.21	104.067	<.001	0.183
Interaction weather:degrees	2.36	80.3	114.3	<.001	0.211
maxPower					
Weather	1	34	282.972	<.001	0.129
Degrees	2.7	91.68	78.23	<.001	0.092
Interaction weather:degrees	2.32	78.99	79.294	<.001	0.111
propPower					
Weather	1	34	9.168	<.01	0.004
Degrees	4	136	6.308	<.001	0.007
Interaction weather:degrees	3.09	105.22	2.767	<.05	0.004

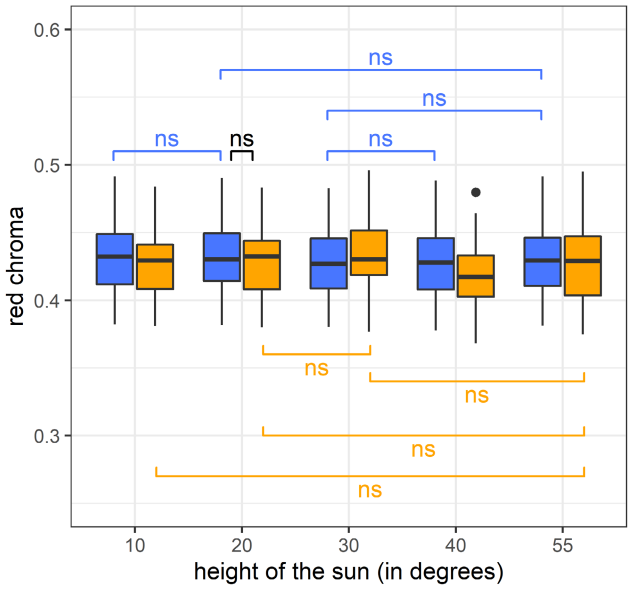
TABLE 3 Results of repeated-measures ANOVA for granularity analysis traits for a set of 36 Japanese quail *Coturnix japonica* eggs photographed in different weather conditions and elevation angles of the sun ('degrees'). Generalised eta square (*ges*) is the amount of variability due to the within-subjects factor.

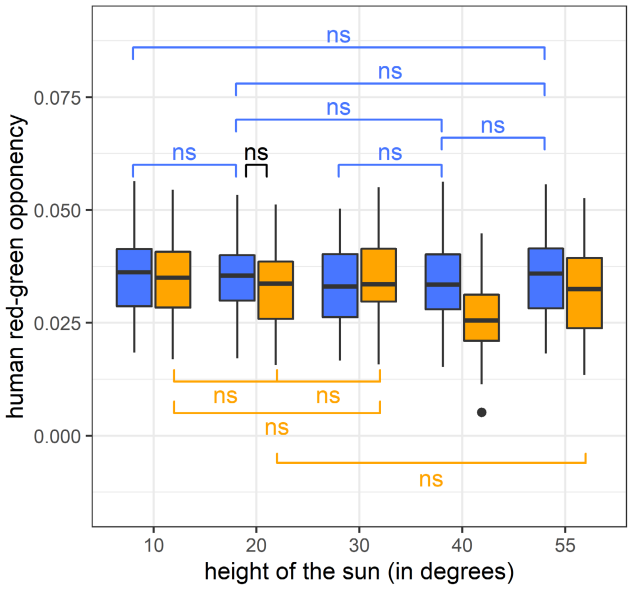
Weather	Effect	DFn	DFd	*F-value*	*p*.adj	*ges*
Brightness						
Cloud	Degrees	2.38	83.38	23.866	<.001	0.04
Sun	Degrees	2.88	100.8	143.846	<.001	0.204
Red chroma						
Cloud	Degrees	2.55	89.13	12.59	<.001	0.003
Sun	Degrees	2.66	93.2	32.276	<.001	0.05
Human red-green opponency						
Cloud	Degrees	2.9	101.37	7.832	<.001	0.008
Sun	Degrees	2.69	94.08	47.907	<.001	0.14
sumPower						
Cloud	Degrees	2.07	70.29	37.722	<.001	0.168
Sun	Degrees	2.7	91.7	268.136	<.001	0.47
maxPower						
Cloud	Degrees	2.13	72.32	29.143	<.001	0.078
Sun	Degrees	2.53	86.13	146.127	<.001	0.292
propPower						
Cloud	Degrees	4	136	4.447	<.01	0.011
Sun	Degrees	2.84	96.67	4.167	<.05	0.01

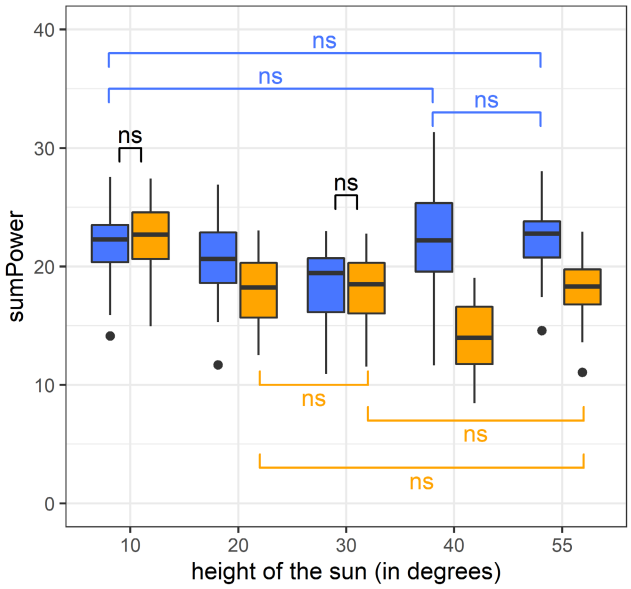
TABLE 4 Effect of the elevation angle of the sun ('degrees') at each level of the weather variable for a set of 36 Japanese quail *Coturnix japonica* eggs photographed in different weather conditions and elevation angles of the sun. *p*-value was adjusted with Bonferroni correction. Generalised eta square (*ges*) is the amount of variability due to the within-subjects factor.

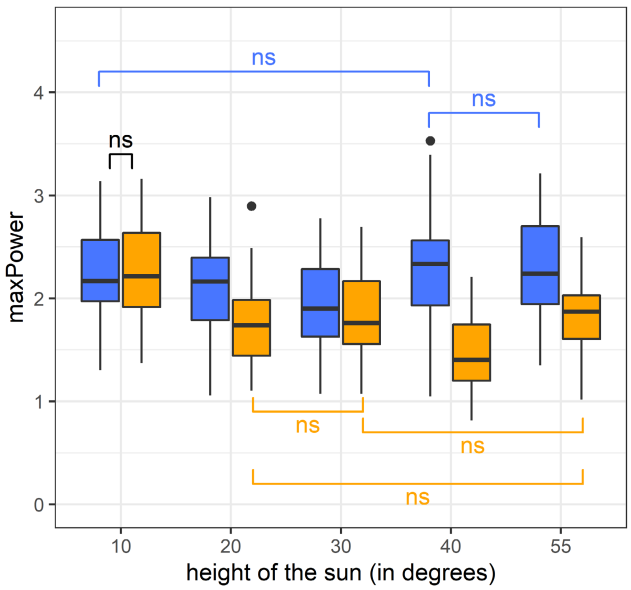
FIGURE 2 Results of the repeated-measures ANOVA. The same set of 36 Japanese quail *Coturnix japonica* eggs was photographed in sunny (orange boxes) and cloudy (blue boxes) conditions at five different elevation angles of the sun above the horizon. For brightness, red chroma, human red-green opponency, *sumPower* and *maxPower*, non-significant pairwise comparisons are marked. Blue lines above boxplots connect pairs of measurements taken in cloudy conditions; orange lines pairs of measurements taken in sunny conditions; and black lines pairs of measurements taken in different weather conditions but at the same elevation angles of the sun. In the case of *propPower*, significant pairs are shown for clarity (there were too many non-significant comparisons), and the meaning of the colour is the same as for the rest of the plots, $^{*}p < .05$; $^{**}p < .01$.

