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Research article

Heavy metal pollution exposure affects egg coloration but not male provisioning effort in the pied flycatcher *Ficedula hypoleuca*

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Heavy metal pollution is known to negatively affect numerous traits in birds, including foraging, metabolism, immunity, and reproductive success. In this study, our primary aim was to assess the impact of metal pollution exposure on the visual appearance of the pied flycatcher *Ficedula hypoleuca* eggs. Specifically, we focused on blue-green biliverdin-based coloration, a trait expected to function as a signal of female quality to males. In line with the sexually selected egg coloration (SSEC) hypothesis, which posits that males respond to more intensely colored eggs by increasing their provisioning effort, our second objective was to investigate whether metal pollution exposure affects this specific signaling mechanism and subsequent male behavior. Our results showed that although coloration did not correlate with female quality or male provisioning effort, egg blue-green coloration decreased in polluted areas compared to non-polluted control areas. Our analysis of reflectance data revealed that this difference was due to an increased ultraviolet reflectance of eggs from polluted areas, likely caused by changes in eggshell microstructure (e.g. porosity). We therefore propose that metal pollution exposure may compromise crucial color signals of bird eggs. Avian visual modeling indicated that eggs laid by different flycatcher females are generally very similar, making discrimination by males challenging and perhaps impossible especially in dark cavities. Overall, our results suggest that the SSEC hypothesis may lack adaptive relevance for the pied flycatcher in northern Europe, even in environments influenced by anthropogenic activities.

Keywords: Biliverdin, pied flycatcher, pollution, reproductive investment, sexual selection

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Introduction

Terrestrial metal pollution is a global and persistent concern to wildlife that has permanently altered ecosystem functioning. Potentially toxic metals typically originate from point sources such as metallurgic smelters, mines and other industrial sources, and can remain in the soil even long after industrial emissions have decreased (Berglund and Nyholm 2011, Eeva and Lehikoinen 2015, Barton et al. 2023). Birds are highly vulnerable to metal pollution due to inevitable exposure and accumulation through the food chain. Notably, heavy metals can affect adult foraging behavior (Brotons et al. 1998, Eeva et al. 2005), impair the survival and growth of nestlings (Eeva and Lehikoinen 2015), impact metabolism, hormone levels, immune function and induce oxidative stress (Berglund et al. 2010, Koivula and Eeva 2010, Espín et al. 2016, Barton et al. 2023 for review).

A number of pollution-affected traits can be measured non-invasively and used as bioindicators of exposure, as is the case for plumage (Eeva et al. 2008, Dauwe and Eens 2008, Chatelain et al. 2014, Lifshitz and St. Clair 2019) or bill coloration (Spickler et al. 2020) but also egg quality (Ding et al. 2019). It is now well known that metal pollution can induce eggshell thinning and an increase in porosity of eggshells (e.g. in raptors: Carson 2002, in passerines: Eeva and Lehikoinen 1995). More recently, studies in metal polluted areas have investigated variation in other eggshell characteristics, including color, pigment content and patterning (Dauwe et al. 2004, Hargitai et al. 2016a, Ding et al. 2019). Since the natural diversity of avian egg coloration has been suggested to serve critical functions such as camouflage (Troscianko et al. 2016), mimicry (Štětková et al. 2023), thermoregulation (Wisocki et al. 2020), eggshell strengthening and even sexual signaling (Kilner 2006 for review), changes in coloration due to anthropogenic impact could be concerning for population persistence and are essential to investigate.

According to the sexually selected eggshell coloration (SSEC) hypothesis, biliverdin IXα, the pigment responsible for the blue-green coloration of eggs, is an important post-mating signal for females (Moreno and Osorno 2003). Due to the antioxidant properties of biliverdin, its deposition into the eggshell imposes an important cost on females, making it an honest indicator of female quality: in that sense, only high-quality females would be able to afford removing antioxidants from their system to invest them into their eggs (Moreno and Osorno 2003). As such, the blue-green eggshell color, positively associated with biliverdin concentration (Morales et al. 2013), is assumed to serve as a signal of the female's immune capacity (Moreno et al. 2005) and antioxidant status (Hanley et al. 2008), which should promote in return higher contributions from the male partner in nest defense and nestling provisioning effort (Moreno and Osorno 2003). Over the past 15 years, numerous studies have been carried out to test the hypothesis, though overall evidence in favor of the SSEC hypothesis remains inconsistent. Some investigators have found that blue-green coloration intensity correlates of female body condition and/or antioxidant capacity (Moreno et al. 2005, 2006b, Hanley et al. 2008, Hargitai et al. 2016b) and increased male investment in response (Soler et al. 2008), while others failed to establish any connections between the different variables (Johnsen et al. 2011, Krištofík et al. 2013, Dehnhard et al. 2015, D'Arpa et al. 2022). Although the SSEC hypothesis has been quite extensively studied, the validity of the hypothesis has to this day never been assessed among environments in which egg color could be affected by an anthropogenic stressor.

