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Avian Behavior, Ecology, and Evolution

Variation of iris color in Spotted Towhees (*Pipilo maculatus*) in urban areas in the lower-mainland region of British Columbia, Canada

Variación del color del iris en *Pipilo maculatus* en zonas urbanas de la región del Lower Mainland de Columbia Británica, Canadá

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ABSTRACT. It has been suggested that the eye color of Spotted Towhees (*Pipilo maculatus*) is indicative of age, with adult birds characteristically having bright red irises. However, during field work at four different study sites within urban green spaces of the Lower Mainland of British Columbia, we noted a surprising level of variation in Spotted Towhee iris color, from pale yellow to dark maroon. Using digital photographs of Spotted Towhee eyes, we assessed several hypotheses about what factors influence iris color during the 2022–2023 breeding (n = 133) and 2022–2024 non-migratory non-breeding periods (n = 72). Iris lightness and red saturation differed significantly between sexes (p = 0.01 and p = 0.001, respectively) and years (p = 0.008 and p = 0.03, respectively), but only during the breeding season. During the non-breeding season, we found there to be a significant difference only in iris red saturation between after hatch year and hatch year males (p = 0.0007). Study site and age alone did not significantly influence iris color during either period (p > 0.05). The extensive overlap between age and sex categories within each seasonal period suggests that iris color is unreliable for aging and sexing towhees in the hand while banding. Additional research is needed to determine the underlying mechanisms responsible for iris color in Spotted Towhees. Our findings suggest unless iris variation has been thoroughly examined in a species, it should not be used as a method for aging or sexing passerines. We also encourage re-examination of presumed links between age and sex and iris color for other species.

RESUMEN. Se ha sugerido que el color de los ojos de *Pipilo maculatus* es indicador de la edad, siendo característico en individuos adultos la presencia de un iris de color rojo brillante. Sin embargo, durante el trabajo de campo realizado en cuatro lugares diferentes dentro de los espacios verdes urbanos de la región del Lower Mainland en Columbia Británica, observamos un sorprendente nivel de variación en el color del iris de *P. maculatus*, desde el amarillo pálido hasta el marrón oscuro. Utilizando fotografías digitales de los ojos de *P. maculatus*, evaluamos varias hipótesis sobre los factores que influyen en el color del iris durante la temporada reproductiva 2022–2023 (n = 133) y en los periodos no reproductivos y no migratorios entre 2022 y 2024 (n = 72). La luminosidad del iris y la saturación del rojo difirieron significativamente entre sexos (p = 0,01 y p = 0,001, respectivamente) y años (p = 0,008 y p = 0,03, respectivamente), pero solo durante la temporada reproductiva. Durante la temporada no reproductiva, solo encontramos una diferencia significativa en la saturación del rojo del iris entre los machos nacidos después del año de eclosión y los nacidos en el año de eclosión (p = 0,0007). El lugar de estudio y la edad por sí solos no influyeron significativamente en el color del iris durante ninguno de los dos periodos (p > 0,05). La amplia superposición entre las categorías de edad y sexo dentro de cada período estacional sugiere que el color del iris no es criterio confiable para determinar la edad y el sexo de *P. maculatus* en mano durante el anillamiento. Se necesitan más investigaciones para determinar los mecanismos subyacentes responsables del color del iris en *P. maculatus*. Nuestros hallazgos sugieren que, a menos que se haya evaluado exhaustivamente la variación en el iris de una especie, no debería utilizarse como método para determinar la edad o el sexo en paseriformes. Asimismo, alentamos la reevaluación de los supuestos vínculos entre color del iris, edad y sexo en otras especies.

Key Words: *avian coloration; city; color; eye-color; songbird*

INTRODUCTION

Bird iris color is highly diverse and there are several potential mechanisms explaining variation in iris pigmentation and color among and even within species (Corbett et al. 2024). Iris color is the result of varying combinations of pigments and structures such as pteridines, purines, melanins, carotenoids, collagen fibers, lipids, cholesterols, and/or blood vessels (Oehme 1969, Oliphant 1988, Oliphant and Hudon 1993, Hudon and Muir 1996). Even within species, iris color can vary as similar colors can be produced through different mechanisms (Oehme 1969). For example, red iris color is produced by a combination of pteridines and purines in Red-eyed Vireos (*Vireo olivaceus*; Hudon and Muir 1996); by blood vessels, melanins, and cholesterols in Black Swans (*Cygnus atratus*; Oehme 1969); and by carotenoids in Canvasbacks (*Aythya valisineria*; Oliphant 1987, Corbett et al. 2024). Differences in the expression of existing pigments and structures may also contribute to iris color variation, although knowledge of the genetic mechanisms influencing iris color among wild birds is lacking (Price-Waldman and Stoddard 2021, Corbett et al. 2024).

Iris color has been hypothesized to be involved in survival and signaling, with patterns among species with similar life histories. Iris color may be associated with habitat, with colors that increase survival under habitat-specific light conditions being selected for

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(Craig and Hulley 2004). Differences in light conditions impact vision which can subsequently impact signaling, foraging, and camouflage abilities (Endler 1993). Relative to cavity nesters, open-nesting species tend to have more cryptic and dark irises, possibly because bright irises (e.g., orange, yellow, white, blue, and pink; Worthy 1997) would make open-nesting species more conspicuous to predators (Davidson et al. 2017). Bright irises might help in close-range signaling among conspecifics. For example, bright irises aid in deterring conspecific nest intruders in colonial breeding Jackdaws (*Coloeus monedula*) that compete for nest sites (Davidson et al. 2014).

Intraspecific color variation remains poorly understood but may vary with several endogenous and exogenous factors. Iris color may be flexible within individuals, changing based on external factors such as environmental conditions. Dietary variation may contribute to color variation because of the energetic demands of producing and depositing pigments (Tyczkowski and Hamilton 1986). For carotenoid-based pigments, which cannot be produced endogenously but instead must be consumed, color depends on both the availability of carotenoid-rich resources, as well as energy to process the pigments (Hill 2000). Environmental pollutants may also impact color production and perception (Peneaux et al. 2021). For example, carotenoid-based iris pigmentation in American Kestrels (*Falco sparverius*) was affected by experimental exposure to polychlorinated biphenyls (Bortolotti et al. 2003). Landscape changes and artificial light associated with urbanization are known to impact production, perception, and function of integumentary color among a wide range of taxa (Koneru and Caro 2022). Differences between habitats in urban areas may occur if iris color is also influenced by anthropogenic alterations to light conditions (i.e., landscape changes, artificial light, pollutants; Koneru and Caro 2022).

Further, intraspecific color variation may be a developmental byproduct of aging or influenced by hormones varying between sexes and/or seasons. Distinctive sex differences in iris color exist in some species. The irises of female Sri Lanka Bush Warblers (*Elaphrornis palliseri*) are ivory-cream while males have bright-red irises (Krishan and Seneviratne 2021), and among Black Oystercatchers (*Haematopus bachmani*), females have full eye flecks (dark specks) while males have slight or no eye flecks at all (Guzzetti et al. 2008). Further, several studies have found iris color to change at different rates in males and females. In Humboldt Penguins (*Spheniscus humboldti*; Scholten 1999), Sparrowhawks (*Accipiter nisus*; Newton and Marquiss 1982), and Cooper's Hawks (*Astur cooperii*; Rosenfield and Bielefeldt 1997) iris color changed at earlier ages in males relative to females. Age was found to be the most common influencing factor of iris color variation among birds with immatures typically having dark irises that may lighten with maturation (Polakowski et al. 2020). Many juvenile birds have dark irises because of a melanin-rich layer and gradually acquire their adult iris color because of the loss of an anterior melanin layer or as an inner melanin layer becomes covered by pigmented outer layers (Oehme 1969, Sweijd and Craig 1991, Hudon and Oliphant 1995). Seasonal changes in iris color have also been observed. Consistent variation among individuals between non-breeding and breeding seasons were seen in the iris color and patterns of species such as Mangrove Cuckoos (*Coccyzus minor*; Frieze and Lloyd 2017), Common Mynas (*Acridotheres tristis*; Feare et al. 2015), and Humboldt Penguins (Scholten 1999). It is believed that these patterns, as well as age- and sex-related variation in iris color are driven by hormonal changes (Trauger 1974).

Despite a lack of clear scientific understanding surrounding the variation, causes, and mechanisms of iris color in birds, even within a species, iris color differences are a commonly used method for distinguishing age and sex of numerous species during bird banding operations around the world (Lowe 1989, de Beer et al. 2001, Pyle et al. 2015, DeSante et al. 2021, Pyle 2022). One such species for which iris color has been suggested to be helpful for aging is the Spotted Towhee (*Pipilo maculatus*), a large New World sparrow common throughout much of western and central North American from southern Canada to Guatemala (Davis 1957a, Smith and Greenlaw 2020). Spotted Towhees are most associated with shrubby habitats, build nests on or close to the ground, and are dietary generalists, foraging on arthropods, seeds, and berries (Davis 1957b, 1960, Smith and Greenlaw 2020). They have sexually dichromatic plumage and show sexual differences in breeding behavior (Smith and Greenlaw 2020, Pyle 2022). Spotted Towhees have dark brown/black irises as nestlings and are typically shown in bird guides with bright red irises as adults (Sibley 2016, Beehler 2024). Pyle (2022) indicates that hatch year/second year (HY/SY) towhees may be aged based on their irises being gray-brown to dull red from November until at least March or later, while the irises of after hatch year/after second years (AHY/ASY) are bright red. While banding Spotted Towhees (subspecies *P. m. oregonus*; Paynter and Storer 1970) as part of a project studying songbirds in urban parks and a National Wildlife Area in the Lower Mainland region of British Columbia, Canada, we visually noticed that there was considerable variation in the iris color of towhees that did not correspond to the age-related descriptions of iris color in Pyle (2022).