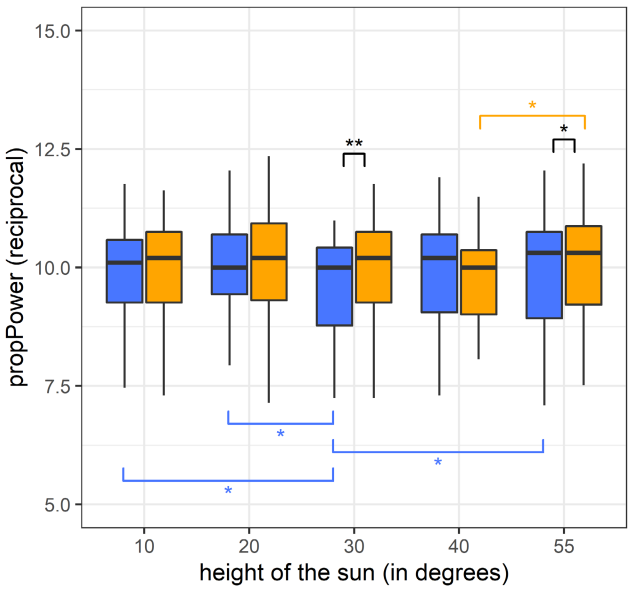












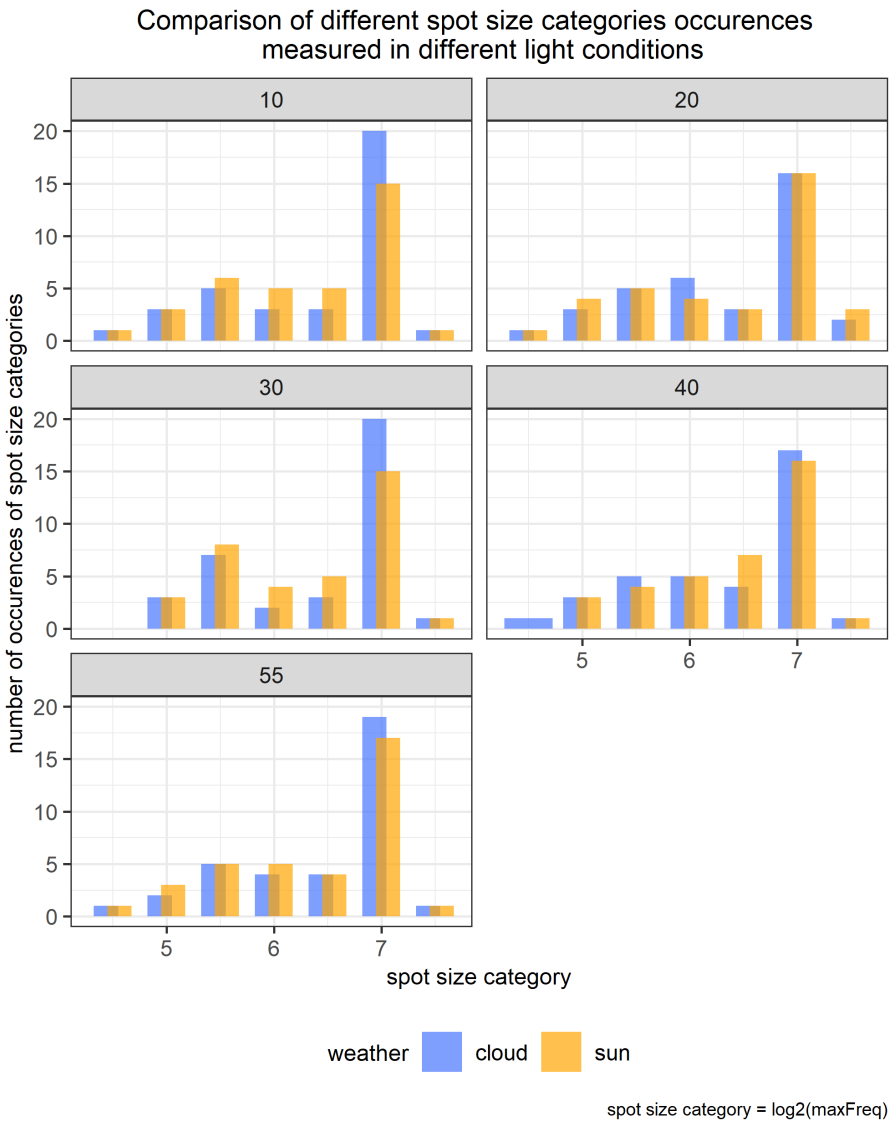


FIGURE 3 Histograms with comparison of occurrences of spot size categories in different light conditions. The same set of 36 Japanese quail *Coturnix japonica* eggs was photographed in sunny and cloudy conditions at five different elevation angles of the sun. Numbers above facets indicate elevation angle of the sun above the horizon (in degrees), and different weather conditions are expressed as colour. Spot size category is *maxFreq*, value from the granularity analysis after log2 transformation to acquire linear scale of spot size categories.

CI: 0.175–0.494) and the highest for red chroma measured in cloudy conditions ($R=0.984$, 95% CI: 0.971–0.990). In fact, the repeatability of red chroma measured in cloudy conditions in different elevations of the sun was almost as high as repeatability of red chroma measured twice in constant artificial light. Except for *propPower*, in all cases, the repeatability of measurements taken in cloudy conditions was higher than the repeatability of measurements taken in sunny conditions or than repeatability calculated for the entire dataset (combined data for sunny and cloudy conditions). Moreover, for brightness, red chroma and human red-green opponency, 95% confidence intervals of repeatability at both weather conditions did not overlap. Instead, for *propPower*, 95% confidence intervals of repeatability of measurements taken in both weather conditions highly overlapped, with repeatability for sunny conditions being slightly higher (Figure 4; Table S2).

In constant artificial light, all measured traits had repeatability higher than 0.9, the highest for red chroma ($R=0.998$, 95% CI: 0.995–0.999) and the lowest for *propPower* ($R=0.907$, 95% CI: 0.759–0.959; Table S2).

In 13 out of 22 reviewed articles, pictures were taken in natural light. Among these, in five cases photographs were taken only under sunny conditions, whereas in the remaining eight the authors did not provide details about the weather. There were no studies where pictures were taken only under cloudy conditions. In next three cases, artificial light was used for taking photographs, and in the remaining six papers, no information was provided on whether pictures were taken under natural or artificial light. Furthermore, among 13 studies that photographed in natural light conditions, only in 6 articles authors restricted their measurements to selected parts of the day (and thus, to certain elevation angles of the sun). Mostly, they avoided early morning and late evening hours when the sun is low above the horizon. Finally, in three studies, photographs were taken in direct light; in six, the authors used a diffuser to diffuse light; and in four, the authors took pictures in the shade. In the remaining nine cases, there was no information on whether the light was direct or diffused (Table S1).

Validation using X-Rite's four grey patches and red, green and blue patches (see Appendix S1 for details) showed a similar pattern of change as for the eggs (Tables S3 and S4).