In this study, we aimed to identify whether metal pollution affects egg coloration and parental provisioning effort in the pied flycatcher *Ficedula hypoleuca*. First, we validated whether blue-green egg coloration correlated with traits indicative of female quality, and whether this relationship differed between metal-polluted and control areas. Since metal pollution has been associated with changes in body condition index of females and reduction in clutch size (Berglund et al. 2010, 2011, Rainio et al. 2017), we predicted that females from polluted areas would lay eggs with a less intense blue-green coloration. Additionally, heavy metal pollution can increase eggshell porosity (Eeva and Lehikoinen 1995) which can, in turn, enhance the egg structural UV reflectance (Ladouce et al. 2020). Therefore, we further predicted that UV coloration would be higher in eggs laid in polluted areas. Alternatively, it has been demonstrated that eggshell porosity may not only be linked with structural UV coloration but also correlates with overall blue-green coloration (Morales et al. 2013). Thus, we may predict that eggs laid in polluted areas could paradoxically exhibit higher blue-green coloration compared to eggs laid in control areas. Since pied flycatcher eggs often vary in coloration within the clutch, with the last or penultimate eggs being paler (Moreno et al. 2005, Johnsen et al. 2011), we investigated whether this variation is related to female condition. We predicted that the ability of females to sustain the same coloration levels across the entire clutch would be impaired in polluted areas, therefore causing higher color variation. Second, we investigated the relationship between female egg coloration and male provisioning effort in polluted versus non-polluted areas. Consistent with the SSEC hypothesis, we predicted that males would increase their provisioning effort in response to higher and more consistent blue-green coloration, leading to higher parental effort in non-polluted areas. Lastly, we used avian color perception models to investigate the magnitude of egg color variation between females and whether this variation is large enough to be detected by males.

Material and methods

Study site and nest-box monitoring

This study was conducted in May and June 2023 in the area of Harjavalta (southwestern Finland, 61°20′N, 22°10′E) where 600 wooden nest-boxes (Lambrechts et al. 2010 for technical description) have been established in 1991 around a metal smelter and along a pollution gradient in direction of northwest, southwest and southeast. The main metals emitted by the smelter are nickel and copper, but arsenic, zinc and lead levels are also considerably higher close to the emissions source compared to the background levels of remote areas (Berglund et al. 2011). Although the emissions have largely decreased since the 1990s, there are still marked differences in the reproductive success of pied flycatchers that breed closer to the smelter today (Eeva and Lehikoinen 2015). The area has been monitored yearly since its creation and as of 2023, the study site comprises four polluted localities in the vicinity of the smelter (< 2 km) and nine unpolluted (also referred to as control) localities > 5 km from the smelter. The data for this study were collected at the four polluted localities and four unpolluted localities (5–10 km from the smelter). Nest-boxes occupied by pied flycatchers were monitored weekly for nesting progression. For 24 clutches (158 eggs), we monitored nest-boxes daily after the first egg was laid and numbered each new egg to obtain the precise laying order of the clutch. Including these 24 nests for which we recorded the laying sequence, we photographed a total of 71 clutches (Egg photography section below). Incubating females were caught at the nest-box and ringed during the first week of incubation, weighed and measured for wing size to the nearest 0.5 mm by using the maximum method (Svensson 1992).

Parental provisioning behavior

We monitored the provisioning of nestlings at 4–5 days of age (mean ± SD: 4.51 ± 0.81 days) in 63 of the 71 nests for which we had photographed the eggs. We video-recorded the activity of adults at their nest-box for 90 minutes between 07:00 and 17:00 as previous studies showed pied flycatchers do not exhibit diurnal variation in provisioning rates (Sisask et al. 2010) and 60 minute recordings provide representative estimates of provisioning (Murphy et al. 2015). We used camcorders (Sony Handycam HDR-CX405) mounted at nest-box height at five to ten meters away from the nest-box. Recordings only took place when weather conditions were favorable (no rainfall). Videos were analyzed by a single observer blind to the area of recording (LM). Males were distinguished from females based on color dimorphism and on the size of the forehead and wing patches as visible when visiting a nest-box. We recorded the entering and exiting the nest-box as a feeding visit. We calculated provisioning rates as the number of nest visits per hour (male visits, female visits and total), as well as the male relative contribution to provisioning (as the number of visits of the focal parent divided by the total number of visits). Although we do not report the type of prey brought to the nest because prey identification was difficult on most videos, a recent study in the closely related collared flycatcher *Ficedula albicollis* showed that provisioning rates accurately reflect the total biomass of food items delivered to nestlings (Cauchard et al. 2021). At two nests (one in a control area and the other in a polluted area), no male was observed for the entire duration of our recording, which could suggest adult mortality or polygyny. Thus, these two nests were excluded from further analysis of provisioning effort.