Here we examine variation in iris color by testing hypotheses explaining intraspecific iris color variation in Spotted Towhees during the breeding and non-breeding seasons. First, if iris color is a reliable indicator of age in Spotted Towhees, as is suggested in existing literature, then we expected there to be consistent visual and statistical age-related differences in iris color with minimal overlap between age categories (i.e., ASY birds with bright red irises and SYs with more gray-brown or dull red irises during breeding season and AHY birds with red irises and HYs with gray-brown irises during non-breeding season; Pyle 2022). It is also possible that iris color in Spotted Towhees is related to factors that have not been explored prior. Spotted Towhees have sexually dichromatic plumage, and if this extends to iris color as well, we expect consistent differences visually and statistically in iris color between males and females. Next, we examined potential interactive effects of age and sex because effects of these factors are not necessarily mutually exclusive. Finally, if iris color varied with external factors such as habitat or environmental conditions, then differences in iris color would exist between field sites that varied in structural habitat and vegetation. In this study, we did not assess subspecies level geographic variation in iris color because there is only one subspecies within our study region. Further, we were unable to investigate the effect of repeated measures across seasons or time of season on iris color (i.e., how iris color may change within an individual over time) because we presently lack sufficient recapture data.

MATERIALS AND METHODS

Study sites

We captured Spotted Towhees during two breeding seasons (20 April to 16 August 2022 and 27 March to 2 August 2023) and three non-breeding seasons (1 November to 5 December 2022; 31 October to 12 December 2023; and 14 November to 11 December 2024). Study sites included four different urban green spaces in the heavily populated Lower Mainland region of southwestern British Columbia, Canada: Alaksen National Wildlife Area and George C. Reifel Migratory Bird Sanctuary in Delta (Alaksen/Reifel; 49°6′3.6″N 123°10′22.8″W), Fleetwood Park in Surrey (Fleetwood; 49°8′42″N 122°46′58.8″W), Tynehead Regional Park in Surrey (Tynehead; 49°11′2.4″N 122°46′12″W), and Terra Nova Rural Park in Richmond (Terra Nova; 49°10′19.2″N 123°11′45.6″W). All four sites were used during the breeding season, but only Alaksen/Reifel and Terra Nova were used in the non-breeding seasons because we did not conduct field work/catch birds at Fleetwood or Tynehead during the non-breeding season for logistical reasons.

Each site varied in the amounts and types of habitats, level of anthropogenic related features and activities, as well as weather patterns (additional details about sites are available in Appendix 3, Table S1). Alaksen and Reifel are overlapping wildlife reserve/sanctuary areas within the Fraser River Delta that feature a mix of forest, shrubland, agriculture, and restored grasslands. Terra Nova is predominantly shrubland, hedgerows, restored grasslands, and fields. The park comprises two main areas: Terra Nova Rural Park, which also contains a community garden and a large playground, and Terra Nova Natural Area, which is a shrubland and forested slough accessible to the public only via a small perimeter path. Fleetwood is an urban park located in a low-density urban neighborhood. It is surrounded by residential roads, homes, a high school, and agricultural land on one side. The majority of Fleetwood is a second-growth mixed ~ 90–100-year-old forest while the remaining area contains scenic gardens, various sports fields and courts, a spray park, picnic areas, and a playground (Table S1). Tynehead is a large urban park created to preserve salmon spawning habitat along the Serpentine River that runs through the park. It is bordered by major roads to the north and east and a low-medium-density residential development to the south and west. Habitat in the park is mostly western red cedar (*Thuja plicata*) and western hemlock (*Tsuga heterophylla*) forest and field/restored grassland/meadows (Table S1; GRVD 2004).

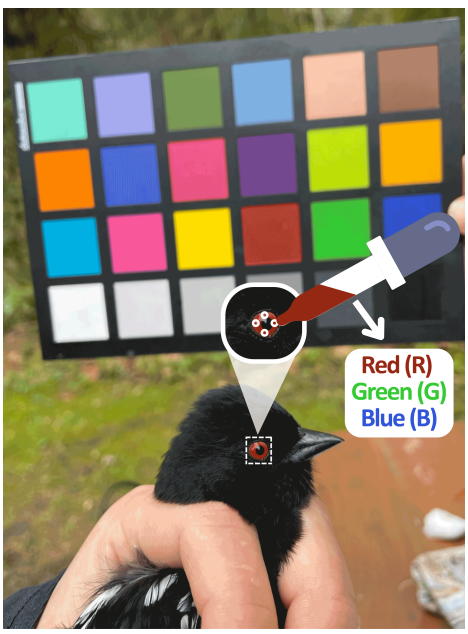
Field methods

Birds were captured using mist nets passively (breeding and non-breeding seasons), using baited potters/sparrow traps (non-breeding seasons only), or actively with conspecific audio playbacks paired with a cardboard decoy painted to resemble a male Spotted Towhee (breeding seasons only). Each bird was banded with a federal aluminum band as well as a unique color band combination, sexed by plumage (~99% accuracy; *unpublished data*), measured (tarsus, wing chord, bill length, bill width, bill depth), and weighed. Birds were aged as SY or ASY during breeding and HY or AHY during the non-breeding season using a combination of criteria including the quality of remiges, coverts, and rectrices, as well as gape color (non-breeding only) as per guidelines (Pyle 2022). We took photographs of each captured bird's bands, wing, rectrices, and eye. Photographs were taken with a cellphone in natural lighting (i.e., without a flash or external artificial lighting) with attention paid to ensuring homogeneous lighting throughout the entire photograph. Photographs had a neutral-colored background during the 2022 breeding season and a color matching palette was added during the 2022 non-breeding season and subsequent seasons to enable assessment of color accuracy in photographs.

Analyses

We examined Spotted Towhee iris color using a two-step process in which we first identified the red, green, blue (RGB) values of the iris. All photographs were processed in Adobe Photoshop (Adobe Inc. 2019a; Appendix 1). The color selector eyedropper was set to output the average value of a 5x5 pixel area of the inner iris adjacent to the pupil (Fig. 1). We obtained RGB values from three to four evenly spaced locations throughout the iris (above, below, anterior, and posterior to the pupil) without reflection or shadow to capture variation that exists within an iris (Fig. 1). RGB values range from 0 to 255, with 0 values having less of the color, and 255 having more of the color. We calculated the mean RGB values for each iris from these points. We conducted a Principal Component Analysis (PCA) involving the three standardized RGB values to derive a single iris value (the PC score from retained principal components axes) for each bird using the package "FactoMineR" (Lê et al. 2008). We used a PCA, an unconstrained ordination, because we assumed the RGB values would form linear relationships, and we did not have prior assumptions about the structure of these data. We visualized the results with a scree plot and biplot (Fig. S1).

Fig. 1. Visual summary of the methodology used to extract RGB values from digital photographs of Spotted Towhees (*Pipilo maculatus*) taken under field conditions at the study's four study sites in the lower mainland of British Columbia, Canada during the 2022–2023 breeding and 2022–2024 non-breeding seasons.



To assess differences in iris RGB values between raw and color corrected photographs we used a paired t-test to compare principal component values. We used a subset (N = 25) of photographs that all contained an unobstructed color calibration palette (not blocked by a bird's head). We obtained RGB values from the photograph in its original, "raw" form and then we color calibrated each photograph using Adobe Lightroom Classic (Adobe Inc. 2019b) according to the instructions of use for the Datacolor Spyder CHECKR 24 (Spyder Checkr 1.6; Appendix 1A). This comparison also allowed us to assess if methodological differences in iris color were likely between 2022 (when color correction palettes were not included) and subsequent seasons. We estimated replicability of color selection by having two individuals independently obtain RGB values from 3–4 points in the iris of a subset of photographs (N = 25; the same subset used in color validation of raw versus calibrated photographs). We compared both the average RGB values and PC1 values using a paired t-test.

We assessed whether iris color (PC score) was influenced by the predictor variables of age, sex, and study site, and their interactions using general linear models (GLMs; Tredennick et al. 2021). Prior to running the GLMs, we tested for violation of model assumptions, using the package "performance" to assess the assumption of no multi-collinearity among predictors (Lüdecke et al. 2021). We tested for a general seasonal effect by conducting a t-test with only towhees that were more than one year old: AHY (representative of the non-breeding season) and ASY birds (representative of the breeding season). We were unable to analyze the effects of season within a main model involving all birds because Spotted Towhee age categories are season specific themselves (i.e., a bird can be ASY or SY during the breeding season, and AHY or HY during the non-breeding season), thus we had to model the seasons separately. Further, one of the aims of this study is to test if iris color is useful in distinguishing between the two possible age categories within a season. We did not have sufficient recapture data to examine how iris color changes within individuals between or within seasons. Year was included as a fixed factor effect in all models to account for any potential variation by year. We obtained *p*-values and 95% confidence intervals (CI) using a type III sum of squares analysis of variance (ANOVA) with the package "car," comparing the fit of the full GLM to a reduced model (Fox and Weisberg 2019). We used the package "emmeans" to calculate estimated marginal means and conduct pairwise post hoc tests, when necessary, to compare multi-level differences (Lenth 2024). We also ran the same GLMs with only the red value of each iris to assess if iris redness alone could be informative. All analyses were conducted in R 4.3.1 (R Core Team 2024).

Ethics and permits

Capturing, handling, and banding birds was approved by the Western and Northern Animal Care Committee (22EG01; 23EG01; 24EG01) and conducted with the appropriate banding permit (10961), and migratory bird scientific permit (SC-BC-2023-0068). Each study site required a separate research permit from the managing jurisdiction, which were obtained prior to conducting research. Additional permits for conducting research on federal lands, Alaksen National Wildlife Area (NF-BC-2022-0088; NF-BC-2023-0088; NF-BC-2024-0088) and George C. Reifel Migratory Bird Sanctuary (MM-BC-2022-0088; MM-BC-2023-0088; MM-BC-2024-0088) were also obtained.

RESULTS

We captured a total of 257 Spotted Towhees during the 2022–2023 breeding and 2022–2024 non-breeding seasons. After excluding 65 birds, for reasons including absence of photographs, poor photograph quality, or unclear age/sex, we had sample sizes of 38 HY and 34 AHY Spotted Towhees from the non-breeding season and 63 SY and 70 ASY towhees from the breeding season (Table 1).

Table 1. The number of Spotted Towhees (*Pipilo maculatus*) of each sex (female or male) and age second year or after second year, hatch year or after hatch year demographic captured at each of the four study sites within the lower mainland of British Columbia, Canada during the breeding and non-breeding seasons. Research was not conducted at Fleetwood or Tynehead during the non-breeding season, thus sample sizes are denoted as "-." Additional details regarding year-specific sample sizes can be found in Table S2.