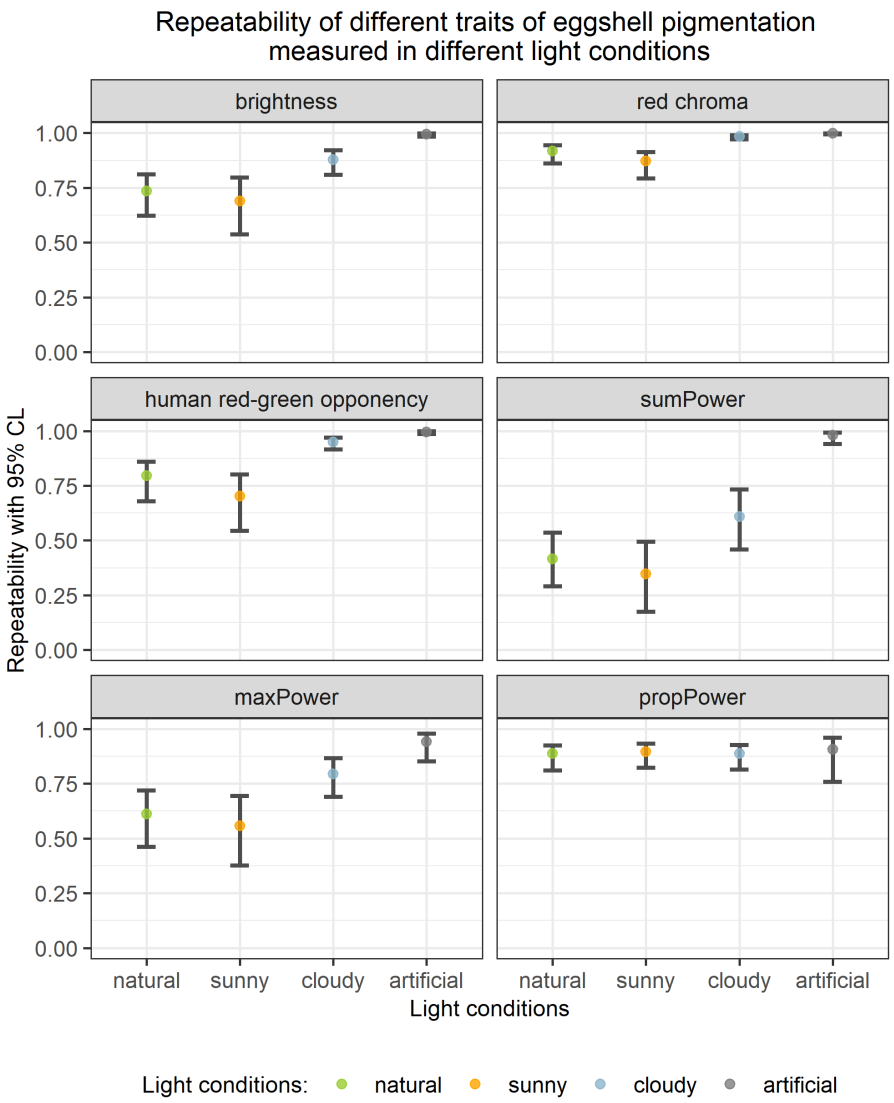


FIGURE 4 LMM-based repeatability and 95% confidence limits of measurements of six eggshells' colour and pattern traits. The same set of 36 Japanese quail *Coturnix japonica* eggs was photographed in sunny and cloudy conditions at five different elevations angles of the sun. Category 'natural' is a combination of sunny and cloudy categories. Additionally, a subset of 18 eggs was photographed twice in constant artificial light conditions ('artificial' category) to measure the noise that arose from putting the eggs on the egg-holder and selecting the eggs' edges on the picture.

4 | DISCUSSION

The light intensity and spectrum are highly variable in the course of a day and due to weather (Condit & Grum, 1964; Cronin et al., 2014), and our results show that the normalisation process is not fully capable to cope with such differences. This confirms that measurements should be taken in uniform weather conditions, as Troscianko and Stevens (2015b) advised. Furthermore, we show that there is another, often overlooked, factor that affects the measurements and that cannot be fully controlled for by the normalisation, namely the elevation angle of the sun. Our analysis indicated that the elevation angle of the sun and weather had a similar effect on brightness and granularity analysis traits. Instead, in the case of red chroma and human red-green opponency the elevation angle of the sun affected the measurements even to a greater degree than the weather.

The excellent repeatability (nearly 1.0) of measurements taken in artificial light clearly shows that we managed to minimise error due to the arrangement of eggs on the egg-holder. Thus, we always measured the same side of every egg and accurately selected eggs' edges in the MICA Toolbox software. Hence, the ambient light was the only factor that accounted for differences between our measurement series. However, to prevent eggs from shaking due to wind, we always surrounded our setup with a brownish-grey cardboard box (Figure 1). Even though the box was rather dull in coloration, it could contribute to some extent to smaller consistency of measurements in red channel comparing with, for example, green channel (Table S4; Figures S2 and S3). We also did not control for the colour of our clothes when taking photographs. However, since we photographed eggs in our shade, we were always turned with the back to the sun. Therefore, no direct light could reflect from our clothes and reach the eggs.

Behind the normalisation process, there lay assumptions that grey standards are positioned at the same distance as the measured object and that the angle between the light source and the sample, as well as between the light source and the grey standards, match (Troscianko & Stevens, 2015b). While it is possible to align standards and flat objects such as for example skin patches (Bergman & Beehner, 2008; DeLacey et al., 2022), it is impossible to do so with a tri-dimensional object such as egg, and thus, this assumption will never be met. The curvature of the eggs results in a non-uniform

reflection of light, depending on where a point is localised on the egg surface: the closer the point is to the visible edge of an egg, the reflection is lower (Gómez & Liñán-Cembrano, 2017).

It is worth underlining here that, except *propPower*, measurements of all analysed traits were more repeatable when sky was overcast compared to sunny conditions. In particular, the repeatability of red chroma under cloudy conditions was almost as high as repeatability of measurements from pictures taken in artificial light conditions (Figure 4). This is consistent with the ANOVA results that also show the variance introduced by the elevation of sun was lower in the cloudy conditions. It is important to note that we were not able to control for the cloud layer thickness. One could expect that this variable would introduce some noise into data and that sunny conditions would be more uniform, but our results showed the opposite. Cloud cover diffuses light, compensating for the eggs' curvature and making eggs more uniformly illuminated. It is essential to underline that all photographs in the study were taken in a shade. Thus, the results of our analysis indicate that shading eggs is not enough to diffuse light properly in sunny conditions. Therefore, we recommend taking photographs when the sky is overcast than when it is clear. Another option is to photograph during sunny weather using light diffusers, but unfortunately, we did not include such light conditions in our study design.

Alternatively, a box with an artificial light source was successfully applied in some studies (e.g. Poláček, Griggio, et al., 2017; Poláček, Bartíková, & Hoi, 2017; Ornés et al., 2014; Rahn & Ornés, 2020). Such an approach allows to omit the issue of ambient light variation. However, there are two other pitfalls: the light source should have a broad and flat spectrum that resembles the spectrum of sunlight (Troscianko & Stevens, 2015b) and light should be diffused to avoid glare, over-exposition and, again, illuminate the eggs uniformly.

As the sun goes down, its radiance decreases and shifts to longer wavelengths (Johnsen, 2012). Therefore, we expected the measurement taken at 10° in sunny conditions to stand out. However, only in the case of brightness and two contrast measures (*sumPower* and *maxPower*) this measurement significantly differed from all the other four measurements taken in sunny conditions. For the rest of the traits, at least one pair of measurements did not differ, for example, 10° and 55° for red chroma (Figure 2). It may be explained by the fact that the most extreme changes in illumination occur once the sun is less than 10° above the horizon, whereas we took photographs when the sun was already 10° above the horizon. Also, since we shaded the eggs from direct sunlight, they were illuminated by the skylight. Skylight, which is scattered sunlight, is shifted to shorter wavelengths (Johnsen, 2012). However, when the cloud cover was present, it diffused not only the skylight but also sunlight that indirectly reached the eggs. Consequently, more light (including longer wavelengths) reflected from the eggs and eggs were more evenly illuminated. This is why brightness and red chroma tended to be higher in cloudy conditions (Figure 2).

The effect of inconstant natural light conditions on colour measures obtained with photography was also found in the studies on Gelada's skin patch (Bergman & Beehner, 2008; DeLacey et al., 2022). The results of Bergman and Beehner (2008) showed that measurements taken under cloudy conditions stood out and that time of day (and thus, the elevation angle of the sun) affected the red-green ratio that they used in the study as a measure of colour. Similarly, DeLacey et al. (2022) showed that the measurements taken in different illumination (sun, shade, backlit and cloud) significantly differed, even though the images were transformed into a cone-catch model of the *Papio*, and the measured object was a flat skin patch of uniform colour.