Egg photography

We took human-visible and UV-spectrum photographs of 458 pied flycatcher eggs (laid by 71 females) five days after clutch completion with a Samsung NX 1000 camera converted to full spectrum, fitted with a Nikkor EL 80 mm lens. Human-visible photographs were taken through a Baader UV-IR blocking filter (Baader Planetarium, Mammendorf, Germany), permitting only visible spectrum light from 420 to 680 nm. UV photographs were taken with a Baader UV pass filter permitting ultraviolet light from 320 to 380 nm. All photographs were taken in RAW format under diffuse sunlight conditions between 09:00 and 14:00. All eggs of a given clutch were placed on a dark board and photographed together using a nylon light tent (50 × 50 × 50 cm, Fomei, China) placed in the shade, at the same angle and from the same distance. Photographing in the shade and inside a diffusive light tent should limit the variability that could be introduced by potential changing illumination during the day (see also recommendations by Szala et al. 2023). All photographs were referred to two custom-made polytetrafluoroethylene gray standards reflecting 6% and 89% of the light spectrum (300 nm–700 nm, which includes UV and visible light). Exposure settings were adjusted accordingly with lighting conditions, the ISO value was set constant at 400 and aperture f/8. Image calibration and calculations of average reflectance and cone-catches (below) were performed in ImageJ software (Schneider et al. 2012) using the multispectral image calibration and analysis (MICA) toolbox (Troscianko and Stevens 2015). A scale bar was included in each photo and used to rescale all images to 30 pixels (px) per mm.

Egg coloration analysis

The eggshells of 185 eggs photographed in this study had speckles caused by impurities on their surface (stains from nest material or feces) that could bias our biliverdin-based coloration measurements. As such, we excluded these speckles from color measurements by using a local thresholding function with the Phansalkar method (Phansalkar et al. 2011) and Gaussian blur to correct for uneven illumination of eggs and improve detection of spots (Gómez et al. 2018). We used 50 px radius for thresholding and 2048 px Gaussian blur. To avoid measuring the dark board that eggs were placed on for taking photographs, we decreased selection around every egg by 3% of the egg's width.

To analyze eggshell coloration, we first calculated average reflectance values for four camera channels (UV, blue, green and red) for each egg. To calculate blue-green coloration and discriminability of eggshell colors as potential signals, we used a photoreceptor noise-limited color opponent model of avian color perception (Vorobyev and Osorio 1998). For each egg, we calculated the relative photon catch values of each of the four single cone types in the avian retina that are responsible for color vision (Hart 2001). As cone sensitivities were unavailable for our study species, we computed cone-catches for the most closely related species with accessible information, the European blackbird *Turdus merula*. Single cone sensitivities, such as ultraviolet-sensitive (UVS), short-wavelength-sensitive (SWS), medium-wavelength-sensitive (MWS) and long-wavelength-sensitive (LWS) were derived from (Hart et al. 2000) and sensitivity curves (Supporting information) were generated by using the 'sensmodel' function included in R package 'Pavo' (www.r-project.org, Maia et al. 2019). We used the illuminant D65 which mimics daylight illumination (International Commission on Illumination 2018) and allows the best color perception. The intensity of egg blue-green coloration was determined as blue-green chroma (BGC). BGC was calculated as the ratio of the sum of photon catches performed by SWS and MWS cones divided by the sum of photon catches of all single cones: BGC = (SWS + MWS)/(UVS + SWS + MWS + LWS). This is reasonable because the SWS and MWS cones (responsible for perceiving short- and medium wavelengths) approximately correspond to the reflectance of biliverdin-colored eggshells (for cone sensitivity data, see Supporting information, for the average reflectance of flycatchers eggs, see e.g. Johnsen et al. 2011). Because our calculation of BGC reflects the intensity of blue-green coloration as perceived by bird vision, we chose to refer to it as 'perceived BGC' instead of BGC only, which is instead used for direct reflectance measurements (Supporting information for additional analyses using BGC). To assess the within-clutch consistency of blue-green coloration (referred to as consistency hereon), we calculated the perceived BGC standard deviation (SD) of all eggs of a particular clutch. To compare perceived BGC between clutches of individual females, we averaged the perceived BGC of all eggs from the same clutch.

