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RESEARCH ARTICLE

## Plumage pattern dimorphism in a shorebird exhibiting sex-role reversal (*Actitis macularius*)

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### ABSTRACT

In birds, both males and females can exhibit socially selected traits, but relatively few studies address the role of female ornaments despite their potential importance in competitive female–female interactions and male mate choice. We investigated the melanized plumage pattern of male and female Spotted Sandpipers (*Actitis macularius*), a species with sex-role reversal and a polyandrous mating system. While the sexes overlap in the spottiness metrics, females had fewer, but larger and more irregularly shaped spots that covered a greater percentage of their plumage than did males. Feather mite load best explained the first principal component of plumage pattern (i.e. spot size) in females as well as in males. Sandpipers with lower mite loads had larger spots, but this relationship was less strong in males. Considering the second principal component (i.e. spot shape and percent cover), mass, hematocrit levels, and day captured best explained variation across females. Heavier females with higher hematocrit levels were caught later in the season and had more irregular spots and a higher percentage of melanized plumage cover. Spot pattern in recaptured individuals changed with capture year, indicating that spottiness varies within an individual's life. Overall, these results show that although the differences between the sexes are subtle, spottiness in Spotted Sandpipers is a measurably sexually dimorphic trait with females as the more ornamented sex, and that melanized ornaments can be indicators of female, and possibly male, condition.

**Keywords:** *Actitis macularius*, feather mites, melanin, plumage pattern, polyandry, sex-role reversal, sexual dimorphism, Spotted Sandpiper

### Dimorfismo en los patrones del plumaje en un ave playera con roles sexuales invertidos (*Actitis macularius*)

#### RESUMEN

En las aves, los machos y las hembras pueden exhibir rasgos seleccionados socialmente, pero relativamente pocos estudios abordan el papel de los ornamentos de las hembras a pesar de su importancia potencial en las interacciones competitivas entre hembras y en la escogencia de pareja. Investigamos los patrones melánicos del plumaje de machos y hembras de *Actitis macularius*, una especie con roles sexuales invertidos y un sistema de apareamiento poliándrico. Aunque ambos sexos se superponen en las medidas del moteado de su plumaje, las hembras tienen menos manchas pero de mayor tamaño y más irregulares en su forma, que cubren un mayor porcentaje del plumaje que en los machos. La carga de ácaros en el plumaje fue el parámetro que mejor explicó el primer componente principal del patrón del plumaje (i.e. tamaño de las manchas) en hembras y machos. Las aves con menores cargas de ácaros tenían manchas más grandes, pero esta relación fue menos fuerte en los machos. Al considerar el segundo componente principal (i.e. forma de las manchas y porcentaje de cobertura), los parámetros que mejor explicaron la variación en las hembras fueron la masa, los niveles de hematocrito y el día de captura. Las hembras más pesadas y con niveles más altos de hematocrito fueron capturadas más tarde en la temporada, y tuvieron más manchas irregulares y un mayor porcentaje de cobertura de plumaje melánico. El patrón de las manchas en los individuos recapturados cambió con el año de captura, indicando que el moteado varía a lo largo la vida de los individuos. En general, estos resultados muestran que aunque las diferencias entre los sexos son sutiles, el grado de moteado del plumaje de *A. macularius* es un carácter sexualmente dimórfico medible, en el que las hembras son el sexo más ornamentado, y en el que los ornamentos melánicos pueden ser indicadores de la condición física de las hembras y posiblemente de los machos.

**Palabras clave:** *Actitis macularius*, dimorfismo sexual, ácaros de las plumas, melanina, patrones del plumaje, inversión de roles sexuales

## INTRODUCTION

From the tails of peacocks to the horns of rhinoceros beetles, sexually dimorphic traits have evolved repeatedly. Most frequently, it is the male that is ornamented (e.g., Hill 1993, Møller 1993, Cuervo et al. 1996, Amundsen 2000) due to intrasexual competition, mate choice, and/or mate stimulation (Andersson 1994, Servedio et al. 2013, Tazzyman et al. 2014). Selection can be equally important in driving the evolution of female ornaments (Amundsen 2000), and as females may also compete for mates (e.g., Gwynne 1981, Berglund et al. 1996, Swenson 1997, Eens and Pinxten 2000, Härdling et al. 2008), sexual selection pressures could influence exaggerated traits in females. Alternatively, females may exhibit an ornamented trait present in males, but in a less exaggerated form as a genetic by-product of selection on males (Lande 1980), a stimulant for male provisioning of offspring (e.g., Matessi et al. 2009), or mutual mate choice (e.g., Kraaijeveld et al. 2007).