Site	Breeding season				Non-breeding season				Total
	Second year		After second year		Hatch year		After hatch year		
	Female	Male	Female	Male	Female	Male	Female	Male	
Alaksen/ Reifel	5	15	10	18	2	13	4	11	78
Fleetwood	6	9	4	10	-	-	-	-	29
Terra Nova	8	10	5	12	10	13	4	15	77
Tynehead	6	4	2	9	-	-	-	-	21
Total (Sex)	25	38	21	49	12	26	8	26	
Total (Age)	63		70		38		34		205

Iris color

Spotted Towhee irises ranged from gray-yellow to dark red or maroon. Towhee irises had average red values from 62 to 241 (mean = 147.7 ± 33.2 standard deviations [SD]), green from 2 to 135 (mean = 54.9 ± 25.1 SD), and blue from 1 to 95 (mean = 32.1 ± 16.5 SD).

We created an iris color index using a PCA with RGB values. PC1 (PC1) accounted for 75.59% of the variance (eigenvalue = 2.27) while PC2 accounted for 18.22% of the variance (eigenvalue = 0.55; Fig. S1A). All colors were positively loaded on PC1 (red = 0.53, green = 0.62, and blue = 0.57; Fig. S1B). Higher PC1 scores were associated with lighter irises with values of RGBs in the upper aspect of the observed range, while low PC1 iris values were associated with darker irises that corresponded to RGB values in the lower observed ranges for each color (Fig. S2). Red was positively loaded on PC2, but green and blue were negatively loaded on PC2 (red = 0.78, green = -0.11, and blue = -0.61; Fig. S1B). Higher PC2 scores were associated with irises with higher saturation of red, those with high red values and low values of green and blue, while lower PC2 scores were associated with browner irises, those with low red values and high green and blue values (Fig. S2). For the purposes of this study, we use the terms "lightness" and "darkness" to refer to PC1 values, "red saturation" or "red pureness" to refer to PC2 values, and "redness" to refer to R values alone.

We compared a subset (N = 25) of PC1 and PC2 iris colors to estimate the accuracy of colors in the original, raw photographs versus color corrected versions as well as the replicability of color selection. We did not find a significant difference in PC1 or PC2 iris values between raw and color corrected photographs (paired

t-test: PC1: $t < 0.0001$, df = 24, $p = 1$, 95% confidence interval [CI] [-0.19, 0.19]; PC2: $t < 0.0001$, df = 24, $p = 1$, 95% CI [-0.09, 0.09]). Thus, all future analyses used uncorrected photographs because we did not use a color correction palette for most of 2022. We also did not find a significant difference in PC1 or PC2 iris values obtained from average RGB values selected by two independent individuals (paired *t*-test; PC1: $t < 0.0001$, df = 24, $p = 1$, 95% CI [-0.18, 0.18]; PC2: t < 0.0001, df = 24, $p = 1$, 95% CI [-0.12, 0.12]).

Season did not affect iris color, when comparing Spotted Towhees that were more than one year old between the breeding and non-breeding seasons (ASY and AHY, respectively). ASY birds had lighter irises with slightly lower red saturation, but differences in iris color between AHY and ASY towhees were not significant (Welch Two Sample *t*-test; PC1: $t = 1.78$, $p = 0.08$, 95% CI [-0.06,1.02]; PC2: $t = 1.04$, $p = 0.30$, 95% CI [-0.14, 0.45]; Fig. S3).

To assess if iris color differences between age classes are perceivable to the human eye, we conducted a secondary assessment in which three banders independently aged each bird looking at an anonymized photo of each bird's eye with only the capture date known. When only using iris color to age, we found that 55.12% of birds were consistently correctly aged and 20.49% were consistently incorrectly aged by all three banders (15.12% correctly aged by 2/3 banders; 9.27% correctly aged by 1/3 banders; Fig. 2). Banders incorrectly aged birds according to iris color 31.71 ± 2.22% (average ± SD) of instances. Females were more often incorrectly aged than males (37.37 ± 4.34% of females; 29.02 ± 2.71% of males). When separated by season, 39.85 ± 3.07% of birds during the breeding season but only 16.67 ± 1.13% of birds during the non-breeding season were aged incorrectly. Of birds captured during the breeding season, 34.29 ± 9.11% of ASY birds were incorrectly aged as SY, and 46.03 ± 5.18% of SY birds were incorrectly aged as ASY. When separated by sex, ASY males were least often incorrectly aged (only 28.57 ± 11.66%), followed by SY females (40.00 ± 6.66%), ASY females (47.62 ± 16.95%), and then SY males (50.00 ± 5.68%). For the non-breeding season birds, 13.73 ± 1.39% of AHY birds were incorrectly aged as HY, and 19.30 ± 2.48% of HY birds were incorrectly aged as AHY. HY females were most often incorrectly aged (27.78 ± 3.93%), whereas AHY females, and both AHY and HY males were incorrectly aged at similar rates (AHY females: 16.67 ± 5.89%; AHY males: 12.82 ± 1.81; HY males: 15.38 ± 3.14%).

Breeding season

During the breeding season, both towhee iris lightness and red pureness were influenced by sex and year (Table 2). Males had significantly lighter irises with greater red saturation than females (ANOVA Type III; PC1: $p = 0.01$, 95% CI [0.21, 1.63]; PC2 = $p = 0.0001$, 95% CI [0.32, 1.03]; Fig. 3A; Table S3). Individuals captured in 2023 had significantly lighter irises with greater red saturation than those captured in 2022 (ANOVA Type III; PC1: $p = 0.01$, 95% CI [0.17, 1.16]; PC2: $p = 0.03$, 95% CI [-0.52, -0.03]; Fig. 3B; Table S3).

Iris color in Spotted Towhees, both lightness and red saturation, during the breeding season was not influenced by age, study site, nor an interaction between sex and age (Table 2). Older (ASY) birds had darker irises than younger (SY) birds, but this difference was not significant and ASY and SY birds had similarly pure red colored irises (ANOVA Type III; PC1 = $p = 0.23$, 95% CI [-0.32, 1.32]; PC2: $p = 0.07$, 95% CI [-0.03, 0.78; Fig. 3D; Table S3). Additionally, iris color during the breeding season was not influenced by an interacting effect between age and sex (ANOVA Type III; PC1: $p = 0.29$, 95% CI [-1.55, 0.47]; PC2: $p = 0.12$, 95% CI [-0.90, 0.10]; Fig. 3E; Table S3). Both ASY and SY female birds had darker and less pure red irises than male ASY and SY birds. There were minimal site differences in towhee iris color captured during the breeding season (ANOVA Type III; PC1: $p = 0.15$; PC2: $p = 0.60$; Fig. 3C; Table S3). When we carried out a GLM considering only iris redness (only the average red value), the results were comparable to the GLM involving PC1 iris color values (average RGB values). Iris redness differed significantly between male and females but was not influenced by age, year, study site, nor an interaction between age and sex (Appendix 2A).

Fig. 2. Examples of Spotted Towhees (*Pipilo maculatus*) that were consistently aged correctly ("consistent") and incorrectly ("inconsistent") according to iris color from each age and sex class captured during the 2022–2023 breeding and 2022–2024 non-breeding seasons in the lower mainland region of British Columbia, Canada. ASY = After Second Year. SY = Second Year. AHY = After Hatch Year. HY = Hatch Year.

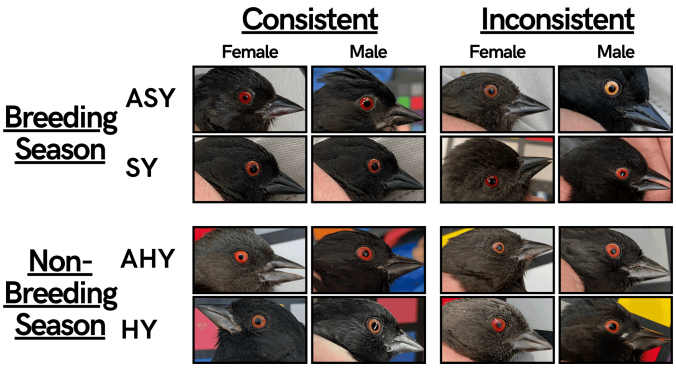
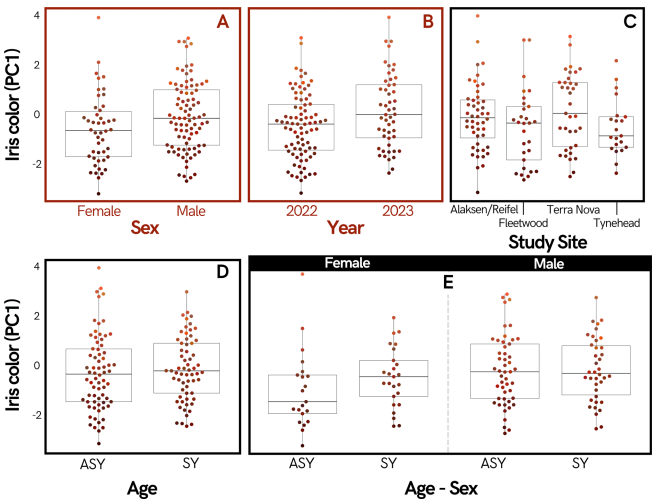


Table 2. Effect of age, sex, site, year, and age × sex on iris color PC1 and PC2 values of Spotted Towhees (*Pipilo maculatus*) captured during the 2022–2023 breeding seasons in the lower mainland region of British Columbia, Canada. SE = standard errors. 95% CI = 95% confidence interval (lower value, upper value).