To investigate color differences between and within clutches of individual females, we calculated chromatic contrast (ΔS) between all egg pairs with the 'Pavo' package (Maia et al. 2019). We determined the relative proportions of the different single-cone types in the retina according to available data (blackbird: UVS cones = 1, SWS cones = 1.78, MWS cones = 2.21 and LWS cones = 1.96 (Hart et al. 2000). Since we did not measure illumination in individual nest boxes, we conservatively used a model with neural noise only. For the high-intensity noise, we used a Weber fraction value of 0.05. To investigate between-clutch color differences, i.e. between eggs of different females, we first calculated ΔS for all possible egg pairs belonging to different females. Second, we calculated the average ΔS for each female pair (accounting for 2485 combinations for 71 females) indicating between-clutch color differences as perceived by birds. Units of ΔS are just noticeable differences (JNDs) which estimate the discriminability between two colors for the chromatic (hue, chroma) aspect of coloration. Values < 1 JND refer to two colors that are impossible to discriminate even under perfect viewing conditions (Vorobyev and Osorio 1998, Siddiqi et al. 2004). However, under suboptimal conditions, which may be the case of flycatchers that nest in dim nest boxes (Langmore et al. 2005, Węgrzyn et al. 2011, Maziarz and Wesołowski 2014), values < 3 JNDs indicate that colors are still very difficult to distinguish (Vorobyev and Osorio 1998, Siddiqi et al. 2004, Endler and Mielke 2005). For male flycatchers to assess the fitness of different females based on the phenotype of their eggs and to allocate parental resources accordingly, the difference in eggshell coloration between clutches should be greater than these thresholds of visual discrimination.

Statistical analysis

All statistical analyses were conducted using R ver. 4.3.1 (www.r-project.org). To investigate whether eggshell characteristics were representative of female condition, we fitted multiple linear regressions with either perceived BGC or UV reflectance (for egg coloration) or SD of perceived BGC (for coloration consistency) as response variables (both averaged per clutch). The coloration-related variables fitted assumptions of normality and were not transformed. We included variables relating to female condition (standardized mass index and clutch size), breeding area (polluted or control) and the interaction between female condition and area as explanatory variables. We calculated standardized mass index (SMI) after Peig and Green (2009) and used the 'smatr' package to fit allometric lines (Warton et al. 2012). We decided not to include laying dates as all nests included in this study were synchronized and laying occurred essentially at the beginning of the flycatcher breeding season (during the second half of May, with only thirteen days between the first and last clutch of this study). To test the relationship between eggshell characteristics and provisioning behavior of males, we fitted a generalized linear model with a quasi-Poisson distribution with the provisioning rate of males (number of hourly visits) as our response variable. We included either perceived BGC, UV reflectance, or SD of perceived BGC (all averaged per clutch), and area (polluted or control), age of nestlings at recording, brood size and female provisioning effort (number of hourly visits) as explanatory terms. Before running models, continuous variables were scaled and categorical variables coded using sum-to-zero contrasts. To assess the performance of our models, we verified the normality and homoscedasticity of residuals for the linear models, and inspected the existence of influential observations and collinearity for all models with the 'performance' package in R (Lüdecke et al. 2021). We did not identify any influential points and collinearity, if present, was low. To estimate the significance of our predictors, we performed pairwise post hoc comparisons with the 'emmeans' package (Lenth 2023). We tested the effect of laying order on blue-green intensity on the subset of clutches for which the laying sequence was known using a linear model with BGC as response variable and the rank of the egg in the sequence as explanatory variables, running one model and pairwise post hoc tests for each clutch size (six, seven and eight eggs). To compare whether female condition, reflectance of eggs, male contribution to feeding and pairwise ΔS differed between areas, we performed Wilcoxon sum rank tests with a continuity correction.

Results

The average perceived blue-green chroma was 0.61 ± 0.017 in control areas and 0.6 ± 0.02 in polluted areas. Our model revealed perceived blue-green chroma was significantly higher in control areas (linear model, $F_{1,69}=6.81$, $p=0.01$; post hoc estimated marginal means, $t=2.39$, $p=0.02$) but was not associated with female body condition ($F_{1,69}=0.00$, $p=0.99$, Fig. 1A) or clutch size ($F_{1,69}=3,25$, $p=0.08$). There was no difference in perceived blue-green consistency between areas ($F_{1,69}=0.08$, $p=0.77$) and no association with female traits ($F_{1,69}=0,50$, $p=0.48$ for female body condition, Fig. 1B, $F_{1,69}=0.83$, $p=0.36$ for clutch size). Female body condition was slightly higher in polluted areas than in control areas (Wilcoxon rank test, $W=425$, $p=0.03$). Perceived blue-green chroma decreased along the laying sequence, with the last egg being paler than the antepenultimate egg in six-egg clutches (rank 4–rank 6 contrast: $t=3.57$, $p < 0.01$, rank 5–rank 6 contrast: $t=2.53$, $p=0.12$), paler than the penultimate egg in seven-egg clutches (rank5–rank7 contrast: $t=3.70$ $p < 0.01$, rank6–rank7 contrast: $t=2.80$ $p < 0.09$) but not in eight-egg clutches (rank 7–rank 8 contrast: $t=1.843$, $p=0.60$).