In rare cases, females are the more ornamented sex (e.g., Johns 1964, Heinsohn et al. 2005, Muck and Goymann 2011, Clutton-Brock and Huchard 2013). The evolution of female-specific ornaments under sexual selection is expected in circumstances where females experience greater competition for mates (i.e. “sex-role reversal”) and therefore greater potential variance in reproductive success (Emlen and Oring 1977, Amundsen 2000). This reversal in typical sex roles is unusual across vertebrates and is usually associated with polyandrous mating systems (Andersson 2005), in which females pair with multiple males in a single breeding season. Polyandry, though uncommon (Andersson 2005), has evolved independently several times in birds (e.g., hawks (Accipitridae), shorebirds (Charadriidae, Jacanidae, Scolopacidae), buttonquail (Turnicidae), and coucals (Cuculidae); Vernon 1971, Andersson 1994, DeLay et al. 1996, Ligon 1999).

Plumage color is one way by which females may be ornamented, and the most abundant pigment in integument is melanin, which predominantly produces black, brown, and gray colors (Jawor and Breitwisch 2003, McGraw 2006). As melanin pigments also function in cryptic coloration (reviewed in Jawor and Breitwisch 2003), this can complicate understanding melanized ornaments. However, there is evidence in females that plumage melanization can positively correlate with greater antibody responses (Tawny Owls, *Strix aluco*; Gasparini et al. 2009), body condition (Northern Flickers, *Colaptes auratus*; Wiebe and Vitousek 2015), immunocompetence, and offspring ectoparasite resistance (Barn Owls, *Tyto albus*; Roulin et al. 2000, 2001).

In this study, we analyzed the spotted plumage of breeding Spotted Sandpipers (*Actitis macularius*). The Spotted Sandpiper is an ideal species in which to study female ornamentation as females are territorial, polyan-

drous, and exhibit sex-role reversal (Oring and Lank 1986, Fivizzani and Oring 1986). It is the most widespread sandpiper species breeding in North America and is a habitat generalist, living along lakes, streams, and wetlands (Oring et al. 1983). Males and females are the same size with respect to wing chord, tail, and tarsus length (Pyle 2008), but females are 20–25% heavier than males (Maxson and Oring 1980). Both sexes are philopatric, returning to the same breeding site every season from their wintering grounds (Oring et al. 1983), which stretch from northern Argentina to the southern United States (Nelson 1939, Hayes 1995).

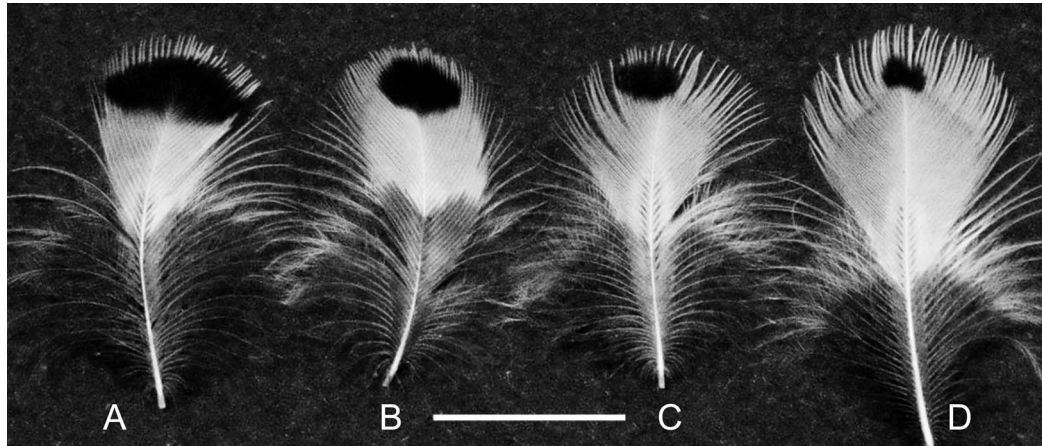
Female Spotted Sandpipers arrive at the breeding ground first and compete for territories and males, once they arrive. Females that are unable to establish and maintain a territory may be excluded from the breeding ground, skewing the operational sex ratio (Maxson and Oring 1980). Both sexes display courtship behavior, but females are more likely to initiate copulation and spend significantly more time in agonistic interactions than males (Maxson and Oring 1980). A clutch of 4 eggs is typically incubated for 21 days by the male, who also cares for the offspring after hatching. After laying eggs in the nest of one male, a female can lay another clutch in the nest of second male in as few as 8 days (Emlen and Oring 1977, Maxson and Oring 1980, Lank et al. 1985). Female reproductive success increases with age and depends on number of clutches laid (i.e. number of mates; Oring et al. 1983, 1991).

When in breeding plumage, the white ventral surface of Spotted Sandpipers is covered in dark brown spots, each one localized to a single feather (Figure 1). These spots vary in number and size across individuals, and females have been reported as “spottier” than males (Mousley 1937, Hays 1972, Oring and Knudson 1972, Reed et al. 2013; see Dwight 1900 for observations of sexual dimorphism when the sexes were misidentified), but this sexual dimorphism has yet to be quantified. In aggressive and courtship interactions, the birds present to the conspecific their throat, chest, and abdomen, the precise areas where the feathers are spotted (Maxson and Oring 1980). Notably, the spotted pattern is entirely absent in juvenile and nonbreeding plumage, suggesting the spots have a role in social interactions specifically during the breeding season. After quantifying the spotted plumage pattern, we considered spottiness relative to phenotypic metrics to explore the possible signaling roles of this plumage pattern and differences between the sexes.

