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RESEARCH ARTICLE

Digital photography quantifies plumage variation and salt marsh melanism among Song Sparrow (*Melospiza melodia*) subspecies of the San Francisco Bay

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ABSTRACT

Local adaptation is often implicated as a driver of speciation and diversity, but measuring local variation within a species can be difficult. Many taxa endemic to salt marshes exhibit a phenotypic trait called salt marsh melanism, in which salt marsh endemics have a darker or grayer integument than their freshwater congeners. The repeated occurrence of salt marsh melanism across distantly related taxa in similar environments suggests a role for local selection in maintaining this trait. We quantitatively explored variation in plumage characteristics for four subspecies of the Song Sparrow (*Melospiza melodia*) in the San Francisco Bay area. These subspecies are restricted to habitats of varying salinity and climate, and are considered a classic example of ecologically based variation on a local scale. To analyze plumage color, we employed a digital photographic technique which was quantitative, able to deal with pattern variation, and independent of a particular visual system. Although no single plumage measure distinguished among all four subspecies, combining the measures allowed reliable assignment of most specimens. Using a discriminant analysis with five measures of plumage color, we were able to classify 75% of specimens to the correct subspecies, well above the 25% correct classification expected due to chance. The three subspecies inhabiting more saline environments (*M. m. pusillula*, *M. m. samuelis*, and *M. m. maxillaris*) were either darker (lower luminance) or grayer (lower red dominance) than the inland subspecies *M. m. gouldii*, supporting the pattern of salt marsh melanism observed in other taxa.

Keywords: digital photography, plumage evolution, salt marsh melanism, Song Sparrow, subspecies

La fotografía digital cuantifica variación en plumaje y melanismo asociado con pantanos salobres entre subspecies de *Melospiza melodia* de la bahía de San Francisco

RESUMEN

La adaptación local frecuentemente está implicada como promotor de especiación y diversidad, pero la medición de la variación local dentro de una especie puede ser difícil. Muchos taxones endémicos de pantanos salobres exhiben un rasgo fenotípico llamado melanismo de pantanos salobres, que consiste en que presentan integumentos más oscuros o más grises que sus congéneres de aguas dulces. La existencia repetida del melanismo de pantanos salobres en taxones lejanamente relacionados de ambientes similares sugiere que selección local mantiene este rasgo. Exploramos cuantitativamente la variación en las características del plumaje en cuatro subspecies de *Melospiza melodia* en el área de la bahía de San Francisco. Estas subspecies están restringidas a hábitats de salinidad y clima variados, y son consideradas un ejemplo clásico de variación ecológica a escala local. Empleamos una técnica cuantitativa de fotografía digital para analizar el color del plumaje que tiene en cuenta la variación en patrón y es independiente de un sistema visual particular. Aunque ninguna medida del plumaje distinguió entre las cuatro subspecies por sí sola, la combinación de las medidas permitió una asignación confiable de la mayoría de los especímenes. Usando un análisis discriminante con cinco medidas del color del plumaje pudimos clasificar 75% de los especímenes en la subespecie correcta, muy por encima del 25% de clasificaciones correctas esperadas por azar. Las tres subspecies que habitan los ambientes más salobres (*M. m. pusillula*, *M. m. samuelis*, y *M. m. maxillaris*) fueron más oscuras (menor luminancia) o más grises (dominancia de rojos bajos) que la subespecie continental *M. m. gouldii*, lo que sustenta el patrón de melanismo de pantanos salobres observado en otros taxones.

Palabras clave: evolución del plumaje, fotografía digital, melanismo de pantanos salobres, subspecies.

INTRODUCTION

Quantifying geographic variation within a species has occupied biologists for over a century. Since Darwin first synthesized his ideas on heritable variation and selection, we have been attempting to incorporate the individual variety of organisms into the typological classification system of Linnaeus. One way of doing so has been to use subspecies and trinomial names for geographically partitioned variation within a species. Originally, the standard method for identifying subspecies was through careful, but subjective, observation of morphological differences, primarily based on geographically partitioned variation in overall size and color. New methods for demarcating distinct groups, especially genetic analyses, have altered our ideas of what constitutes local variation. Molecular advances have given us additional tools with which to evaluate taxonomy, but the morphologically based taxonomic hypotheses of our predecessors are not explicitly wrong, they are merely based on a different set of evidence. In addition to our current knowledge of gene flow and population histories, a rigorous reevaluation of the morphological features used by traditional taxonomists may lead us to a fuller, more exciting understanding of the processes creating and maintaining local diversity.

Song Sparrows (*Melospiza melodia*) are highly morphologically variable, with 25 currently recognized subspecies (Patten and Pruett 2009). We focus particularly on the populations in and around the San Francisco Bay, because they exhibit highly localized plumage variation in stable populations that are clustered within a 100-km radius of one another (Grinnell 1913, Marshall 1948b). The 4 San Francisco Bay subspecies are found in the 3 embayments within the San Francisco Bay, as well as in the surrounding upland (Figure 1), with narrow, stable intergrade zones between populations (Marshall 1948a). The last American Ornithologists' Union checklist that covered subspecies (American Ornithologists' Union 1957) recognized these taxa as subspecies, as did a relatively recent revision of Song Sparrow taxonomy (Patten and Pruett 2009), so we accept them as the starting point for our analyses in this paper (as follows): *Melospiza m. gouldii* is found in the uplands surrounding all three portions of the bay area; *M. m. pusillula* inhabits the surrounds of the south San Francisco Bay; *M. m. samuelis* is located around San Pablo Bay; and *M. m. maxillaris* is found around Suisun Bay (Marshall 1948a, Patten and Pruett 2009). All subspecies will hereafter be referred to solely by their trinomial *gouldii*, *pusillula*, *samuelis*, and *maxillaris*.