	PC1			PC2		
Coefficients	Estimate ± SE	*p*-value	95% CI	Estimate ± SE	*p*-value	95% CI
Intercept	-1.15 ± 0.36	-	-1.85, -0.44	-0.28 ± 0.17	-	-0.62, 0.06
Age	0.52 ± 0.43	0.23	-0.32, 1.35	0.37 ± 0.21	0.07	-0.03, 0.78
Sex	0.94 ± 0.37	0.01	0.21, 1.67	0.68 ± 0.18	0.0001	0.33, 1.03
Site	-	0.15	-	-	0.60	-
Fleetwood	-0.41 ± 0.34	-	-1.07, 0.25	-0.05 ± 0.16	-	-0.37, 0.26
Terra Nova	0.27 ± 0.32	-	-0.35, 0.89	-0.06 ± 0.15	-	-0.36, 0.24
Tynehead	-0.47 ± 0.38	-	-1.21, 0.27	-0.25 ± 0.18	-	-0.61, 0.11
Year	0.68 ± 0.26	0.01	0.17, 1.18	-0.28 ± 0.12	0.03	-0.52, -0.03
Age × Sex	-0.56 ± 0.32	0.29	-1.59, 0.48	-0.40 ± 0.26	0.12	-0.90, 0.10

Fig. 3. Iris color (PC1) of Spotted Towhees (*Pipilo maculatus*) captured during the 2022–2023 breeding seasons in urban green spaces in the lower mainland regional of British Columbia, Canada across different (A) sex, (B) years, (C) study sites, (D) age classes, and (E) age-sex classes. Iris color (PC2) depicted in Fig. S4 and iris color PC2 vs. PC1 in Fig. S5. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. ASY = After Second Year. SY = Second Year.



Non-breeding season

We found that none of the assessed variables had a significant influence on the lightness of towhee irises, but that red saturation was significantly influenced by an interaction of age and sex during the non-breeding season (Fig. 4; Table 3). Although iris lightness (PC1) was not influenced by an interaction between age and sex (ANOVA Type III; $p = 0.31$, 95% CI [-0.74, 2.33]; Table 3), older (AHY) male towhees had irises with significantly greater red saturation (PC2) than younger (HY) males (Pairwise comparison: AHY males - HY males: $p = 0.0007$, 95% CI [0.28,1.30]; Table S4). Female towhees did not show significant difference in red saturation between AHY and HY birds or any age of male towhees (Fig. 4E; Table 3). Older (AHY) birds had darker, more pure red irises than younger (HY) birds, on average, but these differences were not significant (ANOVA Type III; PC1: $p = 0.85$, 95% CI [-1.19, 1.44]; PC2: $p = 0.90$, 95% CI [-0.63, 0.71]; Fig. 4D; Table S3). On average, females had darker irises with lower red saturation than males, but this difference was also not significant during the non-breeding season (ANOVA Type III; PC1: $p = 0.85$, 95% CI [-1.01, 1.44]; PC2: $p = 0.16$, 95% CI [-0.17, 0.99]; Fig. 4A; Table S3). There was also no clear influence of site on iris color (ANOVA Type III; PC1: $p = 0.83$, 95% CI [-0.65, 0.81]; PC2: $p = 0.92$; 95% CI [-0.39, 0.35]; Fig. 4C; Table S3). Birds captured at Alaksen/Reifel had similar colored irises to those captured at Terra Nova (Table S3). The year of capture did not clearly explain iris color variation either (ANOVA Type III; PC1: $p = 0.13$; PC2: $p = 0.11$; Fig. 4B; Table S3). When we carried out a GLM considering only iris redness (only the average red value), the results were again comparable to the GLM involving PC1 iris color values. Iris red value was not significantly influenced by any of the considered factors during the non-breeding period (Appendix 2B).

DISCUSSION

We found that Spotted Towhee iris color was influenced mainly by sex and year during the breeding season, and that during the non-breeding season, AHY and HY males differed greatly in the red saturation of their irises, but females of different ages did not differ. We found no clear influence of study site on towhee iris color during the breeding or non-breeding seasons.

Spotted Towhees exhibit a degree of iris sexual dichromatism. Females had darker irises with lower red saturation than males. Although this sex difference was only clear during the breeding season, we also found a significant sex specific effect of age on the red saturation of irises during the non-breeding season. Importantly, there remained considerable overlap in iris color year-round between males and females, suggesting iris color cannot be reliably used to sex individuals. Other traits, such as plumage (year-round), or presence of a brood patch or cloacal protuberance (breeding season only), are much more reliable (plumage) or definitive (brood patch or cloacal protuberance) indicators of sex for Spotted Towhees (Pyle 2022). The sexually dichromatic nature of Spotted Towhee irises could be related to sex-specific behavioral differences. Male Spotted Towhees defend territories against neighboring males using various agonistic behaviors and displays (Davis 1958, Baumann 1959). For some behaviors, lighter irises may help produce a more conspicuous signal, particularly in contrast to males' glossy black feathers (Courtney 1997, Davidson et al. 2014). In contrast, breeding female Spotted Towhees spend much of their time foraging, building nests, incubating eggs, and brooding nestlings in dense understory (Baumann 1959, Davis 1960). Darker irises may reduce predation risk by helping to camouflage females as they travel to and from the nest site and while they sit on their nests (Davidson et al. 2017, Passarotto et al. 2018). Spotted Towhee iris color may be influenced by factors that differ more between males and females during the breeding period than non-breeding period, such as hormones involved in reproduction (Witschi 1935, Trauger 1974). The sex specific effect of age observed during the non-breeding season, with AHY males having much redder irises than HY males, is suggestive of a possible role of androgens in Spotted Towhee iris color development (Trauger 1974, Corbett et al. 2024). A sex specific mechanism of iris color production is further supported by our finding that there did not appear to be an overarching effect of season on adult towhee iris color. However, the exact function and cause of sexual dichromatic iris color in Spotted Towhees cannot be fully elucidated without understanding the mechanisms involved in color production and how this varies between sexes. Further, differences between the breeding and non-breeding seasons in the relationship between sex and iris color should be considered with a degree of caution because of differences in sample sizes. Future work involving data on iris color of the same bird at different points of the year or even within a season, particularly breeding versus non-breeding seasons, would be informative. However, this would require multiple captures of the same individual between seasons, which can be logistically difficult in field studies where individuals do not necessarily stay in the same area between breeding and non-breeding seasons.

Fig. 4. Iris color (PC2 vs. PC1) of Spotted Towhees (*Pipilo maculatus*) captured during the 2022–2024 non-breeding seasons in urban green spaces in the lower mainland region of British Columbia, Canada across different (A) sex, (B) years, (C) study sites, (D) age classes, and (E) age-sex classes. PC1 and PC2 iris color are individually depicted in Figs. S6-7. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Ellipses enclose all points corresponding to unique categories for each factor. Each figure includes a legend indicating which category correspond to which symbol and line type combinations. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. AHY = After Hatch Year. HY = Hatch Year.

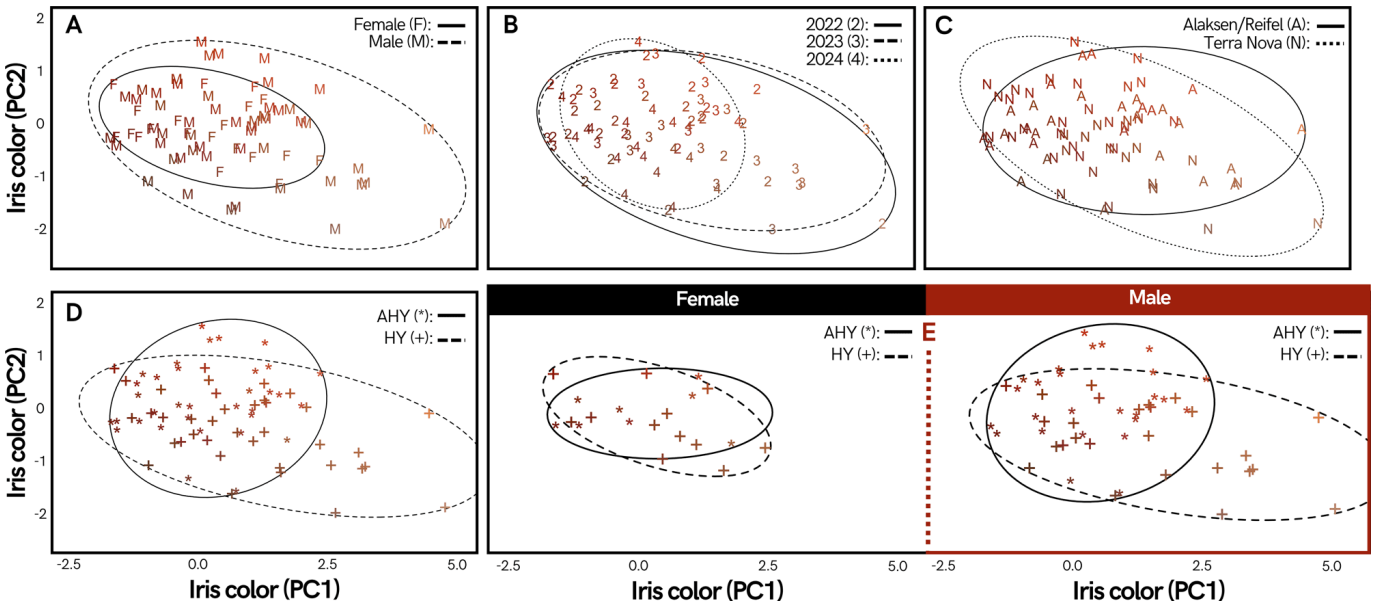


Table 3. Effect of age, sex, site, year, and age × sex on iris color PC1 and PC2 values of Spotted Towhees (*Pipilo maculatus*) captured during the 2022–2024 non-breeding seasons in the lower mainland region of British Columbia, Canada. SE = standard errors. 95% CI = 95% confidence interval (lower value, upper value).