## METHODS

### Study Site

We worked along 20 km of shoreline on the southern and southeastern coast of Beaver Island, Michigan (45.592°N,



**FIGURE 1.** Examples of spotted breast feathers from female (A, B) and male (C, D) Spotted Sandpipers. Scale bar represents 1 cm.

85.518°W). This 144.5 km<sup>2</sup> island is one of several islands in the Beaver Island Archipelago and is the largest island in Lake Michigan. Spotted Sandpipers breed along the shoreline in areas where there is a mixture of rocky coasts, sand dunes, wetlands, and semi-open shrubby vegetation. Much of the land is privately owned, but human presence and activity is seasonal, peaking during the months of July and August. Spotted Sandpipers are the most abundant shorebird nesting at the field site, and 2 other shorebird species, Killdeer (*Charadrius vociferus*) and Wilson's Snipe (*Gallinago delicata*), also nest in the area at lower densities. An estimated 90 adult Spotted Sandpipers breed along the coast of Beaver Island each season, and our study site encompassed the territories of ~45 adults (17 females, 28 males, and 25–35 nests). Nesting density varied along the shoreline from 20 m to 1,875 m between nests.

### Fieldwork

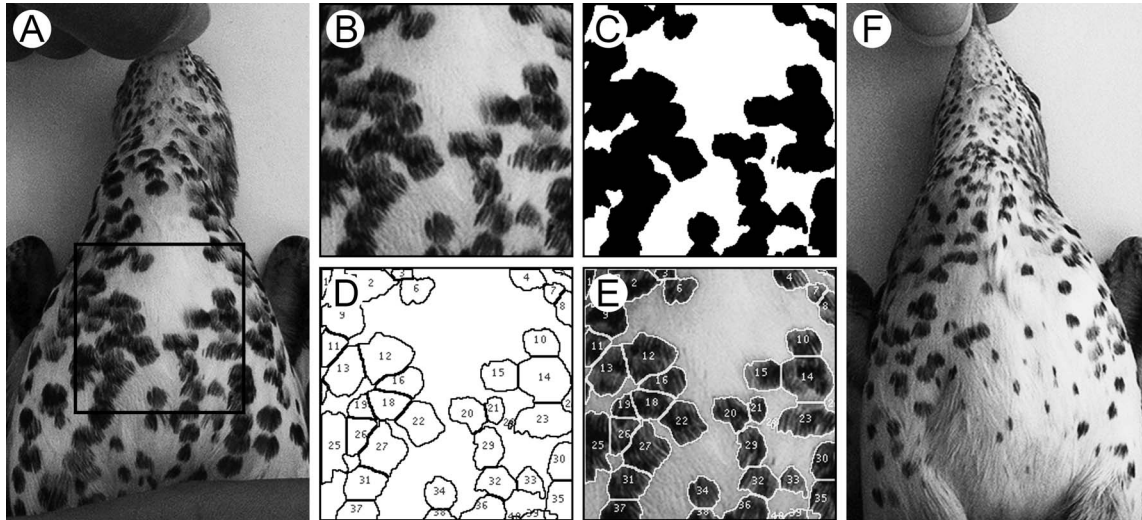
Fieldwork was conducted from early May through late July for 3 consecutive seasons from 2013 to 2015. We captured adult sandpipers during territory establishment and courtship with mist nets and playback calls. Once adults began incubating eggs, birds were caught with a nest trap at least 5 days after nest discovery. In total, we captured 87 adults (32 females, 55 males), 16 of which were recaptured across more than one breeding season (3 females, 13 males). For individual identification in the field, we banded each bird with a permanent metal band and a unique combination of 3 colored plastic bands. We recorded the following phenotypic measures: mass, tarsus length, and feather mite load on the primary and secondary wing feathers using a standard scale of 0 to 4 (Thompson et al. 1997) and took a blood sample from the brachial vein (Owen 2011) to determine sex and hematocrit level. We also recorded probable sex of each adult based on behavioral observations. Genetic analysis (see below) of 11 females and 42 males confirmed our observations. For

analysis of the plumage pattern on the chest and abdomen, each bird was held with the wings gently restrained against the body and the ventral surface was photographed from ~135 cm above the bird (Figure 2A), using the same camera each field season (Nikon D70 with an AF-S Nikkor 55–200 mm telephoto lens; Nikon, Tokyo, Japan). A scale bar, present in every photo, was used to normalize any differences in distance. Between photographs, each bird was adjusted in the hand to smooth the feathers.