Perceived BGC and UV reflectance were negatively correlated (Pearson's product-moment correlation, $r=-0.58$, $p < 0.01$). To disentangle whether the variation in blue-green chroma could be caused by changes in UV, we also analyzed differences between areas in the reflectance of all channels (Fig. 2). Blue, green and red channel reflectances were all similar between both areas. Only the UV channel differed significantly, with its reflectance being higher in polluted areas than in control areas ($W=481$, $p=0.03$).

The mean (±SD) number of overall nest visits was 19.5 ± 5.68 per hour in the control areas and 20.4 ± 5.21 per hour in the polluted areas. Males carried out 11.0 ± 3.01 and 10.9 ± 3.51 visits per hour in the control and polluted areas, respectively. However, male feeding effort did not differ statistically between areas ($\chi^2=0.068$, $p=0.79$), nor did we detect any relationship between perceived BGC and male feeding effort ($\chi^2=0.117$, $p=0.79$, Fig. 3) or between perceived blue-green consistency and male feeding effort ($\chi^2=0.935$, $p=0.33$). Male contribution to overall nest visits did not differ between areas (57.2% and 54.1% in the control and polluted areas, respectively, $W=487.5$, $p=0.43$) and was not linked to perceived BGC ($\chi^2=0.54$, $p=0.46$).

The average between-clutch chromatic contrast was 2.53 ± 0.82 JNDs with 68.7% of values below the 3 JNDs threshold (Fig. 4). The average between-clutch chromatic contrast for clutches in control areas was 2.28 ± 0.62 JNDs and differed significantly from that of polluted areas (2.54 + 0.88 JNDs, $W=163002$, $p < 0.01$), meaning egg discriminability was slightly higher in polluted areas. The average contrast in both groups was significantly lower than 3 JNDs (one-sided Wilcoxon signed rank test: $V=10881$, $p < 0.01$ for control areas, $V=43211$, $p < 0.01$ for polluted areas).

Discussion

Our results did not support our initial prediction that females of higher quality (based on their body condition, estimated with the standardized mass index) would lay bluer and more consistently colored eggs than lower quality females. This is partly surprising because previous studies in favor of the SSEC hypothesis suggested that biliverdin should be costly to deposit in eggshells due to its antioxidant properties which the female cannot mobilize for herself (Moreno and Osorno 2003, Moreno et al. 2006b, Hanley et al. 2008). It is possible

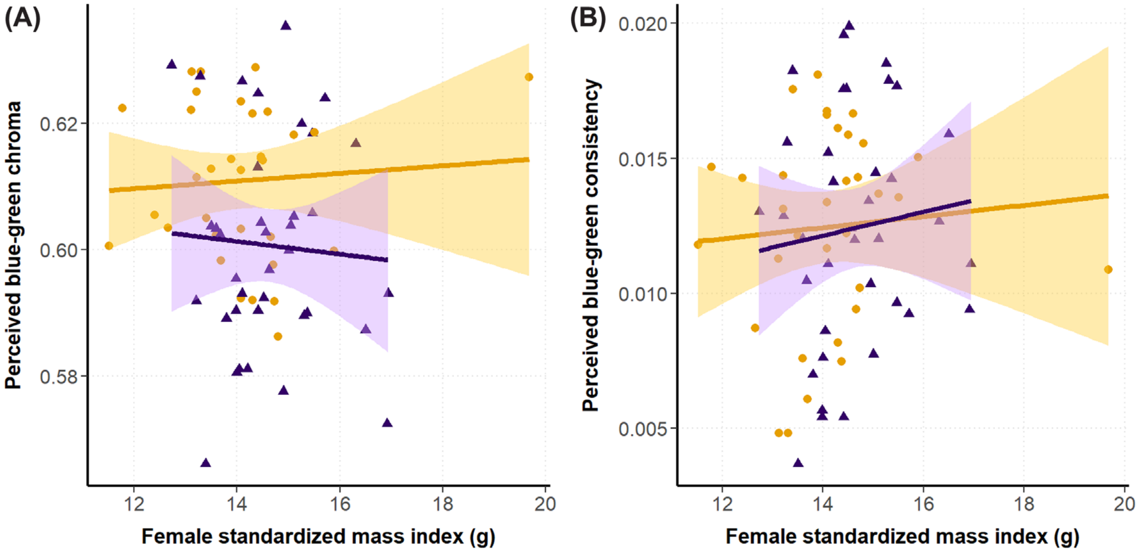

Figure 1. Relationship between eggshell blue-green coloration variables and female standardized mass index (in g) with (A) perceived blue-green chroma and (B) perceived blue-green consistency (standard deviation of blue-green chroma). In yellow, control areas, in purple, polluted areas. Shaded areas show the 95% confidence interval around the regression line. No influential outlier was detected statistically.