For most of the analysed traits, measurements did not significantly differ between photographs taken when the sun was at 40° and 55° above the horizon in overcast conditions (see pairwise comparisons in Figure 2). Therefore, we recommend this timeframe for photographing, as it minimises the noise arising from inconstant natural light conditions. At moderate geographical latitudes of the northern hemisphere in May, this is around 3 h before and after noon, which gives a reasonable amount of time for fieldwork. The higher sun is, the more uniform illumination and the less shade it provides. Thus, measurements taken at elevation angles above 55° would probably bring similar results, but we encourage researchers from lower latitudes to test this idea.

The trait that was highly affected by the light conditions was *sumPower*, especially in sunny conditions. The variability due to weather and elevation of the sun was almost as high as the variability in our sample of eggs for this trait (Figure S4). Therefore, it should be used with caution if the light varies between measurements, especially when pictures are taken in sunny weather. On the other side, red chroma measured in cloudy conditions had very high repeatability and seemed quite resistant to the changes in illumination. Similarly, the Friedman test showed that light conditions had a minor effect on the spot size measurement. However, there is another point that one has to bear in mind when selecting a colour or pattern trait for analyses: whether and how this trait correlates with the content of the pigment in the eggshells of the studied species. The more a colour or a pattern trait correlates with pigment content or concentration, the better (e.g. Gómez et al., 2019; Wegmann et al., 2015, but see Butler & Waite, 2016). Such an approach is often used in studies on mechanical and structural functions of eggshell pigmentation, as it does not assume any specific observer and seeks to objectively measure the amount of the pigments. Alternatively, in the studies on signalling functions of the eggs' appearance, one should transform the image into an appropriate visual model of a signal receiver, for example, a predator for camouflage function and a host species for anti-brood-parasite function. In such a case, variable light is of lower importance, and instead, one should put effort into taking photographs of eggs in conditions in which potential observer would see them (Stevens, 2011; Troscianko, Wilson-Aggarwal, Stevens, & Spottiswoode, 2016).

To conclude, first, we would like to encourage researchers to work out a uniform protocol to align grey standards and measured object(s) when planning the fieldwork. Second, it is essential to keep in mind that the normalisation process cannot compensate for all changes in illumination. Therefore, one should limit

photographing to selected weather conditions, preferably overcast, or use a light diffuser, and restrict fieldwork to a certain timeframe. Alternatively, one can use a box with artificial light source to eliminate the noise that natural lighting conditions can introduce into data. Finally, we encourage other authors to provide more details about the conditions in which photographs were taken, so results of different projects can be compared, as proposed by Kemp et al. (2015).

AUTHOR CONTRIBUTIONS

Klaudia Szala: Conceptualisation (equal); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (equal); methodology (equal); visualisation (lead); writing—original draft (lead); writing—review and editing (equal). **Marcin Tobolka:** Conceptualisation (equal); formal analysis (supporting); funding acquisition (supporting); methodology (equal); supervision (equal); writing—review and editing (equal). **Adrian Surmacki:** Conceptualisation (equal); investigation (equal); methodology (equal); supervision (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the GitHub repository (https://github.com/KlaudiaSzala/eggshell-photography-variable-light) and archived on Zenodo (Szala et al., 2023, https://doi.org/10.5281/zenodo.7976182).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Appendix

Szala K., Tobolka M., Surmacki A. (2023) Presence of the cloud cover and elevation angle of the sun affect measurements of eggshell coloration and patterning obtained from calibrated digital images. Ecology and Evolution 13(7): e10170.

Table A1. Review of light conditions in the original studies that used calibrated digital photographs to measure the eggshell pigmentation. "Sun" in the "light" column means that the authors photographed only in sunny conditions, while "natural" means that photos were taken in outdoor conditions with no further information about the weather. The column "part of day" presents if the authors restricted measurements to a selected part of day (and thus elevation angles of the sun). Hours are in the 24-hour time notation; NA = not applicable. The rightmost column contains information about light diffusing technique applied in the studies (direct light, shade or diffused light). We looked for all papers that cited the paper of Troscianko and Stevens (2015a) in Google Scholar (last accessed on 21.10.2022). We found 334 records and filtered them searching for the word „egg" within the text. Basing on abstracts or/and methods section, we selected articles that were original research papers focused on avian eggs and that used digital photographs to measure eggshell pigmentation trait(s).

first author	year	title	light	part of day	direct light / diffused light / shade
Abernathy, V.E.	2022	Empirical evidence of coevolution between the channel-billed cuckoo and its host, the pied currawong	no info	no info	no info
Dixit, T.	2022	Visual complexity of egg patterns predicts egg rejection according to Weber's law	natural	no info	shade
Šulc, M.	2022	Automatic identification of bird females using egg phenotype	sun	no info	diffused light
Attisano, A.	2021	Discrimination and ejection of eggs and nestlings by the fan-tailed gerygone from New Caledonia	no info	no info	no info
Birkhead, T.	2021	Exceptional variation in the appearance of Common Murre eggs reveals their potential as identity signals	natural	no info	direct light
Lund, J.	2021	Coevolutionary causes and consequences of high-fidelity mimicry by a specialist brood parasite	natural	no info	shade
Liñán-Cembrano, G.	2021	Quail eggs in artificial nests change their coloration when exposed to ambient conditions: implication for studies on nest predation	natural	no info	no info
Nahid, M.I.	2021	No evidence of host-specific egg mimicry in Asian koels	no info	no info	no info

Quach, L.	2021	Egg patterns as identity signals in colonial seabirds: a comparison of four alcid species	artificial	NA	diffused light
Abernathy, V.E.	2020	Empirical evidence of different egg morphs that match host eggs in the brush cuckoo (*Cacomantis variolosus*)	no info	no info	no info
Stoddard, M.C.	2019	Higher-level pattern features provide additional information to birds when recognizing and rejecting parasitic eggs	no info	no info	no info
Štětková	2019	Factors affecting response of the host towards parasitic egg	natural	no info	diffused light
Šulc, M.	2019	Mimicry cannot explain rejection type in a hostebrood parasite system	natural	no info	diffused light
Gómez, J.	2018	Individual egg camouflage is influenced by microhabitat selection and use of nest materials in ground-nesting birds	natural	between 9:00 and 11:00	no info
Gómez, J.	2018	Latitudinal variation in biophysical characteristics of avian eggshells to cope with differential effects of solar radiation	artificial	NA	diffused light
Hwang , I.	2018	Effects of eggshell coloration on egg cannibalism among Glaucous-winged Gulls	artificial	NA	no info
Abernathy, V.E.	2017	Egg mimicry by the pacific koel: mimicry of one host facilitates exploitation of other hosts with similar egg types	no info	no info	no info
Stevens, M.	2017	Improvement of individual camouflage through background choice in ground-nesting birds	sun	two hours after sunrise – two hours before sunset	direct light
Stoddard, M.C.	2016	Camouflage and clutch survival in plovers and terns	natural	6:30 – 9:00	diffused light
Troscianko, J.	2016	Camouflage predicts survival in ground-nesting birds	sun	two hours after sunrise – two hours before sunset	shade
Troscianko, J.	2016	Nest covering in plovers: How modifying the visual environment influences egg camouflage	sun	one hour after sunrise – one hour before sunset	shade
Wilson-Aggarwal, J.K.	2016	Escape distance in ground-nesting birds differs with individual level of camouflage	sun	two hours after sunrise – two hours before sunset	direct light

Literature used in the review

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Detailed description of the assumptions of the repeated-measures ANOVA

To meet the assumptions of normality, we square rooted the brightness. In the case of *sumPower* and *maxPower* we removed observations of one egg (no. 7), from all cells, as it was an outlier. This egg had an additional calcium layer on the very surface of the eggshell, with small very bright dots of calcium, which may be the reason of its exceptionally high contrast value. In the case of *propPower* we found one outlier (egg no. 12 that had exceptionally big spot that covered almost all blunt end of the egg) and applied reciprocal transformation, but despite this, the distribution in two cells still differed from the normal distribution (Shapiro-Wilk test: $W = 0.933$ and 0.935, $p = 0.034$ and 0.039 respectively), and therefore results of repeated-measures ANOVA for *propPower* should be treated with caution. Other variables had normal distribution, with the exception of maxFreq, which is a discrete variable, and therefore we used Friedman test for the latter.