### Labwork and Pattern Analysis

To calculate hematocrit level, blood samples were centrifuged in heparanized microhematocrit capillary tubes and then the length (i.e. volume) of the red blood cells relative to the total amount of blood was measured to the nearest 0.01 mm using calipers. Sex of individuals was verified by PCR amplification of the CHD gene using the P2 and P8 microsatellite primers (Griffiths et al. 1998) after DNA extraction, which followed standard techniques (DNAeasy; Qiagen, Venlo, Netherlands).

We used the following method to quantify spotted plumage pattern so that individual birds could be objectively compared to one another, under the reasonable assumption that our methods correlate with the birds' visual perception. The spotted patterns, photographed in the field, were saved in the raw file format on the camera to maximize information captured and preserve image quality and then converted to uncompressed TIFF files on a computer. We selected one photo of each sandpiper each year based on image clarity, position of the bird, and visibility of the scale bar. We cropped the images first (Photoshop CS6; Adobe Systems, San Jose, California, USA) to remove any identifying marks (i.e. leg bands) and then renamed the files using random numbers to prevent observer bias. Using ImageJ (Rasband 2015), we scaled and rotated the images so that the bird's left and right wrists (i.e. the forearm joint consisting of the ulna, radius, ulnare, and carpometacarpus



**FIGURE 2.** Our method of analyzing the ventral plumage pattern of Spotted Sandpipers using ImageJ (Rasband 2015). (A, F) Photographs of each adult were taken in the field, scaled, and cropped to  $2.7 \text{ cm} \times 2.7 \text{ cm}$ , represented in (A) by a black outline and also (B). These images were converted into (C) binary files, to which we applied (D) the watershed algorithm to separate overlapping spots. (E) The outline drawings were overlaid onto the original photograph to check for accuracy, and then analyzed using particle analysis. (A) is a female Spotted Sandpiper and (F) is a male.

bones) were aligned horizontally. The images were cropped to a final size of  $2.7 \times 2.7 \text{ cm}$  to maximize the area of plumage analyzed relative to individual size (Figure 2A and 2B). The cropped square was centered on the chest and only encompassed ventral plumage, with the top edge level with the wrists. We transformed all images to grayscale and applied a Gaussian blur to smooth the edges and reduce noise. We then adjusted the threshold to convert the image into an 8-bit binary image necessary for particle measurement on ImageJ (Figure 2C; Schneider et al. 2012). Lastly, we used the watershed algorithm on ImageJ to break apart overlapping spots (Figure 2D). Although this method is not guaranteed to separate all spots whose perimeters intersect, it is a precise mathematical method that first calculates the Euclidean distance map, determines ultimate eroded points, and then expands the points outward (Leymarie and Levine 1992, Schneider et al. 2012). All outline drawings produced by ImageJ were overlaid onto the original  $2.7 \times 2.7 \text{ cm}$  photograph to check for accuracy (Figure 2E).

Lastly, we quantified spottiness of the plumage pattern outline drawings on ImageJ by using particle analysis to determine spot count, size, orientation, shape, and percent cover of spotted plumage. Spot size was measured in terms of area, perimeter, and major and minor axes (i.e. the axes of the fit ellipse, which has the same area, centroid, and orientation as the spot). The major and minor axes were used in lieu of the spots' absolute maximum and minimum diameters, as the former are more robust measures, not influenced by irregularities in the spot's perimeter. However, these variables were strongly correlated with each other: the average major and minor axes correlated,

respectively, to the average spot's true maximum (females:  $r = 0.99$ ,  $df = 30$ ,  $P < 0.001$ ; males:  $r = 0.99$ ,  $df = 53$ ,  $P < 0.001$ ) and minimum diameters (females:  $r = 0.99$ ,  $P < 0.001$ ; males:  $r = 0.99$ ,  $P < 0.001$ ). Spot shape was measured in terms of aspect ratio, solidity, roundness, and circularity. As calculated by ImageJ, aspect ratio is a measure of elongation (major axis/minor axis), solidity is a measure of compactness (area/convex area), roundness is a measure of similarity to a circle based on major axis ( $(4 \times \text{area})/(\pi \times \text{major axis}^2)$ ), and circularity is also a measure of similarity to a circle, but based on perimeter ( $(4 \times \text{area})/\text{perimeter}^2$ ; Rasband 2015). To ascertain the accuracy of the watershed algorithm, one of us (M.A.B.) counted the number of spots in the  $2.7 \text{ cm} \times 2.7 \text{ cm}$  square on the chests of 27 females and 27 males. As the count data were not normal, we used Spearman's rank correlation to verify that spot count by ImageJ significantly correlated with spot count by human eye ( $S = 6,018.3$ ,  $r_s = 0.77$ ,  $P < 0.001$ ). ImageJ tended to underestimate the number of spots (slope of linear regression = 0.79 with count by ImageJ as a function of count by human eye). We also compared our method of quantifying spotted pattern from the binary images with granularity analysis (Stoddard and Stevens 2010), which we ran in MATLAB 7.14 (MathWorks 2012).