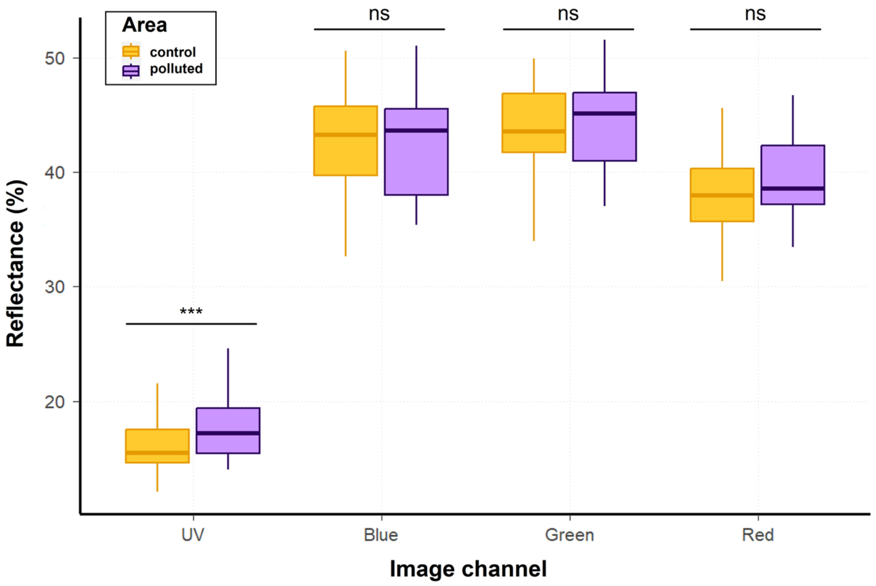

Figure 2. Reflectance of eggs (%) as approximated from the four camera channels and statistical comparisons of each channel's reflectance between control (yellow) and polluted (purple) areas as calculated from Wilcoxon rank sum tests (***: p < 0.01, ns: p>0.05).

that our proxy of female condition is not representative of their physiological status, in which case markers derived directly from blood (such as antioxidant capacity, Hanley et al. 2008) might be more appropriate indicators of the female's condition. However, a number of studies have successfully reported a relationship between female life history traits (i.e. mass indices, clutch size, laying date) and biliverdin-based coloration (Moreno et al. 2005, 2006b, Siefferman et al. 2006, Krist and Grim 2007, Hargitai et al. 2016b). We might also be limited by the lack of seasonal variation of our sample since we collected data rather early in the breeding season, but pied flycatcher females laying early tend to be of higher quality than later-laying females (Järvinen 1991, Fay et al. 2021). On the other hand, prior results collected in a Norwegian population of pied flycatchers during the entire breeding season also run counter to the idea that egg color correlates with female estimates of quality (Johnsen et al. 2011). Alternatively, females also deposit biliverdin into the inner eggshell layers, as such, focusing on the outer eggshell coloration only might poorly reflect the female investment into her eggs, particularly in polluted areas (Morales 2020).

Though focusing on blue-green variability along the precise laying order was not the focus of this study, our results on a rather small sample size (12 clutches of six eggs, nine of

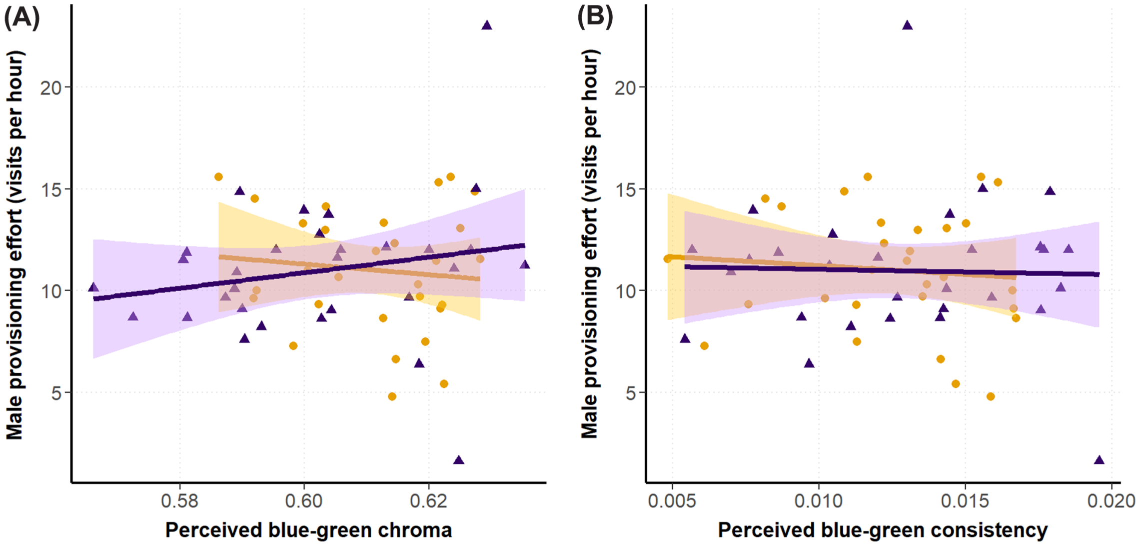

Figure 3. Relationship between eggshell blue-green coloration variables and male provisioning efforts with (A) perceived blue-green chroma and (B) perceived blue-green consistency (standard deviation of blue-green chroma). In yellow, control areas, in purple, polluted areas. Shaded areas show the 95% confidence interval around the regression line.