Table A2. Detailed results of repeatability analysis (inter-correlation coefficient using LMM-basing approach for Gaussian data in *rptR* package for R). Natural light conditions refers to all data set while sunny and cloudy were subset of measurements taken respectively under clear sky and uniformly overcast sky at different height of the sun above the horizon (10, 20, 30, 40 and 55 degrees). Artificial light conditions refers to a subset of 18 eggs measured twice under constant artificial light.

light conditions	trait	repeatability	lower 95% CI	upper 95% CI	p-value
natural	brightness	0.736	0.624	0.812	<0.001
sunny	brightness	0.689	0.537	0.797	<0.001
cloudy	brightness	0.879	0.81	0.921	<0.001
natural	red chroma	0.918	0.862	0.945	<0.001
sunny	red chroma	0.873	0.793	0.914	<0.001
cloudy	red chroma	0.984	0.971	0.99	<0.001
natural	human red-green opponency	0.795	0.68	0.861	<0.001
sunny	human red-green opponency	0.703	0.545	0.801	<0.001
cloudy	human red-green opponency	0.95	0.915	0.969	<0.001
natural	sumPower	0.416	0.292	0.536	<0.001
sunny	sumPower	0.347	0.175	0.494	<0.001
cloudy	sumPower	0.608	0.46	0.733	<0.001
natural	maxPower	0.612	0.462	0.719	<0.001
sunny	maxPower	0.558	0.377	0.695	<0.001
cloudy	maxPower	0.794	0.69	0.867	<0.001
natural	propPower	0.887	0.811	0.925	<0.001
sunny	propPower	0.896	0.822	0.932	<0.001
cloudy	propPower	0.888	0.815	0.927	<0.001
artificial	brightness	0.994	0.985	0.998	<0.001
artificial	red chroma	0.998	0.995	0.999	<0.001
artificial	human red-green opponency	0.995	0.987	0.998	<0.001
artificial	sumPower	0.980	0.94	0.993	<0.001
artificial	maxPower	0.942	0.853	0.978	<0.001
artificial	propPower	0.907	0.759	0.959	<0.001

Validation test using X-Rite ColorChecker four grey and three colour patches

While the brightest and the darkest X-Rite ColorChecker grey patches were used for normalisation, the remaining four grey patches, as well as three colour patches ("red", "green" and "blue" according to producer's names) were used for validation. X-Rite was always photographed six times in every combination of light conditions (weather x elevation angle of sun, in total 10 light conditions).

To be able to compare photographic measurements with reflectance measured with a spectrometer, we calculated reflectance as mean of pixel values in red, green and blue channels for grey patches. Table A3 and Figure A1 below present average values and standard deviation of six measurements in every light conditions of grey patches. Overall, the general pattern of changes in grey squares is similar to the one that emerged for eggs (compare Figure A1 with the pattern for brightness in Figure 2 in the manuscript). Grey standards deviated from the known reflectance values measured with a spectrometer, and while general pattern of changes was the same for all four of them, deviations were smaller for darker and higher for brighter patches (Figure A1).

Using the same approach as for eggs, we calculated brightness, red chroma and human red-green opponency for three colour X-Rite patches: "red", "green" and "blue" (producer's names). Results are presented in Table A4 and Figure A2. We could not calculate other metrics used for eggs (such as sumPower, maxPower, propPower, maxFreq), as they describe pattern features while X-Rite colour patches are homogenous in coloration.

Measurements for red, green and blue patches deviated from mean values and green patch had the smallest deviations, while red patch had the highest. Similarly to eggs, there was a high variability of brightness between measurements, while variability of red chroma was much lower. Green patch had the lowest deviation from mean values, while red patch had the highest (Figure 2). Unfortunately, we cannot provide spectrometric analogues to photographic measurements (as we did for grey patches), because we do not know spectral sensitivity curves of our camera.

We also compared variability (as standard deviation) observed in our sample of 36 Japanese quail eggs with variability generated by weather and by elevation angle of the sun (Figure A4). In some cases, variability generated by weather and elevation angle of the sun is only a small proportion of variability in the sample of eggs (for red chroma, propPower, maxFreq), but in other cases it is almost as high as variability in the sample (for sumPower and human red-green opponency).

Table A3. Comparison of reflectance values for four X-Rite ColorChecker grey patches measured as mean pixel values in red, green and blue channel in varied light conditions: uniformly overcast ("cloud") and sunny ("sun") weather and at five different elevation angles of the sun: 10, 20, 30, 40 and 55 degrees. Standard ID refers to the producer's names of the patches. Bolded rows in the bottom of every section show mean values for measurements taken in all weather conditions and reflectance values measured using spectrometry.

Light conditions	Standard ID	Mean reflectance	sd
cloud10	neutral 8	63.01	0.42
cloud20	neutral 8	62.62	0.9
cloud30	neutral 8	60.33	0.35
cloud40	neutral 8	62.8	1.61
cloud55	neutral 8	62.9	0.56
sun10	neutral 8	61.59	0.39
sun20	neutral 8	60.44	0.57
sun30	neutral 8	60.44	0.1
sun40	neutral 8	59.16	0.66
sun55	neutral 8	63.45	0.36
average value	**neutral 8**	**61.67**	**1.56**
spectrometric measurement	**neutral 8**	**64.51**	**-**
cloud10	neutral 6.5	38.77	0.28
cloud20	neutral 6.5	38.23	1.11
cloud30	neutral 6.5	35.99	0.36
cloud40	neutral 6.5	38.58	1.77
cloud55	neutral 6.5	38.69	0.58
sun10	neutral 6.5	37.36	0.41
sun20	neutral 6.5	36.01	0.75
sun30	neutral 6.5	35.99	0.14
sun40	neutral 6.5	34.2	0.69
sun55	neutral 6.5	39.11	0.35
average value	**neutral 6.5**	**37.29**	**1.74**
spectrometric measurement	**neutral 6.5**	**37.35**	**-**
cloud10	neutral 5	20.2	0.17
cloud20	neutral 5	19.7	0.71
cloud30	neutral 5	18.31	0.25
cloud40	neutral 5	19.95	1.28
cloud55	neutral 5	20.18	0.34
sun10	neutral 5	19.19	0.27
sun20	neutral 5	18.24	0.49
sun30	neutral 5	18.22	0.12
sun40	neutral 5	16.79	0.39
sun55	neutral 5	20.25	0.18
average value	**neutral 5**	**19.10**	**1.22**
spectrometric measurement	**neutral 5**	**19.03**	**-**
cloud10	neutral 3.5	8.29	0.06
cloud20	neutral 3.5	8.07	0.34
cloud30	neutral 3.5	7.64	0.08
cloud40	neutral 3.5	8.22	0.51
cloud55	neutral 3.5	8.26	0.13
sun10	neutral 3.5	8.19	0.1
sun20	neutral 3.5	7.7	0.22

sun30	neutral 3.5	7.72	0.05
sun40	neutral 3.5	6.96	0.16
sun55	neutral 3.5	8.46	0.07
average value	**neutral 3.5**	**7.95**	**0.48**
spectrometric measurement	**neutral 3.5**	**8.52**	**-**