### Statistics

We ran all statistical tests in R 3.3.1 (R Development Core Team 2015). We used a two-way ANOVA to determine differences between the sexes as a fixed effect and across the years as a random effect, with all replicate individuals removed from the data set. We used Mann–

**TABLE 1.** Average body metrics and spottiness variables of Spotted Sandpipers, not including replicates captured across years. Body metrics were measured in the field over 3 years; spottiness variables were calculated using particle analysis on ImageJ (Rasband 2015).

		Females ( <i>n</i> = 32)		Males ( <i>n</i> = 55)	
		$\bar{x}$	SE	$\bar{x}$	SE
Body metrics	Mass (g)	50.28	1.31	39.46	0.38
	Tarsus (mm)	28.12	0.19	27.96	0.13
	Mites (scale)	1.11	0.14	1.19	0.11
	Hematocrit (%)	47.71	0.85	48.63	0.48
Spot metrics	Area (cm <sup>2</sup> )	0.07	0.004	0.04	0.002
	Perimeter (cm)	1.05	0.03	0.75	0.02
	Major axis (cm)	0.34	0.01	0.25	0.005
	Minor axis (cm)	0.23	0.008	0.16	0.004
	Percent cover	38.42	1.88	22.90	1.01
	Spot count	42.56	1.56	48.49	1.80
	Circularity	0.72	0.008	0.74	0.008
	Aspect ratio	1.60	0.03	1.70	0.03
	Roundness	0.67	0.008	0.64	0.007
	Solidity	0.89	0.004	0.89	0.003
Angle (°)	93.15	1.82	87.49	1.33	

Whitney–Wilcoxon tests to verify the results of our particle analysis methods with the results of the granularity analysis. To further examine the differences between the sexes, we used linear discriminant analyses to determine whether individuals could be correctly assigned to their sex and/or year class based on their spottiness metrics. To quantify spottiness and reduce the number of intercorrelated variables (Appendix Tables 5 and 6), we conducted principal component analyses including all plumage metrics on both sexes combined and independently.

We used forward and backward stepwise multiple regression models ('stats' R package) to determine whether the following were explanatory variables for the observed variation in spottiness: log of mass, tarsus length, hematocrit levels, mite score, and day of the season (see Appendix Table 7 for explanatory variable correlations). Day of the season was normalized by counting up from "day 1," the first day a Spotted Sandpiper was observed on the breeding grounds each year. All variables were centered to 0 in the model using the scale function. We used AIC<sub>c</sub> to assess alternative models ('AICcmodavg' R package; Cavanaugh 1997) and ran the regression models on each sex independently. To evaluate how an individual changes among years captured, we used linear mixed-effects models ('nlme' R package) to test whether the variation in PC scores across recaptured individuals could be explained by sex, capture year (i.e. first, second, or third as a categorical variable), or the 2 variables' interaction. Bird identity was included as a random variable (3 females, 13 males), and to account for changes in the sex ratio of

**TABLE 2.** Analysis of variance of spottiness metrics of Spotted Sandpipers (*n* = 87) between the sexes (fixed effect) and across years (random effect), including interactions. With Bonferroni corrections applied to the table, \**P* < 0.05 and \*\*\**P* < 0.001.

	Sex		Sex:Year		
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	
Area	87.19	<0.001	***	0.006	0.94
Perimeter	91.51	<0.001	***	0.07	0.80
Major axis	85.77	<0.001	***	0.002	0.97
Minor axis	74.92	<0.001	***	0.04	0.84
Percent cover	60.14	<0.001	***	0.07	0.79
Spot count	7.01	0.01		0.23	0.64
Circularity	0.34	0.56		0.25	0.62
Aspect ratio	8.49	0.005		0.75	0.39
Roundness	8.74	0.004	*	0.64	0.42
Solidity	3.36	0.07		0.08	0.78
Angle	5.71	0.02		0.18	0.67

captured adults year to year, we used the PC scores calculated separately by sex.