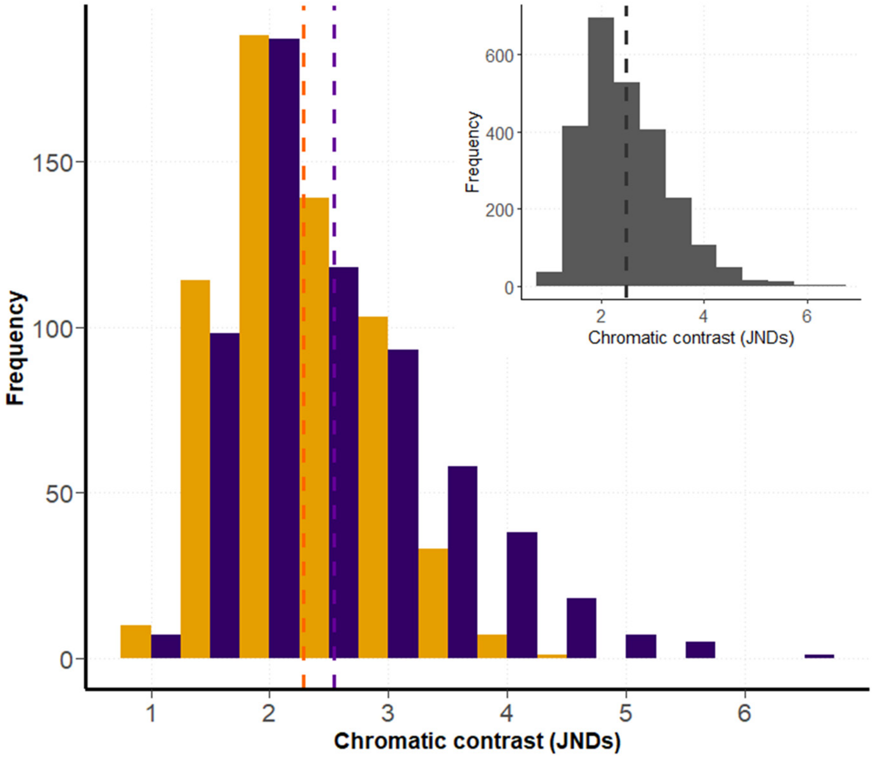

Figure 4. Frequency distribution of pairwise chromatic contrasts between clutches in the control (yellow bars) and polluted (purple bars) area. The inset plot represents the distribution of all possible pairwise ΔS between clutches (including control-control, control-polluted and polluted-polluted pairs). Dashed lines indicate the average between-clutch JNDs for each area.

seven eggs and three of eight eggs) partially confirmed previous findings on the last and/or penultimate eggs being paler than the rest of the egg sequence (Moreno et al. 2005, 2006b, Krist and Grim 2007, Johnsen et al. 2011, Hargitai et al. 2016b). Yet, contrary to our hypothesis, we did not find evidence supporting the idea that females exposed to metal pollution lay less consistent eggs within their clutches in terms of blue-green coloration. This implies that metal pollution exposure may impact the variation of egg coloration uniformly within the clutch.

Interestingly, we found that the perceived BGC was significantly lower in metal polluted areas than in control areas. Since egg BGC and biliverdin content are positively associated in the pied flycatcher (Morales et al. 2013), we could expect this decrease in perceived BGC to be linked to constraints on maternal pigment allocation due to e.g., variation in food resources in the polluted areas (as pied flycatchers indeed rely on alternative food items in such areas, see Eeva and Lehikoinen 2004, Eeva et al. 2005). Eggs from polluted areas could contain less biliverdin overall, or in the outer eggshell layer only, allowing females to concentrate biliverdin deposition in the inner layers to maximize its availability as an antioxidant for the embryo (Morales 2020). In any case, a decreased biliverdin concentration in the eggshell would increase light reflectance especially at UV (300–400 nm) and long (600–700 nm) wavelengths (compared to reflectance at short and medium wavelengths 400-600 nm), which would visually reduce the relative blue-green color intensity (Węgrzyn et al. 2011). We showed that only UV reflectance varied between areas, but not the reflectance in the other three (red, blue, green) channels. It is as such highly possible that the reduction of perceived BGC in polluted areas stemmed from these differences in UV reflectance. We suggest that structural UV reflectance (Węgrzyn et al. 2011, Ladouce et al. 2020), interacting with the eggshell pigments, could help explain why biliverdin pigment concentration alone is sometimes a poor predictor of eggshell coloration (Butler and Waite 2016, Hargitai et al. 2018).