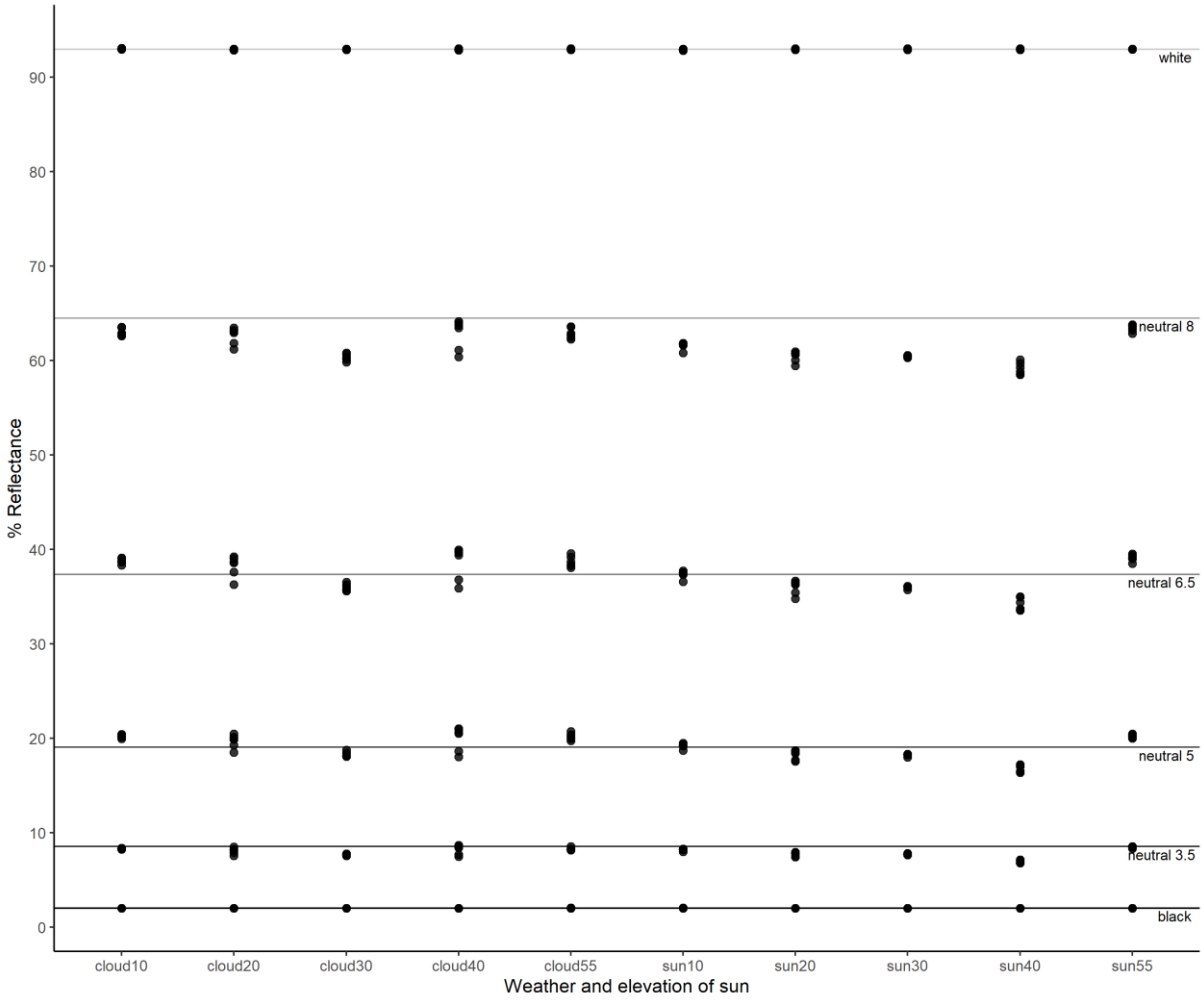

Figure A1. Reflectance of six grey patches of XRite ColorChecker measured by means of photography. Every dot represents reflectance measured as average pixels values in red, green and blue channel. We took six measurements in each combination of weather x elevation angle of sun (in total 60 measurements of every grey patch). Horizontal lines are reflectance for every grey patch measured using spectrometer. Top and bottom lines are for the brightest "white" and the darkest "black" grey standard respectively. These two standards were used for normalisation of the image and this is the reason why all measurements lie exactly on the line of spectrometric reflectance measurement. Names below the lines on right part of the plot are the producer's names for grey patches.

Table A4. Comparison of reflectance values for three X-Rite ColorChecker patches: "red", "green", and "blue" (producer's names) measured using photographic method in different lighting conditions: sunny and overcast weather and at different elevation angles of sun (10, 20, 30, 40 and 55 degrees). We calculated brightness (Br) as sum of pixel values in red, green, and blue channels, red chroma (RC) as pixel values in red channel divided by brightness and human red-green opponency (RGop) as (lw – mw) / (lw + mw). Standards were measured six times in every light conditions. Bolded rows presents average values for all light conditions for every X-Rite patch separately.

Light conditions	standardID	Mean Br	SD Br	Mean RC	SD RC	Mean RGop	Sd RGop
cloud10	red	57.57	0.39	0.76	0.002	0.27	0.002
cloud20	red	55.85	1.228	0.76	0.006	0.27	0.006
cloud30	red	53.93	0.809	0.74	0.002	0.25	0.003
cloud40	red	55.96	1.523	0.76	0.014	0.27	0.014
cloud55	red	56.23	0.654	0.76	0.003	0.27	0.003
sun10	red	49.66	0.211	0.76	0.002	0.27	0.002
sun20	red	45.97	0.971	0.76	0.004	0.26	0.004
sun30	red	46.12	0.34	0.75	0.001	0.26	0.001
sun40	red	48.1	1.445	0.74	0.004	0.24	0.005
sun55	red	54.45	0.937	0.77	0.001	0.28	0.002
average	**red**	**52.38**	**4.36**	**0.75**	**0.011**	**0.26**	**0.012**
cloud10	green	47.11	0.314	0.24	0.001	-0.05	0.001
cloud20	green	46.65	0.495	0.24	0.002	-0.05	0.001
cloud30	green	46.5	0.47	0.24	0.003	-0.05	0.001
cloud40	green	46.27	0.9	0.24	0.006	-0.05	0.002
cloud55	green	46.09	0.338	0.23	0.001	-0.05	0.001
sun10	green	45.05	0.128	0.25	0.001	-0.04	0.001
sun20	green	44.24	0.191	0.26	0.001	-0.04	0.001
sun30	green	44.98	0.258	0.26	0.001	-0.04	0.000
sun40	green	46.17	0.561	0.25	0.002	-0.05	0.001
sun55	green	47.66	0.343	0.24	0.001	-0.05	0.001
average	**green**	**46.07**	**1.081**	**0.24**	**0.009**	**-0.05**	**0.003**
cloud10	blue	38.28	0.272	0.13	0.001	-0.17	0.002
cloud20	blue	38.13	0.69	0.13	0.003	-0.17	0.007
cloud30	blue	39.66	0.743	0.14	0.003	-0.16	0.005
cloud40	blue	37.06	2.463	0.13	0.013	-0.18	0.022
cloud55	blue	37.29	0.36	0.13	0.002	-0.18	0.005
sun10	blue	41.18	0.302	0.15	0.003	-0.13	0.003
sun20	blue	42.49	0.301	0.16	0.002	-0.13	0.003
sun30	blue	43.66	0.333	0.16	0.002	-0.13	0.002
sun40	blue	42.9	0.509	0.16	0.004	-0.14	0.007
sun55	blue	40.34	0.292	0.13	0.002	-0.17	0.003
average	**blue**	**40.10**	**2.440**	**0.14**	**0.013**	**-0.16**	**0.020**