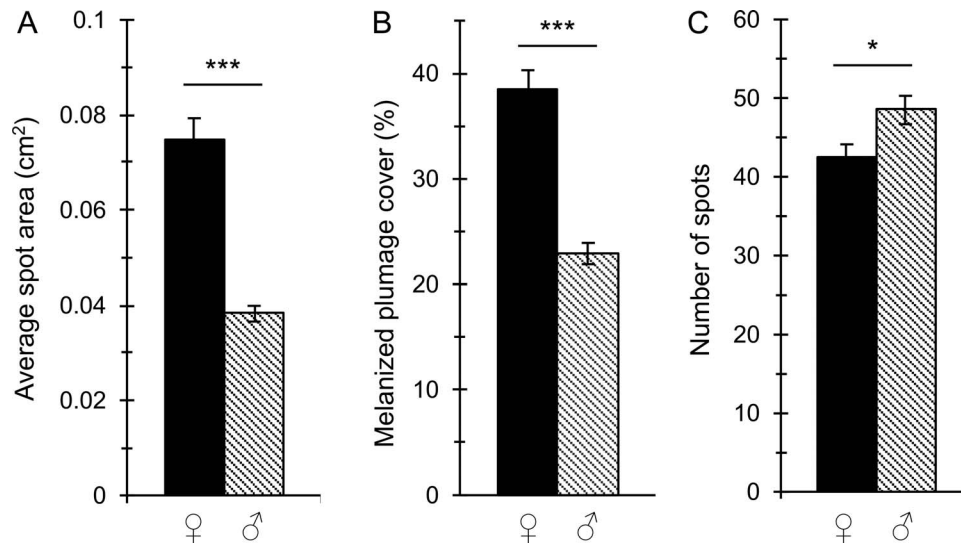
## RESULTS

### Quantifying Spottiness

Females had a greater percentage of plumage covered by spots, but had fewer total spots than males (Tables 1 and 2, Figure 3). Thus, female spots were larger than those of males, as measured by average spot area and perimeter, as well as the major and minor axes. Females had more elongated spots (i.e. larger aspect ratios) that were less round and tended to be less solid than those of males. However, circularity of spots did not differ significantly between the sexes. The interaction between sex and year was not significant for any of the metrics (Table 2). Our particle analysis metrics were correlated to the granularity analysis results (spot area vs. maximum energy peak in the spectrum:  $U = 2173$ ,  $P < 0.001$ ; spot cover vs. total energy in the spectrum:  $U = 2258$ ,  $P < 0.001$ ).

Based on the 11 spottiness metrics, 82% of adult Spotted Sandpipers (69% of females and 89% of males) were correctly assigned to their true sex by linear discriminant analyses (Wilks'  $\lambda = 0.41$ ,  $P < 0.001$ ). Focusing on year, but with the sexes separated, 66% of females were correctly matched to their year of capture (2013: 89%; 2014: 50%; 2015: 62%; Wilks'  $\lambda = 0.29$ ,  $P < 0.002$ ) and 89% of males were correctly matched to their year of capture (2013: 96%; 2014: 73%; 2015: 92%; Wilks'  $\lambda = 0.32$ ,  $P < 0.001$ ). Furthermore, with the sexes combined, the linear discriminant analysis properly matched 68% of individuals to their combined sex and year class (2013: females = 56%, males = 93%; 2014: females = 20%, males = 73%; 2015: females = 38%, males = 85%; Wilks'  $\lambda = 0.03$ ,  $P < 0.001$ ).

For females and males combined, variables associated primarily with spot size (area, perimeter, major axis, and



**FIGURE 3.** Sex differences in (A) spot area, (B) percentage of melanized plumage, and (C) number of spots (error bar: mean  $\pm$  SE). Females: black, males: hatched (ANOVA: \*  $P < 0.05$ , \*\*\*  $P < 0.001$ ).

minor axis), and also shape (aspect ratio and roundness) and percent cover loaded most heavily on PC 1 (Table 3). PC 2 was most heavily loaded by spot shape metrics (circularity and solidity) and percent cover. Individuals with larger, rounder, and less elongated spots had greater percent cover and lower PC 1 scores; individuals with more irregularly shaped spots based on circularity and solidity and greater percent cover had higher PC 2 scores (Figure 4). PC 3 was most heavily weighted by angle, but neither sex clustered within the range of scores.

### Explanatory Variables of Spottiness

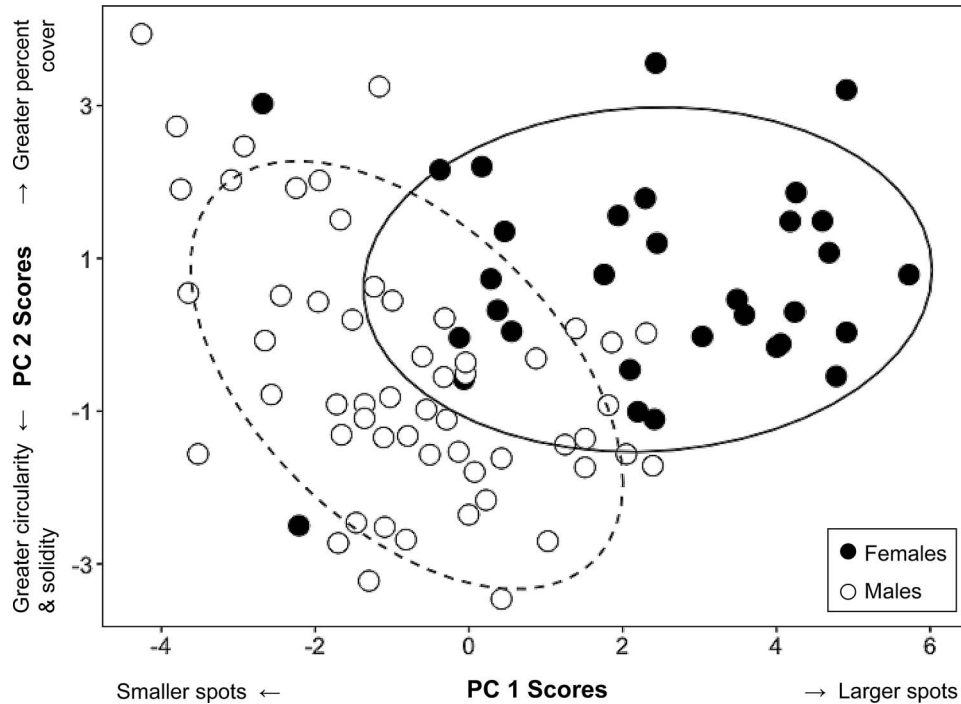
When the sexes were analyzed separately, the first and second PC scores loaded the 11 variables similarly for males and females (Pearson correlation: PC 1:  $r = 0.99$ ,  $df =$