Like most North American birds, the San Francisco Bay subspecies were first described based on plumage variation (although Grinnell [1909] noted the relatively large bill of *M. m. maxillaris*). Marshall (1948a) conducted an expansive qualitative analysis of color which involved

sorting over 2,000 individuals into plumage classes, and found that different plumage morphotypes dominated the different Song Sparrow subspecies of the region. However, Marshall's (1948a) work was limited by the technology of his time, when the most objective method for analyzing color variation was by matching skins to color cards, a method that is inherently observer-biased. Since Marshall (1948a), many studies have evaluated these populations for shape, size, and genetics, but there has been no quantitative exploration of variation in the trait that originally defined the San Francisco Bay Song Sparrows, plumage.

Song Sparrows have been extensively evaluated for patterns of neutral genetic variation, with particular focus on populations in western North America (Zink and Dittmann 1993, Fry and Zink 1998, Chan and Arcese 2002, 2003, Patten et al. 2004, Pruett et al. 2008a, 2008b, Patten and Pruett 2009, Wilson et al. 2011). The resulting body of work suggests that the extensive phenotypic variation in this species complex is likely due to nonneutral processes. Song Sparrows show mitochondrial haplotype sharing among current morphological subspecies, which is likely due to recent shared ancestry among subspecies (Zink and Dittmann 1993, Fry and Zink 1998). In contrast, nuclear microsatellite analyses have revealed mixed support for genetically distinct subspecies, with roughly half of the western subspecies corresponding to genetic groups, and with evidence for as many as 4–12 immigrants per generation in some populations (Chan and Arcese 2002, Pruett et al. 2008a, 2008b, Wilson et al. 2011). Among the San Francisco Bay populations specifically, only 1–2% of genetic variation is explained by subspecies, with evidence of microspatial genetic structuring in patch sizes of 2–10 km (Chan and Arcese 2002, Wilson et al. 2011). Given the extensive evidence for shared ancestry and gene flow among named subspecies, it is remarkable that phenotypic variation persists, and yet 25 Song Sparrow subspecies can be quantitatively diagnosed based on morphological measurements of size and shape, as well as plumage color and pattern (Chan and Arcese 2003, Patten and Pruett 2009). Taken together with the observation that most morphological subspecies of Song Sparrow roughly correspond to biome distributions in North America (Patten and Pruett 2009), these results suggest that the Song Sparrow complex warrants continued scrutiny, although it is unclear whether the force maintaining phenotypic diversity is selective or an expression of phenotypic plasticity.

The maintenance of phenotypic variation in the face of ongoing and recent gene flow among Song Sparrow populations is especially intriguing in the case of the San Francisco Bay populations. In this system, four subspecies inhabit distinct areas of the bay with varying salinity, and salinity is correlated with variation in body shape and size

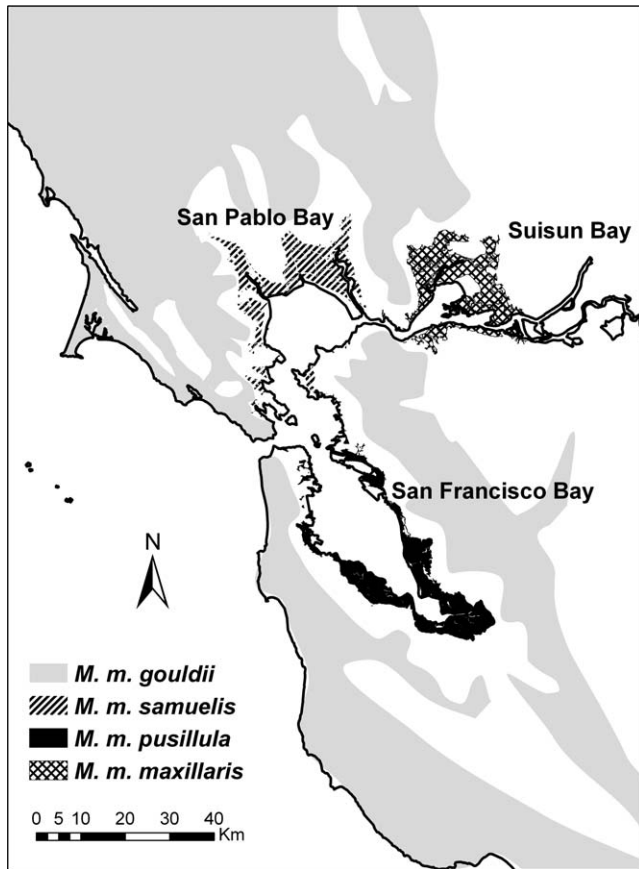


FIGURE 1. Historic ranges of the San Francisco Bay Song Sparrow (*Melospiza melodia*) subspecies (range extents from Marshall 1948a). Historic ranges represent maximum distribution during the last century. The present extent of suitable marsh habitat in all embayments has been severely reduced (California Wetlands Monitoring Workgroup 2013).

among subspecies (Chan and Arcese 2003). Given this pattern, Song Sparrows of the San Francisco Bay may provide insights into the relationship between coloration and the upland–salt marsh ecotone. Salinity is one of the major factors driving changes in species composition and biotic diversity within and among marsh communities (Malamund-Roam et al. 2006, Engels 2010). Adaptations to a specific saline environment may effectively isolate populations, even among closely related taxa such as the King Rail–Clapper Rail (*Rallus elegans*–*Rallus crepitans*) complex along the United States Gulf Coast, where a genetic cline exists that coincides with the change from fresh to saline marsh (Maley 2012).