	PC1			PC2		
Coefficients	Estimate ± SE	*p*-value	95% CI	Estimate ± SE	*p*-value	95% CI
Intercept	-0.14 ± 0.51	-	-1.14, 0.86	0.10 ± 0.26	-	-0.40, 0.61
Age	0.12 ± 0.67	0.85	-1.19, 1.44	0.04 ± 0.34	0.90	-0.63, 0.71
Sex	0.13 ± 0.58	0.83	-1.01, 1.26	0.41 ± 0.29	0.16	0.17, 0.99
Site	0.08 ± 0.37	0.83	-0.65, 0.81	-0.02 ± 0.19	0.92	-0.39, 0.35
Year	-	0.13	-	-	0.11	-
2023	0.67 ± 0.41	-	-0.12, 1.46	-0.30 ± 0.21	-	-0.70, 0.11
2024	-0.12 ± 0.46	-	-1.03, 0.78	-0.47 ± 0.23	-	-0.93, -0.01
Age × Sex	0.80 ± 0.78	0.31	-0.74, 2.33	-0.83 ± 0.40	0.04	-1.61, -0.05

The extensive overlap between, as well as variation within, age classes in iris color, shows it to be an unreliable metric for aging Spotted Towhees during banding. Although older birds had darker irises with greater red saturation, iris color did not differ significantly between age categories during either season. Indeed, when three experienced banders attempted to independently age towhees using only iris color and capture date, only about half of the birds were correctly aged and nearly a quarter were incorrectly aged, consistently. Distinguishing between HY and AHY towhees by iris color during the non-breeding season, particularly for males, is slightly more reliable than attempting to distinguish between ASY and SY birds during the breeding season, however there remained several ambiguous cases within each age category. Given these findings we stress caution in using iris color for aging other species until research has been conducted examining iris color variation in that species. Many juvenile birds have dark irises, due to melanin-rich pigment epithelium, and gradually acquire their adult iris color as outer iris layers become pigmented or as outer melanin-rich layers are lost (Oehme 1969, Sweijd and Craig 1991, Hudon and Oliphant 1995). Dark, relatively brown irises are likely beneficial to young towhees (i.e., nestlings, fledglings, and juveniles incapable of prolonged flight) helping them to remain inconspicuous to predators (Passarotto et al. 2018). Overlap between and variation within age categories of Spotted Towhees could be related to variable rates of pigment acquisition, which may be under hormonal influences (Trauger 1974). Spotted Towhees in southern British Columbia can have up to three broods per breeding season with the first brood hatching as early as mid-April and the third brood hatching as late as mid-August (*unpublished data*). Individuals grouped into the same age category could be separated in true age by as much as four months. Further, our findings show that even among AHY/ASYs, there does not appear to be one single definitive iris color to which non-adult colors can be compared for aging purposes. Even other studies that have found statistically significant differences in iris color between ages in other species have concluded that iris color cannot be reliably used to age individuals in the field because of considerable between and within age variation (Trauger 1974, Newton and Marquiss 1982, Rosenfield and Bielefeldt 1997, Scholten 1999, Frieze and Lloyd 2017).

We did not find clear statistical evidence to support iris color being influenced by habitat characteristics, at the site level. We did note minor differences of iris color in Spotted Towhees captured at the two coastal sites (Alaksen/Reifel and Terra Nova) where captured individuals had lighter irises than those captured at the two inland sites (Fleetwood and Tynehead) during the breeding season. Between site iris color variation could be the result of differences in habitat and/or individual physiology, which might co-vary among sites because of differences in resources, pollutants, and/or other stressors (Newton and Marquiss 1982, Bortolotti et al. 2003, Kristiansen et al. 2006, Passarotto et al. 2020, Koneru and Caro 2022). Although the sites in this study do vary with respect to several features, our findings suggest that environmental differences are not contributing to iris color, the degree of differences between sites is not great enough to elicit clear iris color differences, and/or the study sites do not show differences in environmental factors that have the potential to impact iris color. Future work across even more geographically separated study sites may provide clearer insight into the potential relationship, or lack thereof, among habitat quality, habitat structure, bird condition, and iris color of Spotted Towhees and other species.

We included year as a fixed effect in our models to account for year-related differences in methodology as the project progressed. Although we found iris color to differ significantly between years during the breeding season, we did not find this same clear pattern during the non-breeding season, nor when considering only iris red value. This seasonal inconsistency, along with our confirmation that color calibration did not substantially alter iris colors, suggests that methodological differences in RGB value extraction were not contributing to the iris color variation observed. However, it is possible that the difference in findings regarding the effect of year is the result of other methodological artifacts that we could not control for or sample size differences between the seasons, between age classes, or between sex classes. Indeed, the 2022 breeding season sample comprised a greater proportion of males than the 2023 season (reflective of differences in capture rates, not a change in population sex ratio) and our findings show that both males and the 2022 breeding season were associated with lighter irises with greater red saturation. Alternatively, it may be that iris color differed during the 2022 and 2023 breeding seasons because of some form of interannual variation, such as diet (McGraw 2006, Wails et al. 2018). Additional years of sampling, interannual comparison of recaptured individuals, or experimental manipulation of diet or other environmental conditions could be useful avenues for future research.

Our study also has applications for the validity of digital photographs taken on cellphones for capturing fine scale color variation under variable field conditions. It was identified as early as 1957 that knowledge of the extent of iris color variation in Spotted Towhees was minimal because of a lack of documentation (Davis 1957a). In the time since, there has been little improvement in the documentation of soft part colors, which has hindered research progress (Corbett et al. 2024, Joseph et al. 2024). Much of the color variation in the natural world remains unexplored because pigments and color structures may be altered upon death, be difficult to preserve, or degrade over time even when preserved (Erichsen 1985, Joseph et al. 2024). Previous studies have shown that even highly heterogeneous digital photographs can provide insight into color variation (Trauger 1974, Cake 2019). Our study shows how photographs taken with cellphones can be a valid method for analyzing fine scale color variation when care is taken to minimize lighting variation, with iris principal component values obtained from raw photographs taken under natural lighting in the field with cellphones found to be virtually identical to the values from color corrected versions of the same photographs. Cellphones are less costly and more easily carried during field work than specialized photography equipment. However, caution must be exercised when inferring function or consequences of color variation from digital photographs that only document the visible spectrum of light (Burns et al. 2017).

CONCLUSION

We found that iris color of Spotted Towhees in the Lower Mainland of British Columbia, Canada is influenced by sex, and sex-specific age differences. However, iris color between age and sex categories overlapped to such an extent that the usefulness of iris color to age or sex individual birds in the hand is minimal. Our findings highlight a need for re-examination of aging and sexing assumptions for birds. We have also identified a need to investigate the mechanisms contributing to the red color of Spotted Towhee irises to better understand why sex differences were observed in this study. Further, we provide evidence of the validity of cellphone photography as a methodology for capturing fine-scale color variation under natural lighting in the field if care is taken to obtain clear photographs with homogeneous lighting throughout.

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Data Availability:

The data and code that support the findings of this study are openly available in figshare repository at https://doi.org/10.6084/m9.figshare.25345768.

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Appendix 1. Photograph Processing Instructions:

A. <u>For calibrating photograph colors (SpyderCheckr 1.6):</u>

1) Import photo to Adobe Lightroom Classic
2) Enter Develop Mode
3) Crop around the edge of the palette
    a. Cropping is done with the Lightroom crop tool. Crop to just outside the color patches. Use the rotate function of the crop tool to straighten the target image.
4) Adjusting the Target Shot
    a. Any of the light or medium gray patches can be used to gray balance/white balance your image in Lightroom, ACR, or Phocus. The 20% gray patch (E2, or the gray patch adjacent to the white patch) is recommended. Use the white balance eyedropper tool in Lightroom's Develop mode.
    b. Next look at RGB values or Percentages of the White patch (E1). Adjust the exposure slider until the white patch lists as approximately 90% in Lightroom. Next check the black patch (E6). The Blacks adjustment (and in some cases also the Shadows adjustment) is used to set the black value to 4% in Lightroom.
5) Right click photo -> edit in -> SpyderCheckr -> Edit a copy with lightroom presets/adjustments -> SpyderCheckr automatically launches
6) Processing target shot
    a. The sampling squares will be pretty well placed within the correct patches of your Target shot if you shot and cropped appropriately. If not, you can drag on any edge or corner of the image area to adjust the fit.
    b. The colors inside the sampling squares should be a somewhat less saturated version of the patch colors. If the patch and sample colors are of different colors, check that your target image is not upside down or inverted (48 patch SpyderCheckr only; the SpyderCheckr24 is automatically rotated in software).
7) Colorimetric (offers most literal results and is best when attempting to reproduce artwork or product colors) -> Save to lightroom -> save calibration -> make sure to save it under identifying name
8) Close SpyderCheckr AND Lightroom
9) Reopen Lightroom, wait for presets to load then import original photo of interest
10) Open original photo in develop modem -> click on the appropriate preset on the left side of the screen
11) Right click photo -> export -> save under identifying name and in correct location

B. <u>For extracting RGB values from all photographs:</u>

12) Open Adobe Photoshop -> import photographs
13) Click individual photo to open
14) Zoom 200%
15) Set eyedropper tool to select average of 5x5 pixels (right click)
16) Points to select colors are located above, below, anterior, and posterior to the center of the pupil. Points are selected from the inner iris adjacent to the pupil.
17) Record the RGB values from each of the four points
    a. If one of these four points has reflection or shadow, do not select this location.
18) Calculate the average and standard deviation for each color value – these are what will be utilized in the analysis.

Appendix 2. Results of General Linear Model with Redness Value

A) Breeding Season

Iris redness differed significantly between sexes, with males having redder irises than females (ANOVA Type III; $p < 0.0001$, 95% CI [19.19, 53.90]). Iris redness did not differ significantly between age categories (ANOVA Type III; $p = 0.08$, 95% CI [-2.18, 37.82]). Likewise, iris redness was not significantly influenced by an interaction between age and sex (ANOVA Type III; $p = 0.09$, 95% CI [-46.26,3.10]). Males had lighter, redder irises than females regardless of age. Differences in iris redness among study sites were not significant (ANOVA Type III; $p = 0.23$). In contrast to the GLMs involving PC1 and PC2 iris color values, we found that the red value of irises did not vary significantly between 2022 and 2023 (ANOVA Type III: p 0.33; 95% CI [-6.05,18.05]).

B) Non-Breeding Season

Males had redder irises than female but this difference in redness was not significant (ANOVA Type III: $p = 0.18$, 95% CI [-7.01, 36.97]). AHY birds had redder irises than HYs, but this difference was also not significant (ANOVA Type III; $p = 0.93$, 95% CI [26.58, 24.45]). Iris redness was also not influenced by any interaction between age and sex (ANOVA Type III, $p = 0.56$, 95% CI [-38.63, 20.78]). Males had lighter, redder irises than females regardless of age. Iris redness was not significantly different between birds captured at Alaksen/Reifel compared to those captured at Terra Nova (ANOVA Type III, $p = 0.65$, 95% CI [-10.88, 17.33]). Finally, iris redness was not different among years (ANOVA Type III, $p = 0.15$).