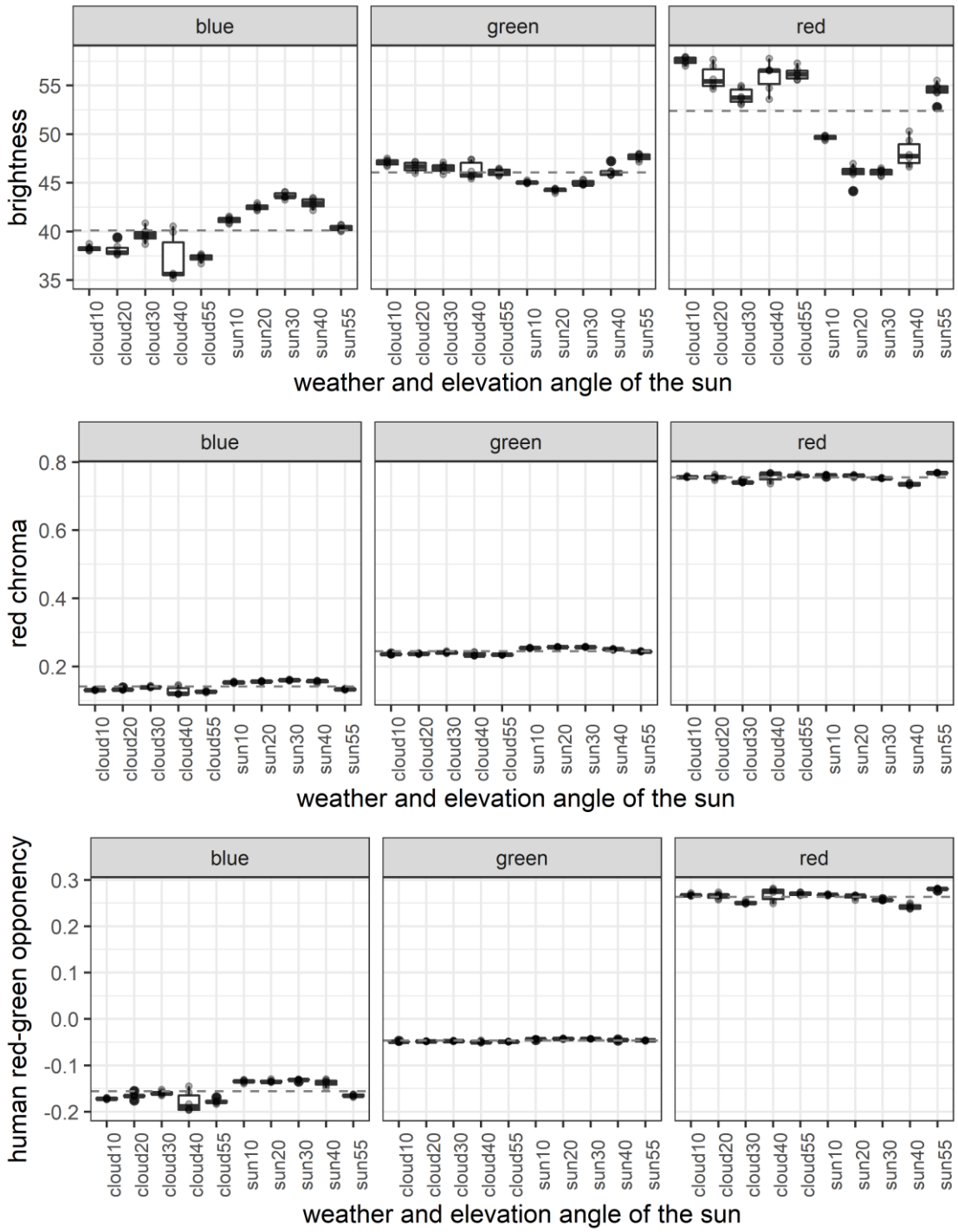

Figure A2. Comparison of brightness (sum of pixel values in red, green and blue channels), red chroma (pixel values in red channel divided by brightness) and human red-green opponency (lw – mw) / (lw + mw) for three X-Rite ColorChecker patches: "red", "green" and "blue" measured using photographic method in different light conditions: sunny ("sun") and overcast ("cloud") weather and at different elevation angles of the sun (10, 20, 30, 40 and 55 degrees). Dashed grey lines represents mean value for all light conditions.

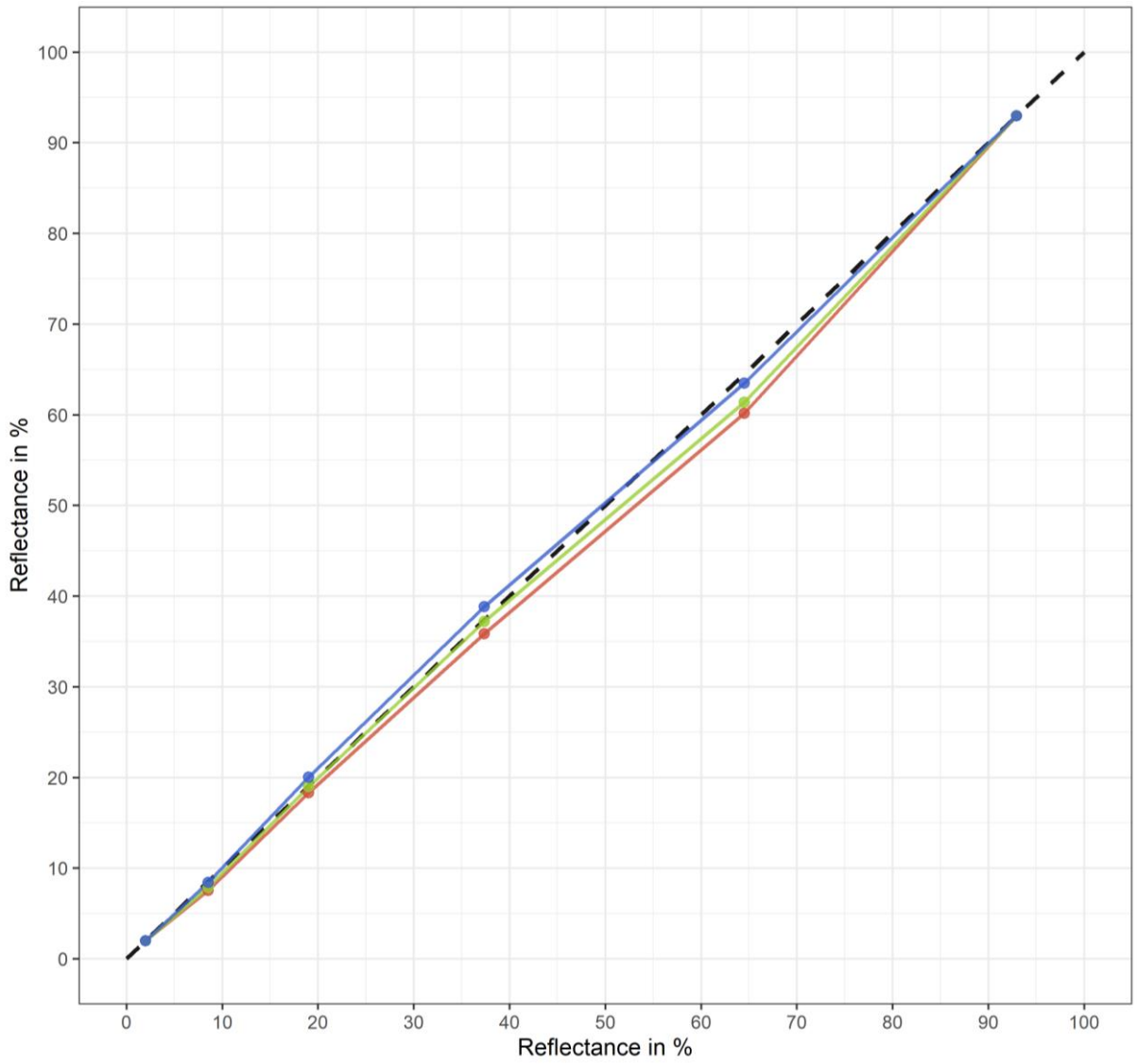

Figure A3. Relationship between reflectance of six X-Rite grey patches measured using spectrometry and calibrated digital photographs (mean values of all measurements). Dashed line represents linear response, while solid lines show pixel values of grey standards measured using photographic method (colours represent camera's channels – red, green and blue respectively). The reflectance values of the darkest and the brightest lie directly on the dashed line, because these two standards were used for normalisation.