Beyond physiological adaptations to salinity (Goldstein 1999, 2006), vertebrate taxa in salt marshes show variation in the coloration of the integument compared with their freshwater marsh congeners, a phenomenon known as salt marsh melanism (Grinnell 1913, Von Bloeker 1932, Greenberg and Maldonado 2006). In salt marsh melanism,

salt marsh taxa are grayer or blacker than their upland relatives (Greenberg and Droege 1990). Qualitatively, Song Sparrow populations of the San Francisco Bay area show a trend toward salt marsh melanism. However, no one has attempted to quantify the pattern and luminosity of the Song Sparrow subspecies because their highly patterned plumage is difficult to systematically sample using standard spectrophotometric measures. The objective of this study was to quantify plumage variation and patterns among four subspecies of Song Sparrow in the San Francisco Bay, to test whether the populations were statistically diagnosable based on plumage traits, and to determine whether the salt marsh populations were darker or had more black in their plumage as would be predicted if salt marsh melanism were present in this group.

METHODS

We used museum specimens from the Smithsonian Institution National Museum of Natural History, the California Academy of Sciences, and the University of California–Berkeley Museum of Vertebrate Zoology to evaluate color in Song Sparrows of the San Francisco Bay. All specimens were adult birds, both male and female, collected during the nonbreeding season from 1876 to 1963. The distributions of the coastal marsh subspecies are quite restricted, but *gouldii* has an extensive range in central California. For the purposes of this study, we selected *gouldii* specimens from counties surrounding the San Francisco Bay, including Alameda, Contra Costa, Marin, Napa, San Francisco, San Mateo, Santa Clara, Santa Cruz, and Sonoma.

Photographic Methods

We applied standardized digital photography analysis for evaluating the back and wing plumage of all specimens (McKay 2013). Using an unbiased measure of color analysis, such as digital photography, is crucial when considering the biological relevance of population variation, because the human visual system is different from the visual systems of many other animals (Stevens et al. 2007, Osorio and Vorobyev 2008). Often, human bias is avoided by using a spectrophotometer to analyze the specific wavelengths present in a color. However, obtaining an average spectrophotometric measure for an animal is most useful when testing a large, uniform patch of color. Many birds have finely patterned plumage, with breaks in color smaller than the diameter of the light beam sensed by the spectrophotometer. Spectrophotometric measures of these patterns are subject to serious error. To account for the fine patterning in the plumage of Song Sparrows and still obtain an accurate and unbiased measure of their overall color, we developed a technique using standardized digital

photographs and the image processing software ImageJ (Abràmoff et al. 2004).

We photographed the dorsal and lateral portions of each specimen against a gray color standard (QpCard 101; QpCard, Helsingborg, Sweden). We used a Canon EOS Rebel Xsi digital camera, with a 60 mm macro lens, an MR-14EX ring flash set to 1/16 power, and a tripod to stabilize the camera at 0.44 m focal distance from the specimen (Canon USA, Melville, NY, USA). The camera was set in manual mode with settings of ISO 100, $f/11$, and $1/250$ s, and images were saved as RAW files. The RAW files of each photograph were standardized to the gray standard by linearizing the red, green, and blue channels, and by adjusting the brightness of each image. The color channels were linearized by selecting a small portion of the gray standard in the image and then adjusting the red, blue, and green sliders in ImageJ to equalize the channels. The brightness was adjusted so that the white square on the gray standard card was set to a luminance value of 230 (± 1.5) and the gray square had a luminance value of 100 (± 1.5).

From the standardized images we created both grayscale and color histograms (red, blue, and green) for each body region of each bird. We used the polygon feature to select a portion of the plumage (either the back, including the scapulars, or the wing, including the primaries and secondaries) for each bird. We recorded means and standard deviations from the histograms of each color channel, as well as the total number of pixels in each bin of the grayscale histogram. From these raw histogram values we calculated luminance (mean luminance in grayscale, indicative of total lightness of plumage), red dominance (mean red luminance / [mean blue luminance + mean green luminance]; a measure of how rusty the plumage is), and percent black (percentage of pixels below a threshold luminance value of 20) for the back and wing. Using our luminance data we calculated the coefficient of variation (CV) of luminance as a measure of the overall patterning of the plumage (solid-colored plumage will have a low CV, highly contrasting patterns will have a high CV).

Statistical Methods

We visually examined plumage measures of luminance, red dominance, percent black, and CV of luminance in two body regions (back and wing) using histograms and bivariate scatter plots. We found that percent black of the wing was uninformative (most values were below 1% for all subspecies). Red dominance of the back and wing and percent black of the back were not normally distributed. To obtain a normal distribution, we transformed red dominance of the wing and back using a modified log transformation ($x_1 = \log_{10}(x - 1.2)$), and percent black using a square root arcsine transformation ($x_1 = \arcsin\sqrt{x}$). All of our statistical analyses were

performed using the transformed data in R (R Development Core Team 2010) unless stated otherwise. We began by testing for differences in population means among subspecies of Song Sparrow from the San Francisco Bay area, as any two populations whose means do not differ cannot be diagnosed. Initially, we tested for correlations of red dominance with luminance and percent black with luminance, because the overall amount of light reflected by the feathers should affect measures of both red dominance and percent black. As expected, both the data for red dominance of the back and percent black of the back were tightly correlated with luminance, but a partial correlation analysis revealed that percent black of the back and red dominance of the back were not correlated with each other when luminance was controlled (Pearson $r = 0.015$, $P = 0.88$). To account for a correlation between luminance and our two color measures, we included luminance as a covariate when testing for variation in population means of red dominance and percent black. We used ANOVAs to test for population mean differences in measures of luminance, and ANCOVAs with luminance as a covariate to test for population mean differences in measures of red dominance and percent black. For the ANCOVAs, we included interaction terms (color measure * subspecies) in our models to test for heterogeneity of slopes among the groups. The interaction term was only significant for the percent black of the back model. A visual inspection of the regressions revealed that this was caused by the *maxillaris* data (Figure 2A). We removed *maxillaris* from the percent black of the back analysis, and the interaction term was no longer significant. All nonsignificant interaction terms were removed from the model. We determined which pairwise mean differences were contributing to significant ANOVA and ANCOVA results using a Tukey's Honest Significant Difference (Tukey's HSD) post hoc analysis.