UV reflectance could also provide an alternative eggshell coloration component to investigate in man-affected environments. In our study area, the reduction of emissions over the past 25 years has resulted in an increase in the breeding success and nestling survival of pied flycatchers, though these parameters have not fully recovered yet (Eeva and Lehikoinen 2015, our unpublished data). The same study reported that the eggshell thickness in polluted areas is now similar to that of control areas. Nonetheless, since UV reflectance is linked to the eggshell structure, particularly to its outer cuticle layer and porosity (Fecheyr-Lippens et al. 2015, Ladouce et al. 2020, but see Hargitai et al. 2011), our results indicate that 1) the relationship between eggshell structure and UV scattering is too complex to be approximated from eggshell thickness only, and 2) flycatcher eggshells of polluted areas likely still differ in their microstructure today. The latter point raises important concerns on the long-term alterations metal exposure inflicts on birds, even long after exposure has been reduced.

Lastly, we did not find support for the SSEC hypothesis as neither BGC nor within-clutch consistency in BGC was associated with the male nestling provisioning effort. This observation joins a larger body of work that did not find a role for egg coloration in modulating male provisioning (e.g. Krist and Grim 2007, Johnsen et al. 2011, Stoddard et al. 2012, Hodges et al. 2020). In flycatchers, changes in male provisioning in the context of the SSEC hypothesis have in fact only been observed in a Spanish population (Moreno et al. 2006a, b, 2008), which could suggest male responses to egg signals are highly population or habitat specific. Yet another likely explanation for the lack of a male response is a limitation in the male's ability to discriminate differences in egg coloration. To test for this possibility, we applied a photoreceptor noise-limited model of avian color perception to assess egg color differences between individual clutches, and used a strict detectability threshold allowing optimal color perception even in a dark nest-box environment. Sexually selected traits are expected to exhibit larger variation than equivalent non-sexually traits (Delhey and Peters 2008). However, we found that the coloration of eggs laid by different females showed minimal variation, and the birds' ability to distinguish between colors was quite low. We found that a significant proportion (68.7%) of between-clutch differences are below the detectability threshold of 3 JNDs (Avilés et al. 2011). This result is in line with the low color variations measured for museum egg collections in 46 species and fresh common blackbird *Turdus merula* and song thrush *Turdus philomelos* eggs, all from the superfamily Muscicapoidea which flycatchers belong to (Cassey et al. 2009). Our findings therefore side with the previously raised concern that a low egg color variation diminishes the likelihood of validating the SSEC hypothesis (Cassey et al. 2009). Furthermore, our observations revealed a lower between-clutch variation in egg appearance in control areas compared to metal-polluted areas, though this variation is too low to be perceived by males. This outcome is consistent with expectations, given the observed association between metal pollution and egg coloration.

To conclude, our study provides evidence that exposure to heavy metal pollution has an impact on the coloration of avian eggshells. While our support for the SSEC hypothesis is weak, egg coloration is known to serve other critical roles (Kilner 2006) that we suggest metal pollution exposure may compromise. Most importantly, we found that it was specifically the UV reflectance that differed between polluted and control areas, pinpointing potential changes in eggshell microstructure. Birds use all four types of cones simultaneously to perceive colors (Olsson 2016), meaning changes in one part of the spectrum (e.g. UV) inevitably influence the overall perception of colors (e.g. blue-green coloration). Future studies should directly explore the relationship among pigmentary and structural colorations and combine measurements of eggshell microstructure and pigment concentrations. We did not find a positive correlation between female condition and intensity of blue-green coloration of their eggs. It is thus likely that the condition index based on body size we used is not relevant to the female ability to metabolize biliverdin. Markers of the female physiological status at the time of laying might be more relevant to use in future research. Despite the pied flycatcher being a key study species for testing the SSEC hypothesis (e.g. Moreno et al. 2005, 2006a, Johnsen et al. 2011), our results did not support this theory. Our conservative visual model showed that color differences between clutches of individual females may be insufficient to be perceived by birds, especially in dark cavities (Avilés et al. 2011). Thus, our findings imply that the SSEC hypothesis may lack adaptive relevance for the pied flycatchers even in anthropogenic environments.

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Author contributions

Lisandrina Mari: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Writing – original draft (equal); Writing – review and editing (equal). **Michal Šulc**: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Writing – original draft (equal); Writing – review and editing (equal). **Klaudia Szala**: Data curation (supporting); Software (supporting); Writing – review and editing (equal). **Jolyon Troscianko**: Resources (equal); Writing – review and editing (supporting). **Tapio Eeva**: Data curation (supporting); Funding acquisition (equal); Investigation (supporting); Project administration (equal); Resources (equal); Writing – review and editing (equal). **Suvi Ruuskanen**: Funding acquisition (equal); Project administration (equal); Resources (equal); Writing – review and editing (equal).

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.2280gb61k (Mari et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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