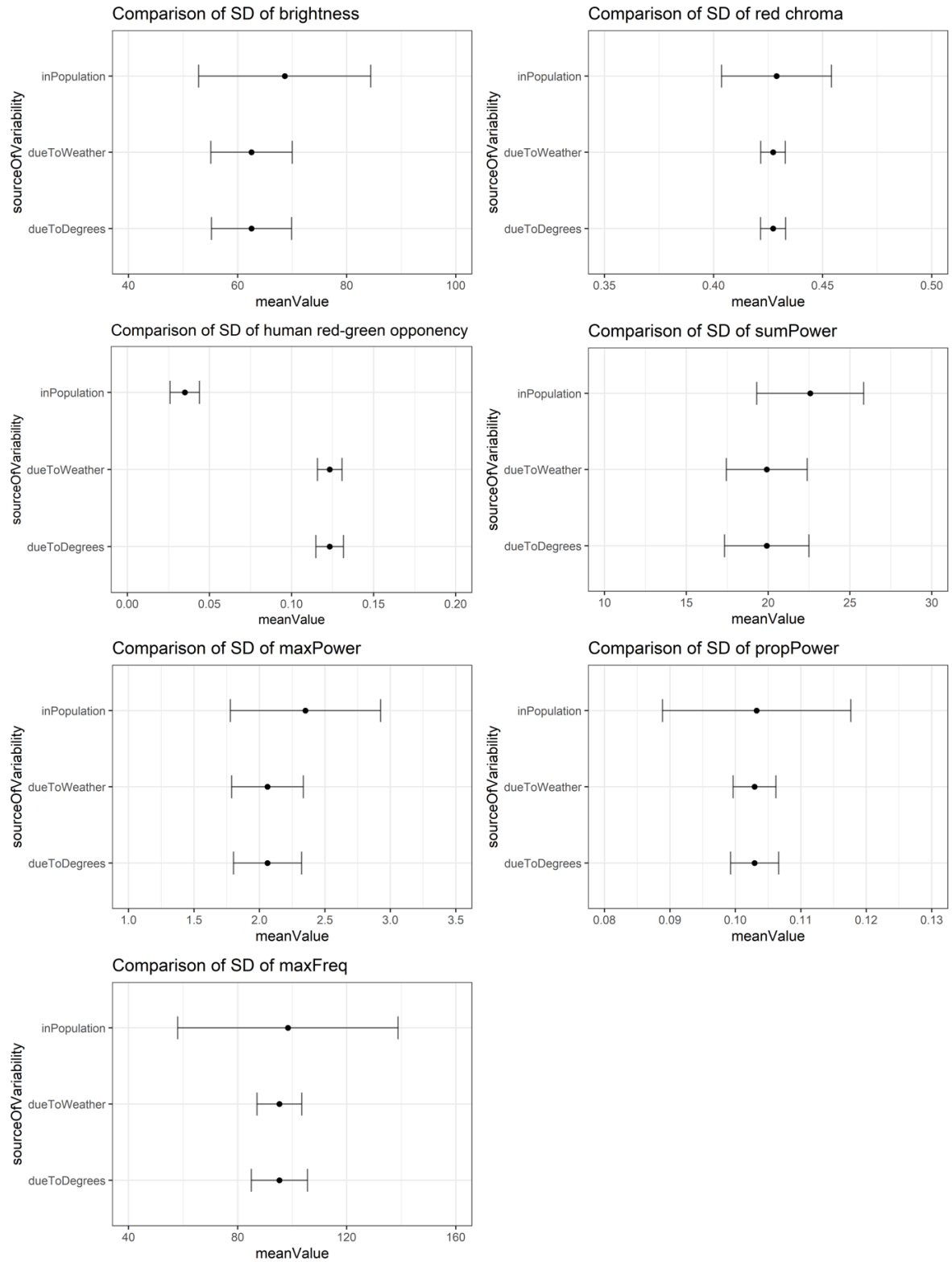

Figure A4. Comparison of sources of variability (standard deviation) for different traits of the eggshell pigmentation in the set of 36 eggs of Japanese quail Coturnix c. japonica photographed in different natural light condition – at different height of the sun above the horizon at both sunny and uniformly overcast day. Variability in population was calculated under cloudy conditions when the sun was 55 degrees above the horizon – this is the variability of pigmentation traits in a set of 36 eggs (every egg was from different female). Variability due to weather was mean standard deviation between two levels of weather conditions at the same level of the height of the sun. Variability due to degrees was mean standard deviation among five levels of height of the sun above the horizon at the same level of the weather. Tails represents +/- 1 SD.

Wnioski i podsumowanie pracy

Przeprowadzone badania pozwoliły mi na wyciągnięcie następujących wniosków. Po pierwsze, chociaż modele wizualne wskazywały, że samce gąsiorka dostrzegają różnice w jasności i ubarwieniu jaj z różnych lęgów w badanej populacji, to samce nie dostosowywały wielkości swojej inwestycji rodzicielskiej do żadnej ze zmierzonych cech wyglądu jaj. To oznacza, że nie odpowiadają na sygnał w postaci jasności, koloru i plamkowania skorupek jaj.

Po drugie, jedyną dostępną dla samca informację stanowił zewnętrzny wygląd jaja, który był słabym wskaźnikiem zawartości pigmentu w skorupce. A zatem, choć samice w lepszej kondycji składały jaja o mniej kolorowych plamach, wcale nie musi to oznaczać, że składały one jaja o mniejszej zawartości pigmentu w skorupce. Tym samym wygląd skorupki nie stanowi u gąsiorka rzetelnego sygnału kondycji samicy, ponieważ jest słabo związany z ilością pigmentu w skorupce.

Po trzecie, pisklęta z lęgów o bardziej czerwonych plamach były w lepszej kondycji, jednak ponownie nie miało to związku z wielkością inwestycji samca. Niniejsze badania nie pozwalają wykluczyć, że kolor plam może być związany z wielkością inwestycji samicy w jaja, jednakże nawet jeśli taka zależność istnieje, nie stanowi ona sygnału, na który odpowiadają samce. Podsumowując, chociaż wiele założeń hipotezy SSEC zostało spełnionych u gąsiorka, to w świetle moich badań pigmentacja skorupek jaj nie służy u tego gatunku jako po-kopulacyjny sygnał kondycji samicy wpływający na wielkość inwestycji rodzicielskiej samca.

Po czwarte, skalibrowana fotografia cyfrowa to użyteczne narzędzie do pomiaru kolorów i plamkowania różnych obiektów, w tym skorupek jaj ptaków, które jest szczególnie przydatne w badaniach terenowych. Jednakże, aby pomiary były porównywalne, konieczne jest wzięcie pod uwagę zmiennego naturalnego oświetlenia przy planowaniu prac terenowych i wykonywanie fotografii przy jednolitej pogodzie i o podobnej porze dnia lub użycie cech wyglądu jaj, na które zmienne oświetlenie nie ma znacznego wpływu.

Oświadczenia o autorstwie | Authorship statements

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Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie artykułu: **Szala K., Tobolka M., Surmacki A., Do eggshell coloration or patterning affect provisioning effort of males in a cup-nesting passerine?** (manuskrypt w recenzji w czasopiśmie *Behavioral Ecology*, ID manuskryptu: BEHECO-2024-0174) polegał na: zaprojektowaniu metodyki badań, zebraniu danych w terenie, analizie danych i interpretacji wyników, wizualizacji wyników oraz przygotowaniu oryginalnego manuskryptu. Jestem pierwszym oraz korespondencyjnym autorem powyższego artykułu.

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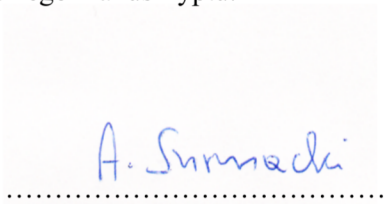

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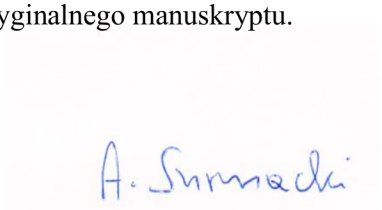

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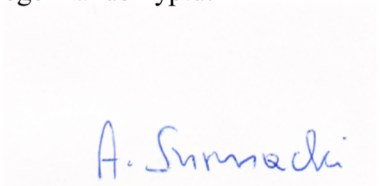

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