Distinguishing among population means demonstrates that the named subspecies may represent phenotypically distinct groups based on plumage. A useful way to test population differentiation is through population diagnosability, or the ability to accurately identify the population of origin for any given individual based on one or more character traits. In order to test for diagnosability among the four subspecies of San Francisco Bay Song Sparrow, we began by employing a discriminant analysis. Discriminant analysis selects traits as predictors of group membership, designs orthogonal functions to discriminate among groups, and then can be used to test the ability of those predictors to assign members into the correct group. We used the untransformed data in a Wilks' lambda method discriminant analysis in Statistica Version 11 (StatSoft 2012), with prior probabilities weighted by original group size, to identify orthogonal functions that predicted subspecies identity, and a jackknifed classification matrix to assign members to subspecies groups. Four individuals

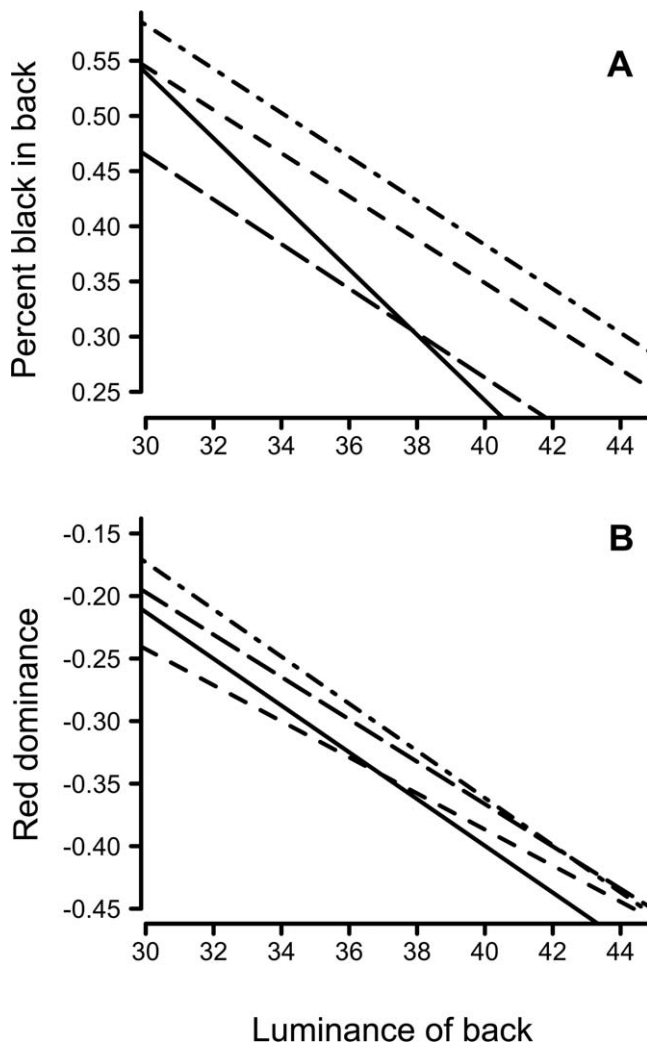


FIGURE 2. Linear regressions of luminance against percent black and red dominance in each subspecies of Song Sparrow (*Melospiza melodia*) used for ANCOVA analysis. Solid lines represent *M. m. maxillaris*, long dashes represent *M. m. gouldii*, short dashes represent *M. m. samuelis*, and dashes-dots represent *M. m. pusillula*. (A) The relationship between luminance and percentage of black in the back plumage in *M. m. maxillaris* differs from that of the other three subspecies, which violates assumptions of an ANCOVA analysis. As a result, data on *M. m. maxillaris* were removed from the ANCOVA analysis of percentage of black in the back plumage. (B) The relationship between luminance and red dominance of the back plumage is similar for all subspecies. Coupled with the information that *M. m. maxillaris* does not show a similar relationship between luminance and percent black of the back, we suggest that *M. m. maxillaris* may be overall more gray than the other three subspecies under consideration.

were excluded from the discriminant analysis because they were missing one body region measurement.

Finally, we performed a pairwise test of diagnosability by using a diagnosability index (*D*-statistic; Patten and Unitt 2002, Patten 2010) based on the “75% rule”: that at least 75% of the distribution of population 2 must lie to the right

of 99% of the distribution of population 1, and vice versa, for the populations to be diagnosable. In this case we tested individual plumage measurement variables separately.