9,  $P < 0.001$ ; PC 2:  $r = 0.98$ ,  $df = 9$ ,  $P < 0.001$ ; PC 3:  $r = 0.04$ ,  $df = 9$ ,  $P = 0.92$ ; Appendix Table 8). Analyzing female PC 1, the best linear regression model included mites as an explanatory variable (Appendix Table 9). Females with smaller mite loads had larger spots (Figure 5A). For PC 2, the best model for females included mass, hematocrit levels, and day of the season captured (Figure 5C, 5D, 5E and Appendix Table 9). Heavier females with higher hematocrit levels (i.e. more red blood cells relative to total blood volume) had more irregular spots in terms of circularity and solidity, greater percent cover, and tended to be caught later in the breeding season.

For males, the best model explaining variation in PC 1 included mites (Appendix Table 9). As with females, males with lower mite loads had larger spots (Figure 5B). For PC 2,

**TABLE 3.** Variable loadings of the first 3 principal components (PC) calculated from spottiness metrics of Spotted Sandpipers, with the sexes combined. Text in bold indicates the most heavily loaded metrics for each corresponding PC ( $r_s < -0.55$  or  $r_s > 0.55$ ).

	PC 1		PC 2		PC 3	
	Loading	$r_s$	Loading	$r_s$	Loading	$r_s$
Minor axis	<b>0.410</b>	<b>0.98</b>	0.091	0.10	-0.074	-0.07
Area	<b>0.392</b>	<b>0.94</b>	0.174	0.21	-0.083	-0.09
Major axis	<b>0.386</b>	<b>0.91</b>	0.174	0.26	-0.140	-0.11
Perimeter	<b>0.380</b>	<b>0.88</b>	0.232	0.36	-0.102	-0.09
Aspect ratio	- <b>0.312</b>	- <b>0.77</b>	0.313	0.46	-0.124	-0.08
Roundness	<b>0.312</b>	<b>0.75</b>	-0.268	-0.42	0.146	0.12
Percent cover	<b>0.311</b>	<b>0.68</b>	<b>0.342</b>	<b>0.57</b>	-0.046	-0.05
Spot count	-0.196	-0.45	0.313	0.47	0.073	0.12
Solidity	0.150	0.36	- <b>0.450</b>	- <b>0.72</b>	-0.115	-0.12
Circularity	0.133	0.30	- <b>0.528</b>	- <b>0.86</b>	0.029	-0.05
Angle	0.119	0.25	0.109	0.19	<b>0.949</b>	<b>0.91</b>
Variance explained	52%		25%		8%	



**FIGURE 4.** The first and second principal components of spottiness calculated by combining the 11 ImageJ spottiness metrics. The first principal component accounts for 52% of the variance among individuals and the second, 25% of variance. Each female is represented by a black circle and each male by an open circle. Ellipses are drawn around 75% confidence intervals for each sex; females: solid line, males: dashed line.