Table S1. Summary of the management practices and ecological characteristics of the urban greenspaces within the Lower Mainland of British Columbia, Canada at which Spotted Towhees were captured in 2022-2023 breeding and 2022-2024 non-breeding seasons.

	Alaksen/Reifel		**Fleetwood**	**Tynehead**	**Terra Nova**
	Alaksen	**Reifel**			
Size (~ hectares)	349	648	49	260	39
	~825 total				
Designated Uses	National Wildlife Area; agriculture (crops and cattle)	Bird Sanctuary	Recreation; urban forest	Preservation of salmon habitat; recreation;	Recreation; agriculture (community garden, orchards, crops); heritage site; ecological preservation
Land Cover	70% agriculture; and 30% mixed forest, shrubland, and restored grasslands (ECCC 2023b)	65% tidal marshes; 10% mixed habitat of grasses, forbs, trees, and shrubs; 5% cultivated land; and 1% tidal mud flats (ECCC 2023a)	~75% forest; and ~25% scenic gardens, sports fields and courts, spray park, picnic areas, and playground	~70% forest; and ~30% field, restored grassland and meadow (GVRD 2004)	~50 forest and shrubland slough; ~20% agriculture; ~15% restored grassland; ~10 managed heritage homestead; and ~5% recreation
Dominant Over Story	Common Hawthorn (*Crataegus monogyna*) and Alder (*Alnus* spp.)	Pacific Crab Apple (*Malus fusca*) and Common Hawthorn	Big Leaf Maple (*Acer macrophyllum*), Red Alder (*Alnus rubra*), and Western Redcedar (*Thuja plicata)*	Western Hemlock (*Tsuga heterophylla*), Mountain Hemlock (*Tsuga mertensiana*), and Western redcedar	Common Hawthorn, Pacific Crab Apple, and Willow (*Salix* spp.)
Dominant Under Story	Himalayan Blackberry (*Rubus bifrons*), Salmonberry (*Rubus spectabilis*), and grasses	Himalayan Blackberry and ferns	Himalayan Blackberry and Salmonberry	Himalayan Blackberry, Salmonberry, ferns, and horsetails	Himalayan Blackberry, grasses, and sedges

Table S2. The number of Spotted Towhees of each sex (female or male) and age (SY or ASY, HY or AHY) demographic captured at each of the four study sites within the lower mainland of British Columbia, Canada during the breeding and non-breeding seasons. The number of birds captured in different years are shown together in one cell, separated by a "/". Research was not conducted at Fleetwood or Tynehead during the non-breeding season, thus sample sizes are denoted as "-".

Site	Breeding Season (2022 / 2023)				Non-Breeding Season (2022 / 2023 / 2024)				TOTAL (2022 / 2023 / 2024)
	Second Year		After Second Year		Hatch Year		After Hatch Year		
	Female	Male	Female	Male	Female	Male	Female	Male	
Alaksen/Reifel	3 / 2	6 / 9	6 / 4	13 / 5	1 / 1 / 0	9 / 4 / 0	4 / 0 / 0	4 / 6 / 1	46 / 31 / 1
Fleetwood	3 / 3	7 / 2	3 / 1	7 / 3	-	-	-	-	20 / 9 / -
Terra Nova	3 / 5	8 / 2	3 / 2	8 / 4	1 / 5 / 4	3 / 5 / 5	3 / 0 / 1	5 / 2 / 8	34 / 25 / 18
Tynehead	3 / 3	3 / 1	1 / 1	6 / 3	-	-	-	-	13 / 8 / -
Total (Sex)	12 /13	24 / 14	13 / 8	34 / 15	2 / 6 / 4	12 / 9 / 5	7 / 0 / 1	9 / 8 / 9	
Total (Age)	36 / 27		47 / 23		14 / 15 / 9		16 / 8 / 10		205

Table S3. Iris color principal component 1 (PC1) and 2 (PC2) estimated marginal mean ± standard error values for Spotted Towhees of varying age, sex, and age-sex categories captured across different sites within the lower mainland of British Columbia, Canada during the 2022-2023 breeding and 2022-2024 non-breeding seasons.

Breeding Season			Non-Breeding Season		
Category	**PC1**	**PC2**	**Category**	**PC1**	**PC2**
	Age			***Age***	
ASY	-0.49 ± 0.20	-0.17 ± 0.10	AHY	0.15 ± 0.29	0.04 ± 0.15
SY	-0.26 ± 0.19	0.01 ± 0.09	HY	0.67 ± 0.25	-0.33 ± 0.13
	Sex			***Sex***	
Female	-0.71 ± 0.21	-0.32 ± 0.10	Female	0.14 ± 0.33	-0.14 ± 0.17
Male	-0.04 ± 0.17	0.16 ± 0.08	Male	0.67 ± 0.19	-0.15 ± 0.10
	Age-Sex			***Age-Sex***	
ASY Female	-0.97 ± 0.32	-0.51 ± 0.15	AHY Female	0.08 ± 0.52	-0.16 ± 0.26
SY Female	-0.45 ± 0.28	-0.13 ± 0.14	HY Female	0.21 ± 0.42	-0.12 ± 0.21
ASY Male	-0.02 ± 0.21	0.17 ± 0.10	AHY Male	0.21 ± 0.27	0.25 ± 0.14
SY Male	-0.07 ± 0.24	0.15 ± 0.12	HY Male	1.13 ± 0.27	-0.54 ± 0.14
	Study Site			***Study Site***	
Alaksen/Reifel	-0.23 ± 0.21	0.01 ± 0.10	Alaksen/Reifel	0.37 ± 0.31	-0.13 ± 0.16
Fleetwood	-0.63 ± 0.27	-0.04 ± 0.13	Fleetwood	-	-
Terra Nova	0.05 ± 0.24	-0.05 ± 0.12	Terra Nova	0.45 ± 0.23	-0.15 ± 0.12
Tynehead	-0.69 ± 0.32	-0.24 ± 0.15	Tynehead	-	-
	Year			***Year***	
2022	-0.72 ± 0.17	0.06 ± 0.08	2022	0.22 ± 0.26	0.11 ± 0.13
2023	-0.04 ± 0.21	-0.22 ± 0.10	2023	0.90 ± 0.31	-0.19 ± 0.16
2024	-	-	2024	0.10 ± 0.38	-0.36 ± 0.19

Table S4. Post-hoc pairwise comparison of estimate marginal mean iris color principal component 1 (PC1) values for Spotted Towhees of varying age-sex categories captured across different sites within the lower mainland of British Columbia, Canada during the 2022-2024 non-breeding seasons. SE = standard errors. CI = confidence intervals.

Contrast	Estimate ± SE	t.ratio	*p*-value	CI	
				2.5%	97.5%
AHY Female – HY Female	-0.04 ± 034	-0.13	1.00	-0.94	0.86
AHY Female – AHY Male	-0.41 ± 0.29	-1.39	0.51	-1.18	0.37
AHY Female – HY Male	0.38 ± 0.29	1.31	0.56	-0.39	1.14
HY Female – AHY Male	-0.37 ± 0.25	-1.48	0.46	-1.02	0.29
HY Female – HY Male	0.42 ± 0.25	1.68	0.34	-0.24	1.08
AHY Male – HY Male	0.79 ± 0.19	4.07	0.0007	0.28	1.30

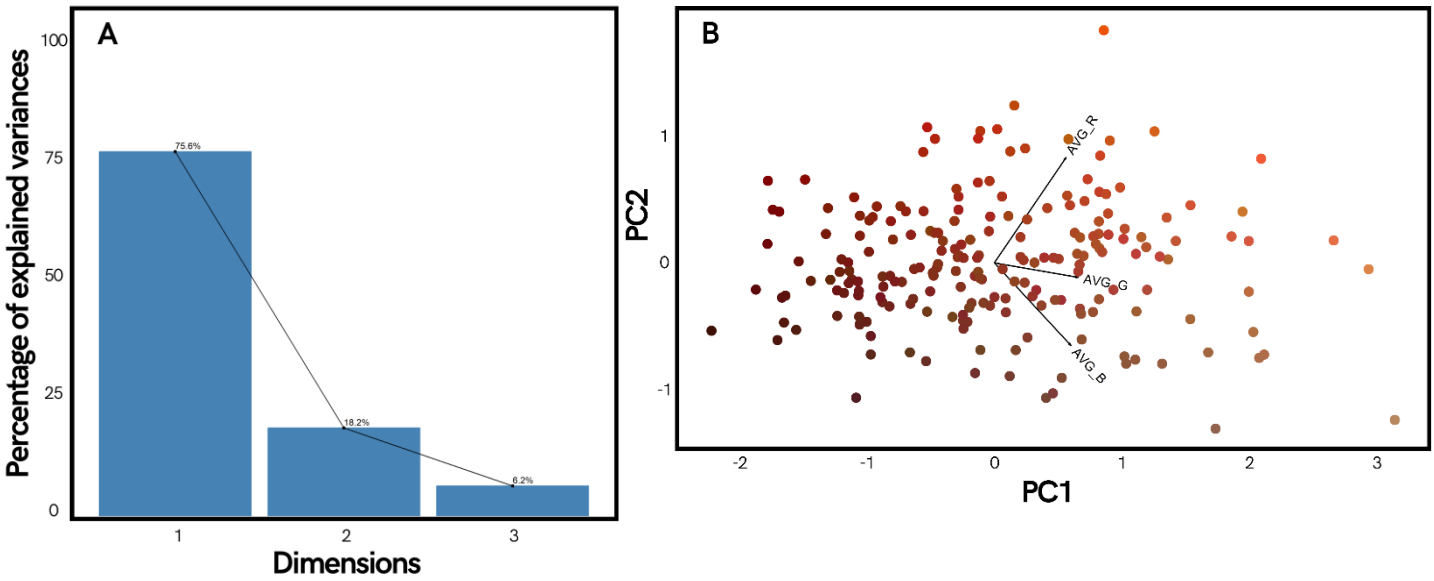

Figure S1. Iris color RGB principal component analysis (A) scree plot and (B) biplot for Spotted Towhees captured during the 2022-2023 breeding and 2022-2024 non-breeding seasons in urban greenspaces in the lower mainland region of British Columbia, Canada. In (B) each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual.