RESULTS

After Bonferroni correction for multiple tests, we found significant mean differences among subspecies in 5 of the 6 plumage characters that we examined: percent black of the back, red dominance of the wing, luminance of the back, luminance of the wing, and CV of luminance of the back. We found significant differences in overall luminance among the subspecies (back ANOVA: $F = 30.2$, $df = 3$, $P \ll 0.001$; wing ANOVA: $F = 6.1$, $df = 3$, $P < 0.001$; Figures 3A, 3B). Tukey’s HSD post hoc analyses showed that, for luminance of the back, all pairwise comparisons were significant except for *samuelis*–*pusillula* and *maxillaris*–*pusillula* (Figure 3A), while for luminance of the wing there were pairwise differences between *maxillaris* and *gouldii* (Figure 3B). We also found significant differences in the CV of back luminance (ANOVA, $F = 30.2$, $df = 3$, $P \ll 0.001$; Figure 3C). Tukey’s HSD for CV of back luminance showed that all pairwise differences were significant except for *samuelis*–*maxillaris*. We used ANCOVAs, rather than ANOVAs, to evaluate percent black of back and red dominance, because each measure was affected by luminance. Percent black of the back (ANCOVA, $F = 45.1$, $df = 2$, $P < 0.001$; *maxillaris* excluded) and red dominance of the wing (ANCOVA, $F = 5.6$, $df = 3$, $P = 0.001$) were both significantly different among subspecies. Tukey’s HSD post hoc analyses showed that, for percent black of the back, pairwise comparisons were significant between *gouldii* and *pusillula* and between *gouldii* and *samuelis*. Pairwise comparisons for red dominance of the wing were significant for *gouldii*–*pusillula*. Only one measure, red dominance of the back, was not significantly different among any subspecies pair (ANCOVA, $F = 2.3$, $df = 3$, $P = 0.089$). See Table 1 for all pairwise comparison *P*-values.

In addition to identifying mean differences among populations, we evaluated the distinctiveness of populations using all traits at once in a discriminant analysis (number of variables in model = 6, Wilks’ lambda = 0.110, $F_{18,263} = 17.30$, $P < 0.001$). Three canonical discriminant functions were identified, but only the first two were significant (Table 2). An assignment test using the canonical functions from the discriminant analysis assigned 75% of the Song Sparrow specimens to the correct subspecies. The ability of the discriminant analysis to classify specimens to the correct group membership varied among subspecies. The discriminant analysis correctly assigned 100% of the *gouldii*, 60% of the *maxillaris*, 76% of the *samuelis*, and 59% of the *pusillula* specimens (Table 3,

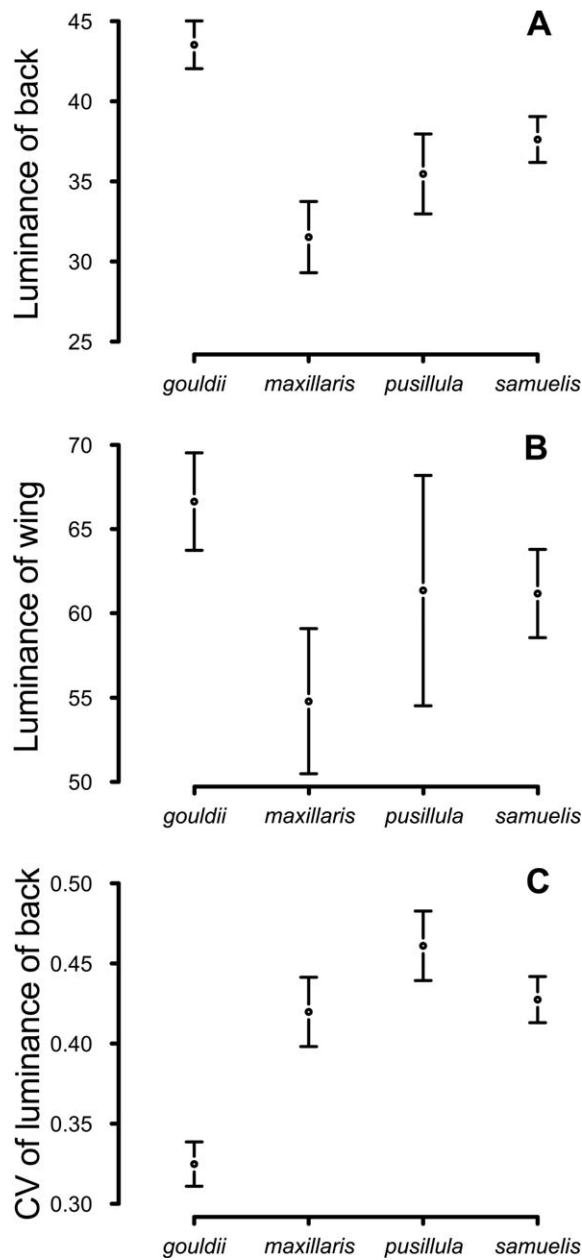


FIGURE 3. Means and 95% confidence intervals for three ANOVA plumage analyses of Song Sparrow (*Melospiza melodia*) subspecies. **(A)** Luminance of the back varies significantly among subspecies ($P \ll 0.001$), with *M. m. gouldii* being lighter in coloration than the three tidal-marsh subspecies. **(B)** Luminance of the wing plumage differs significantly among subspecies ($P < 0.001$), but only *M. m. maxillaris* and *M. m. gouldii* exhibit significant pairwise differences. The upland subspecies *M. m. gouldii* has the lightest coloration of all subspecies based on back and wing luminance measurements. **(C)** Coefficient of variation (CV) of luminance of the back varies significantly among subspecies ($P \ll 0.001$), with *M. m. gouldii* being much less variable than the tidal-marsh subspecies. We used CV as a measure of patterning; thus, *M. m. gouldii* are less patterned than the other San Francisco Bay Song Sparrow subspecies.

Figure 4). Of the 17 incorrect assignments, in no case was an individual assigned to a subspecies from the wrong habitat (i.e. upland rather than salt marsh). Our follow-up tests using the *D*-statistic (Patten and Unitt 2002) found no cases in which a pairwise comparison among subspecies groups was diagnosable using any of the original plumage measurement variables.

DISCUSSION

Based on plumage color measurements of the back and wing, three phenotypic groups of San Francisco Bay Song Sparrow were distinguishable (*gouldii*, *maxillaris*, and *pusillula*–*samuelis*). Distinctiveness varied among subspecies. For example, the *gouldii* and *maxillaris* populations had significant pairwise mean differences when compared with other populations, and *gouldii* was readily distinguishable from all other populations, being correctly classified 100% of the time. In contrast, we found only one significant pairwise mean difference between *pusillula* and *samuelis* (CV of luminance), but were able to distinguish *samuelis* from other populations 76% of the time using an assignment test. Our assignment estimates represent an upper bound on the likelihood of correctly classifying subspecies based on plumage color alone. Using a training subset of data that was different from the data used in the assignment test would have provided a more stringent test of assignment. However, given our sample size, we chose to use all data for both training and classification, and to jackknife the classification matrix. Salt marsh populations could be readily distinguished from freshwater marsh populations based on plumage alone (100% correct discrimination of upland vs. salt marsh subspecies), while discrimination within salt marsh populations likely will require the inclusion of other measures, such as beak size. Indeed, Chan and Arcese (2003) showed that the San Francisco Bay Song Sparrow subspecies are diagnosable based on beak and body size, with an overall correct classification rate of 78%. Furthermore, their canonical analysis readily discriminated *pusillula* from *samuelis*, the two groups that our plumage measures had the most difficulty discriminating between.

Overall, the most useful traits in distinguishing among populations were luminance, CV of luminance (our measure of patterning), and percentage of black on the back. The upland subspecies *gouldii* was significantly lighter and less patterned than the salt marsh subspecies, and, among the salt marsh subspecies, *maxillaris* was the darkest. Although we were not able to statistically compare *maxillaris* with the other subspecies for the trait percent black of back because the data did not comply with the ANCOVA assumption of equal heterogeneity of slopes (Figure 2A), this unsuitability for statistical comparison is in itself interesting. The regression of luminance against

TABLE 1. Summary of mean differences in plumage characteristics for four subspecies of Song Sparrow (*Melospiza melodia*) using ANOVA and ANCOVA analyses. Significant differences based on a Bonferonni-corrected P -value of $P = 0.008$ are indicated by an asterisk (*).

Test	F	df	P -value	Pairwise comparison	Tukey's HSD P -value
ANCOVA percent black back	45.1	2	$<<0.001^*$	<i>gouldii</i> – <i>pusillula</i> <i>samuelis</i> – <i>pusillula</i> <i>samuelis</i> – <i>gouldii</i>	0.001* 0.087 0.001*
ANCOVA red dominance back	2.3	3	0.086	—	—
ANCOVA red dominance wing	5.6	3	0.001*	<i>gouldii</i> – <i>pusillula</i> <i>maxillaris</i> – <i>pusillula</i> <i>samuelis</i> – <i>pusillula</i> <i>maxillaris</i> – <i>gouldii</i> <i>samuelis</i> – <i>gouldii</i> <i>samuelis</i> – <i>maxillaris</i>	0.006* 0.064 0.107 0.454 0.319 0.996
ANOVA luminance of back	30.2	3	$<<0.001^*$	<i>gouldii</i> – <i>pusillula</i> <i>maxillaris</i> – <i>pusillula</i> <i>samuelis</i> – <i>pusillula</i> <i>maxillaris</i> – <i>gouldii</i> <i>samuelis</i> – <i>gouldii</i> <i>samuelis</i> – <i>maxillaris</i>	0.001* 0.023 0.382 0.001* 0.001* 0.001*
ANOVA luminance of wing	6.1	3	$<0.001^*$	<i>gouldii</i> – <i>pusillula</i> <i>maxillaris</i> – <i>pusillula</i> <i>samuelis</i> – <i>pusillula</i> <i>maxillaris</i> – <i>gouldii</i> <i>samuelis</i> – <i>gouldii</i> <i>samuelis</i> – <i>maxillaris</i>	0.264 0.133 0.999 0.001* 0.207 0.131
ANOVA CV of back luminance	30.2	3	$<<0.001^*$	<i>gouldii</i> – <i>pusillula</i> <i>maxillaris</i> – <i>pusillula</i> <i>samuelis</i> – <i>pusillula</i> <i>maxillaris</i> – <i>gouldii</i> <i>samuelis</i> – <i>gouldii</i> <i>samuelis</i> – <i>maxillaris</i>	0.001* 0.008* 0.042 0.001* 0.001* 0.928

percent black of the back plumage in *maxillaris* showed a steeper negative slope than that for the other subspecies, while the relationship between luminance and red dominance was the same among *maxillaris* and the other subspecies (Figure 2B). This suggests that the lower luminance values for *maxillaris* were due to increased

TABLE 2. Standardized coefficients for each plumage trait in three orthogonal functions that define variation in plumage color among San Francisco Bay Song Sparrow (*Melospiza melodia*) subspecies. We identified the orthogonal functions using a Wilks' lambda method discriminant analysis in Statistica Version 11 (StatSoft 2012). Functions 1 and 2 are significant at $P < 0.05$ (indicated by *), and together explain 98.4% of the variation in plumage color among subspecies.