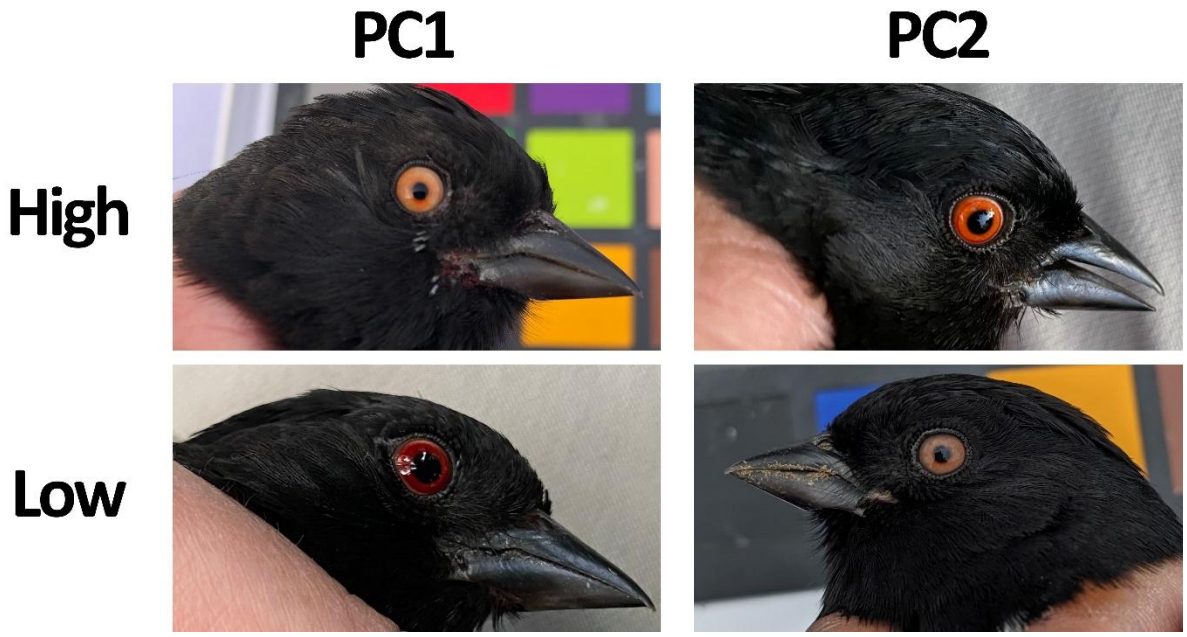

Figure S2. Photograph examples of high and low PC1 and PC2 iris colors for Spotted Towhees captured during the 2022-2023 breeding and 2022-2024 non-breeding seasons in the lower mainland region of British Columbia, Canada. All individuals in this figure are males.

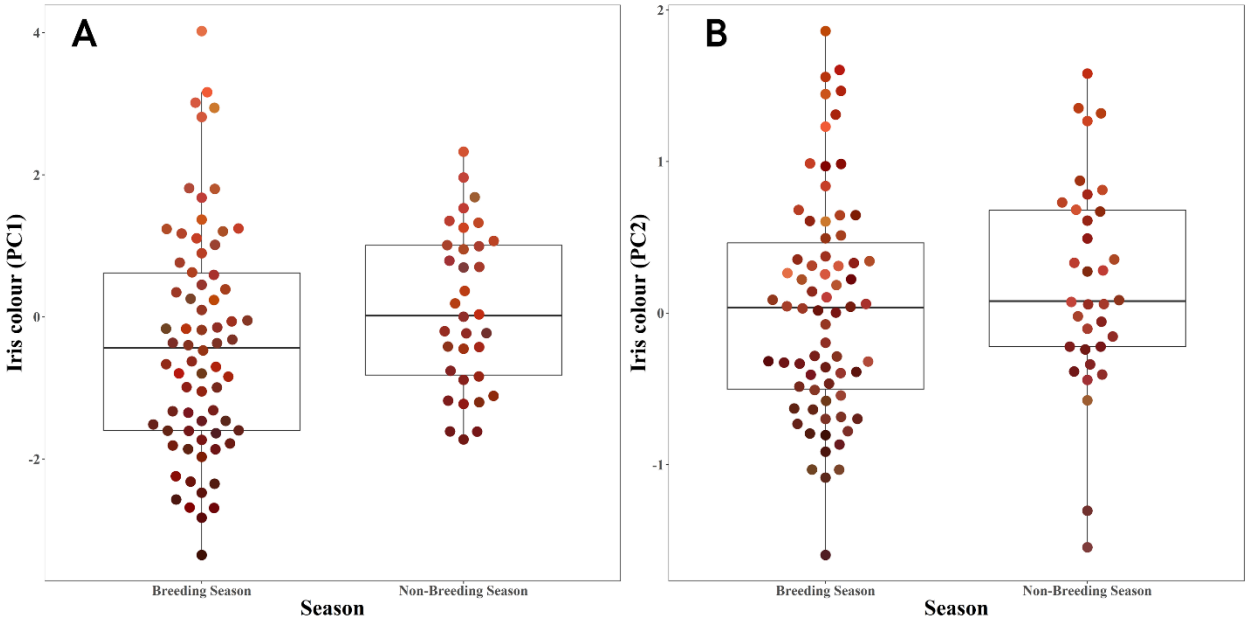

Figure S3. Iris color (A) PC1 and (B) PC2 values of adult Spotted Towhees captured during the 2022-2023 breeding (ASY birds) and 2022-2024 non-breeding seasons (AHY birds) in urban greenspaces in the lower mainland regional of British Columbia, Canada. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories.

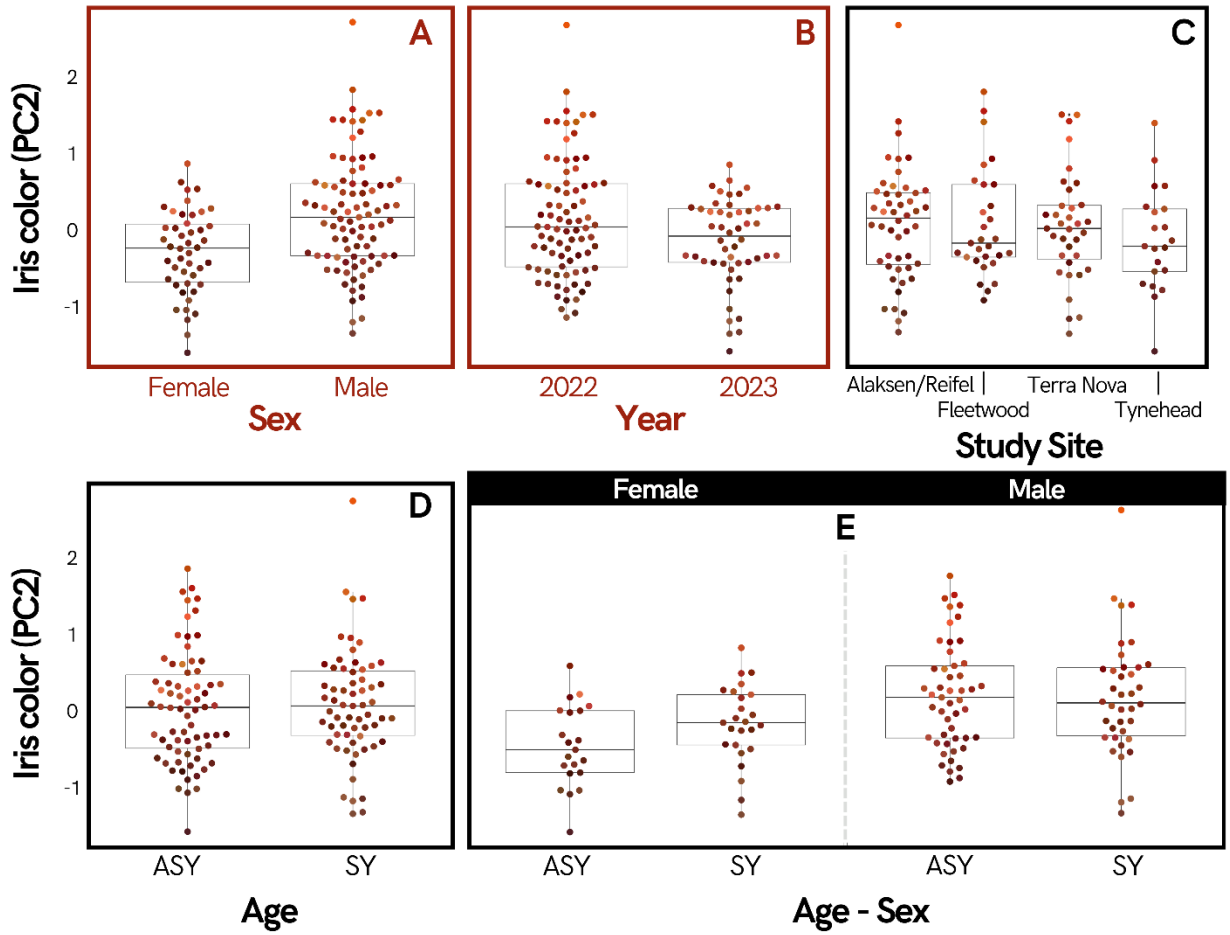

Figure S4. Iris color (PC2) of Spotted Towhees captured during the 2022-2023 breeding seasons in urban greenspaces in the lower mainland regional of British Columbia, Canada across different (A) sex classes, (B) years, (C), study sites, (D) age classes, and (E) age-sex classes. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. ASY = After Second Year. SY = Second Year.