Variable	Function 1	Function 2	Function 3
Red dominance back	−0.135	0.018	0.593
Percent black back	0.580	−1.241	−0.111
Luminance back	1.376	−1.363	−0.010
CV luminance back	−1.124	−0.110	0.000
Red dominance wing	−0.327	−1.138	0.998
Luminance wing	−0.041	−1.276	0.866
Eigenvalue	5.317	0.317	0.091
Cumulative proportion of variation explained	0.929	0.984	1.000
P -value	$<0.001^*$	$<0.001^*$	0.078

amounts of gray plumage (which has equal reflectance in the red, green, and blue channels, and therefore does not affect red dominance); gray plumage is lighter in coloration than our threshold for black plumage. Therefore, the darkness of *maxillaris* is the result of an overall grayer plumage compared with other subspecies, rather than an increase in black plumage. The amount of rust in

TABLE 3. Each individual Song Sparrow (*Melospiza melodia*) specimen measured for this study was blindly assigned to 1 of 4 predicted subspecies groups based on plumage color alone, using a discriminant function assignment test with prior probabilities for group membership weighted by original group size and a jackknifed classification matrix. See Table 2 for orthogonal functions used in this analysis. Overall, 75% of the specimens were assigned to the correct subspecies, with 100% of the upland subspecies (*M. m. gouldii*) being assigned correctly. Total number of specimens for each subspecies used in this analysis are indicated in the rightmost column.

Subspecies	Percent correct	Predicted group membership				Total
		<i>pusillula</i>	<i>maxillaris</i>	<i>gouldii</i>	<i>samuelis</i>	
<i>pusillula</i>	59	13	6	0	4	23
<i>maxillaris</i>	60	5	15	0	2	22
<i>gouldii</i>	100	0	0	30	0	30
<i>samuelis</i>	76	4	4	0	19	27

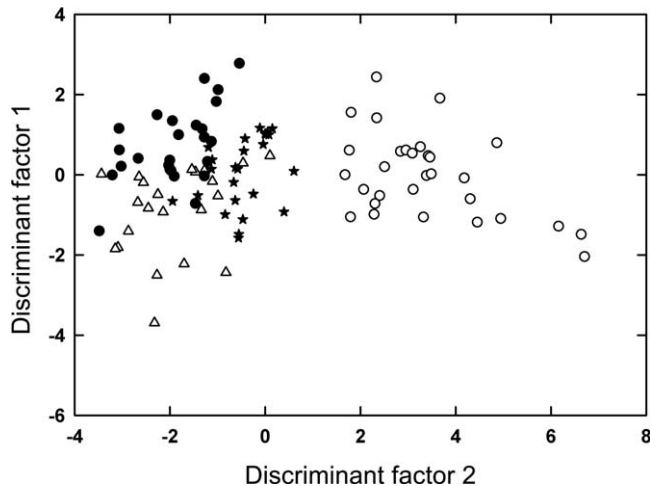


FIGURE 4. A scatterplot of the first two discriminant functions (of five measures of plumage color) demonstrates the reliability of distinguishing the upland Song Sparrow (*Melospiza melodia*) subspecies *M. m. gouldii* from the three saltmarsh subspecies (*M. m. maxillaris*, *M. m. pusillula*, and *M. m. samuelis*). Discriminant factor 1 is principally a measure of the luminance (lightness of coloration) and patterning of the back plumage, while discriminant factor 2 is mainly a measure of overall luminance (lightness of coloration) of the individual. Open circles represent *M. m. gouldii*, closed circles represent *M. m. maxillaris*, open triangles represent *M. m. pusillula*, and stars represent *M. m. samuelis*.

the plumage, measured by red dominance, contributed relatively little to differentiating among populations. Red dominance of the back plumage was not significantly different between any pair of subspecies, while red dominance of the wing plumage was only significantly different between *gouldii* and *pusillula*, with *gouldii* having less rusty wings.

The salt marsh subspecies *pusillula* and *samuelis* had no significant pairwise mean differences in our measures of plumage coloration. However, both Marshall (1948b) and Patten and Pruett (2009) noted that *pusillula* is the only Song Sparrow subspecies with yellow ventral plumage. We did not include any ventral plumage measures in our analyses, so the inclusion of this body region likely would increase the potential for diagnosability of *pusillula* vs. *samuelis*. It is important to note that group distinctions based on mean differences are not equivalent to diagnosability. Indeed, despite 100% of our upland birds being classified correctly based on our discriminant analysis, we found no cases in which plumage measures were able to diagnose pairwise comparisons between groups based on a diagnosability index (Patten and Unitt 2002). Thus, the plumage measures in our study by themselves were not sufficient to diagnose any of the four subspecies.

Despite extensive work demonstrating the phenotypic distinctiveness of Song Sparrow populations (Marshall

1948b, Chan and Arcese 2003, Patten and Pruett 2009, this study), among the western Song Sparrow subspecies, only one subspecies boundary (*M. m. heermanni*–*M. m. fallax*) shows morphological divergence that is unequivocally correlated with genetic divergence (Patten et al. 2004). The overall pattern of genetic evidence in Song Sparrows suggests a recent divergence (Fry and Zink 1998), likely with continued gene flow among many populations, including the San Francisco Bay populations (Chan and Arcese 2002, Pruett et al. 2008a, 2008b). Our data, in conjunction with the data of Chan and Arcese (2003), support the hypothesis that phenotypic differences in plumage, beak size, and body size in Song Sparrow populations are either rapidly evolving, being maintained in the face of gene flow, or both.

Plumage differentiation without marked neutral genetic differentiation has been shown in a number of bird subspecies (e.g., Seutin et al. 1995, Greenberg et al. 1998, Baker et al. 2003, Ödeen and Björklund 2003, Milá et al. 2007, Antoniazza et al. 2010), but mechanisms for the maintenance of plumage divergence are untested, and likely vary among bird taxa. In contrast, the selective pressures that may be acting on beak and body size have been more thoroughly studied. For example, selection acting on beak and body size has been classically shown in the Galapagos finches as a result of feeding ecology (Grant and Grant 1995). Additionally, Greenberg and Danner (2012) have shown that beak size in Song Sparrow subspecies is correlated with annual summer temperatures, and it has been further demonstrated that increased beak size in *M. m. atlantica* allows for the dissipation of “dry” heat while reducing water loss (Greenberg et al. 2012). It is possible that these morphological differences are phenotypically plastic, rather than rapidly evolving traits. However, in a common garden experiment, Greenberg and Droege (1990) found no evidence of plasticity in plumage or beak morphology of a coastal marsh–endemic relative of the Song Sparrow, the Coastal Plains Swamp Sparrow (*Melospiza georgiana nigrescens*).

The plumage patterns identified in our study are consistent with salt marsh melanism, with the upland subspecies being lighter in coloration. This result is supported by the finding of Chan and Arcese (2003) that morphological measurements of San Francisco Bay Song Sparrows vary with salinity. We propose two potential mechanisms for selection of salt marsh melanism in San Francisco Bay Song Sparrows and other salt marsh melanistic taxa. The first is background matching to avoid predation. Tidal marshes have grayer mud than freshwater marshes, because the relatively low oxygen content in the water causes iron to be present as dark iron sulfides, rather than rusty iron oxides (Greenberg and Droege 1990). No studies have tested support for this mechanism, but the occurrence of salt marsh melanism in small mammals and

reptiles as well as birds lends credence to the idea that salt marsh melanism is adaptive via some broadly applicable selective pressure such as predator avoidance. The second possible mechanism is increased melanism to resist feather degradation by bacteria, a selective pressure that is specific to birds. *Bacillus licheniformis*, a naturally occurring feather-degrading bacterium, is found on more individuals and at higher concentrations in the Coastal Plain Swamp Sparrow, a tidal marsh inhabitant, than on inland Eastern Swamp Sparrow (*Melospiza georgiana georgiana*) populations (Peele et al. 2009). *B. licheniformis* is a highly salt-tolerant bacterium, and increased melanin concentration in feathers slows the rate at which it can degrade feathers in vitro (Goldstein et al. 2004). Increased melanin concentration in feathers of salt marsh birds would act as a mechanism to resist bacterial degradation of feathers.

The Song Sparrow subspecies *maxillaris* may be the key to distinguishing between these two hypotheses of selection. It has a steeper negative correlation between luminance and percent black than any of the other three San Francisco Bay subspecies, probably due to an increased amount of dark gray plumage (Figure 2A). *M. m. maxillaris* also inhabits the least saline of the three bays making up the greater San Francisco Bay. Von Bloeker (1932) noted a similar trend among brackish marsh-inhabiting mammals, and argued that background matching with darker soils, rather than salinity, was the selective force maintaining melanism. Specifically testing the San Francisco Bay Song Sparrows for bacterial levels and measuring contrast with background substrate could help to distinguish between these hypotheses.

Alternatively, differences in melanin deposition could result from pleiotropic effects of genes related to osmoregulation. The mechanisms involved in osmoregulation by salt marsh passerines are generally unknown, although one study suggests that modification of the surface area of the intestinal tract may be a key innovation (reviewed in Goldstein 2006). A number of birds inhabiting salt marshes appear to have evolved a tolerance to the ingestion of saline water (Bartholomew and Cade 1963, Poulson 1969, Sabat et al. 2003), including *M. m. pusillula*, but not its nearest upland conspecific, *M. m. gouldii* (Basham and Mewaldt 1987). Establishing a link between integument color and physiological adaptation to salinity would be difficult, but worth investigating.

Regardless of the mechanism behind salt marsh melanism, our study demonstrates that Song Sparrow subspecies in the San Francisco Bay area are another example of this phenomenon. We used digital photography to analyze color, which allowed for a quantitative and objective analysis of highly patterned plumage through calibrated measurement equipment, without bias toward a particular visual system (Stevens et al. 2007, McKay 2013). Plumage traits such as percent black, luminance, and CV

of luminance reliably differentiated upland *gouldii* from tidal marsh *maxillaris*, *pusillula*, and *sameulis* subspecies. Upland birds were on average lighter and less rusty in coloration, and had less contrasting patterning than tidal marsh birds. We hypothesize that the plumage (this study) and morphological differences (Chan and Arcese 2003, Patten and Pruett 2009) demonstrated by Song Sparrows in the San Francisco Bay are the result of local selection on phenotype, especially between upland and tidal marsh populations. Our findings are in agreement with the work of Chan and Arcese (2003), which showed that salinity was correlated with morphological variation in this same group. Furthermore, extensive work on other taxa across upland vs. saltmarsh ecosystems shows that salinity is highly correlated with changes in species composition and diversity in mosquitos, King-Clapper rails, and plants (Roberts and Irving-Bell 1997, Engels 2010, Maley 2012, Sneddon and Steyer 2013). These differences might result from selective pressures for tidal marsh birds to match the grayer background substrate (Greenberg and Droege 1990), for an increase in melanism as a defense against feather-degrading bacteria (Peele et al. 2009), or as a pleiotropic effect related to the physiological demands of osmoregulation in a salt marsh environment. Further studies are needed to provide direct evidence for the mechanism of phenotypic differentiation, but the pattern remains consistent, with salt marsh melanism seen in a wide variety of tidal marsh vertebrates (Greenberg and Maldonado 2006). Many subspecies of birds were originally described based on geographic variation in plumage patterns, but this regional phenotypic variation has often been dismissed by neutral genetic studies that find no clear differentiation among subspecies. Close evaluation of phenotypic variation, to determine when this variation represents diagnosable differences, is clearly warranted.

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