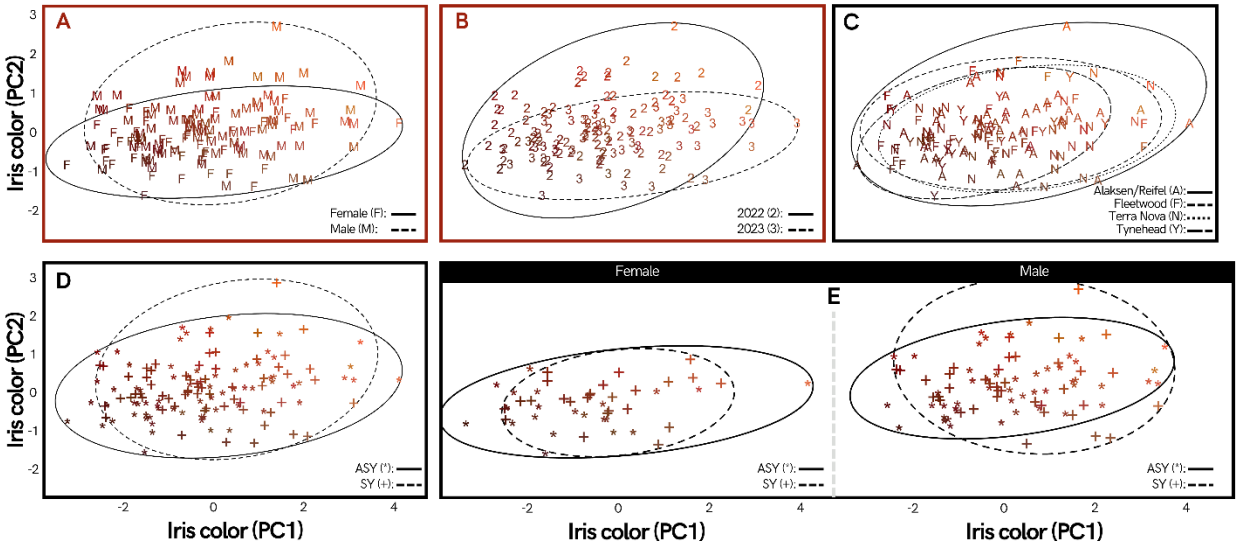

Figure S5. Iris color (PC2 vs. PC1) of Spotted Towhees captured during the 2022-2023 breeding seasons in urban greenspaces in the lower mainland region of British Columbia, Canada across different (A) sex classes, (B) years, (C), study sites, (D) age classes, and (E) age-sex classes. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Ellipses enclose all points corresponding to unique categories for each factor. Each figure includes a legend indicating which category correspond to which symbol and line type combinations. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. ASY = After Second Year. SY = Second Year.

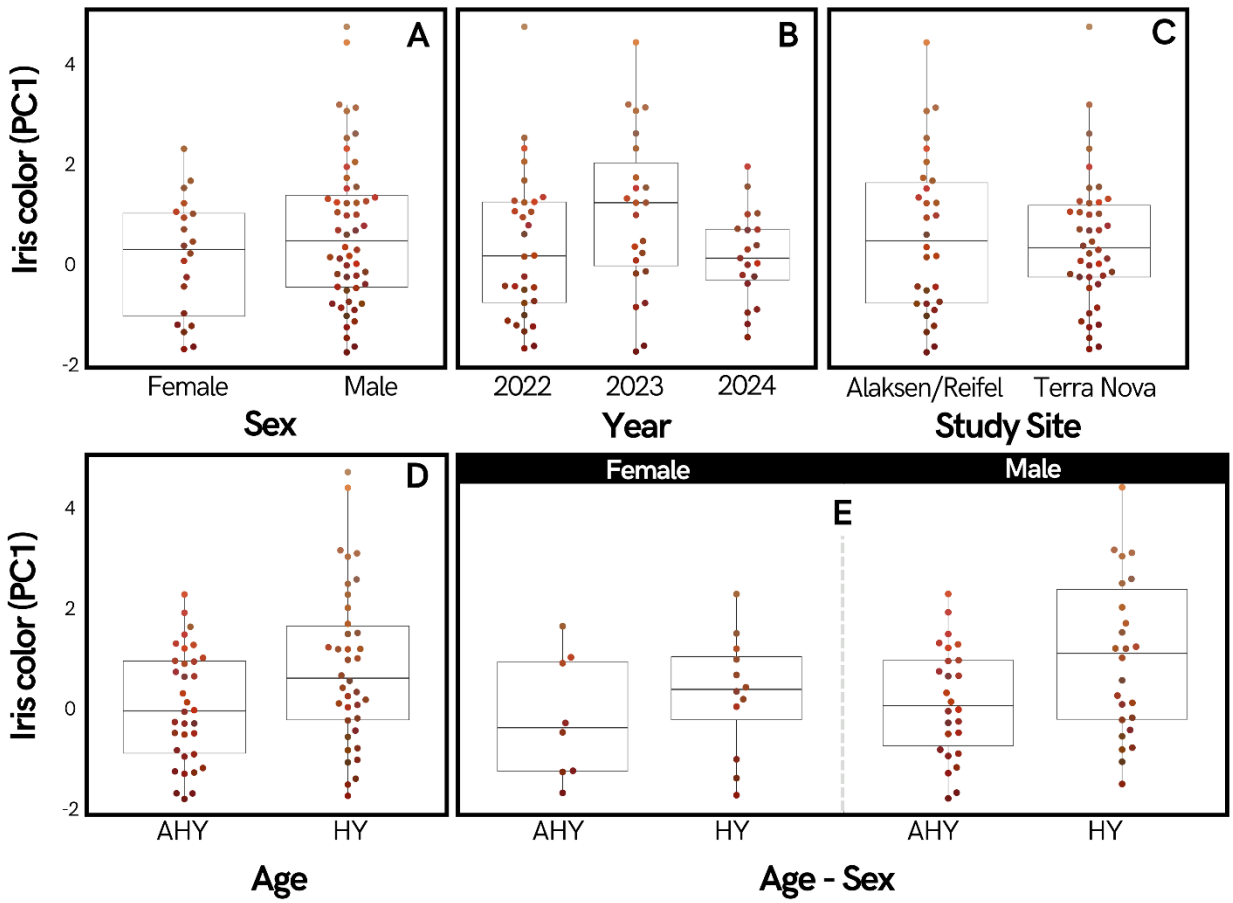

Figure S6. Iris color (PC1) of Spotted Towhees captured during the 2022-2024 non-breeding seasons in urban greenspaces in the lower mainland regional of British Columbia, Canada across different (A) sex classes, (B) years, (C), study sites, (D) age classes, and (E) age-sex classes. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. AHY = After Hatch Year. HY = Hatch Year.

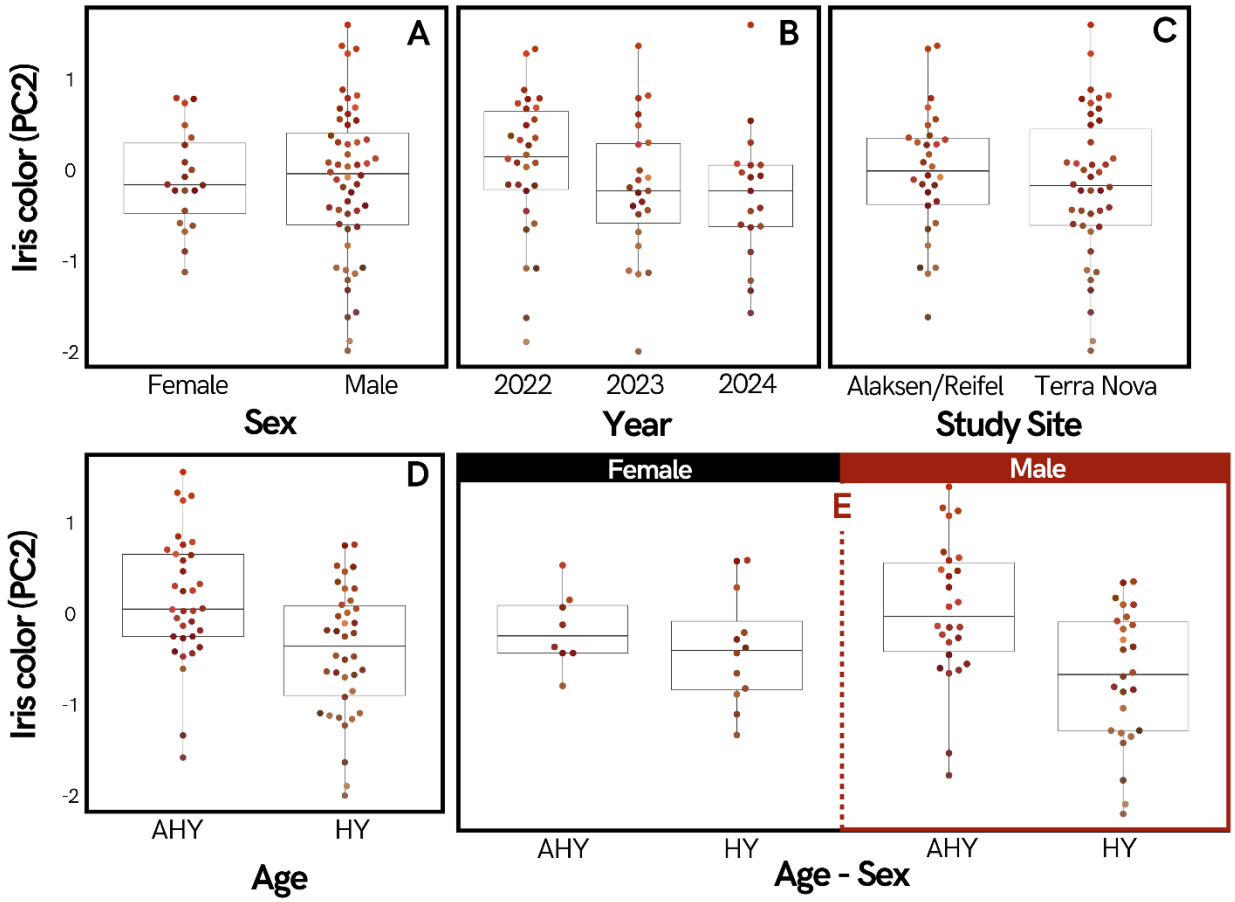

Figure S7. Iris color (PC2) of Spotted Towhees captured during the 2022-2024 non-breeding seasons in urban greenspaces in the lower mainland regional of British Columbia, Canada across different (A) sex classes, (B) years, (C), study sites, (D) age classes, and (E) age-sex classes. Each point is colored according to the hex code corresponding with the average extracted iris RGB values for that individual. Red text, border, and labels are used to highlight factors for which iris color showed significant variation between given categories. AHY = After Hatch Year. HY = Hatch Year.