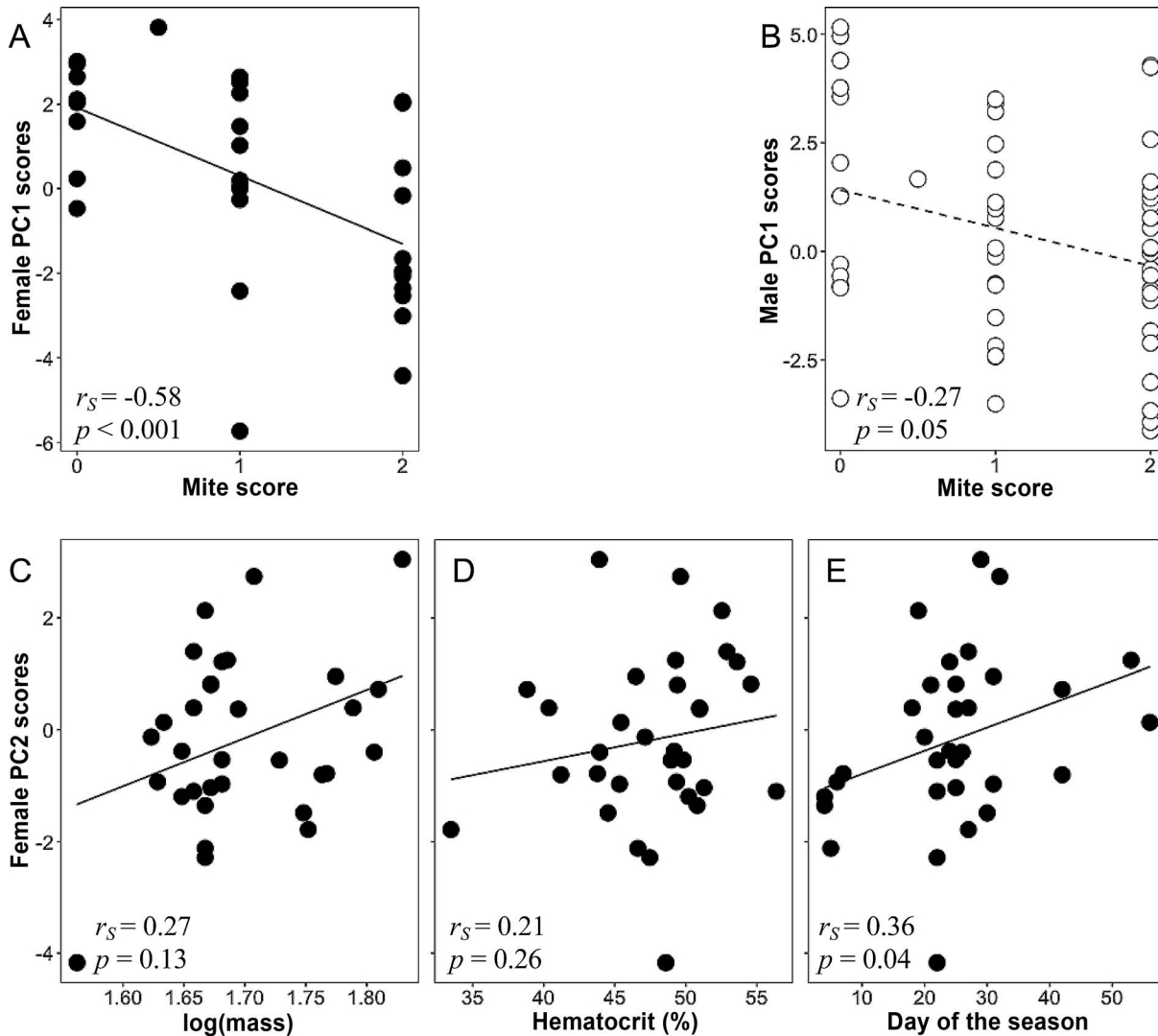
the model including tarsus and day of the season had the lowest AIC<sub>c</sub> score, but day of season had a weak effect within the model. Additionally, ΔAIC<sub>c</sub> is <2 when comparing the null model to the model including tarsus and day of the season or the model including tarsus alone, indicating that the null model also has substantial support in describing the data (Burnham and Anderson 2002). Thus, although males caught earlier in the breeding season and with longer tarsi tended to have greater percent cover and less circular spots, the null model was the most parsimonious model.

In the analysis of individual variation among years, variation in spottiness of recaptured birds was explained by capture year (Table 4). Sex and the interaction between sex and capture year did not significantly explain variation within the models. With the first capture year as the reference, PC 1 significantly decreased in the second capture year and there was a nonsignificant decrease in the third capture year; PC 2 tended to increase in the second capture year and increased significantly in the third capture year (Table 4). In other

**TABLE 4.** Results of linear mixed-effects models explaining variation across the first and second principal components of plumage spottiness of adults (*n* = 16) captured across multiple field seasons. Sex, capture year, and their interaction were included as explanatory variables and identity was included as a random factor (\* *P* < 0.05).

		Estimate	SE	df	<i>t</i>	<i>P</i>	
PC 1:	(Intercept)	1.23	1.31	17	0.93	0.36	
	Sex male	-0.56	1.46	14	-0.38	0.71	
	Capture year second	-5.69	2.63	17	-2.17	0.04	*
	Capture year third	-1.06	2.08	17	-0.51	0.62	
	Sex male:capture year second	4.07	2.79	17	1.46	0.16	
	Sex male:capture year third	-1.04	2.33	17	-0.45	0.66	
PC 2:	(Intercept)	-0.70	0.88	17	-0.79	0.44	
	Sex male	0.21	0.98	14	0.22	0.83	
	Capture year second	2.73	1.41	17	1.94	0.07	
	Capture year third	2.52	1.05	17	2.41	0.03	*
	Sex male:capture year second	-2.49	1.48	17	-1.68	0.11	
	Sex male:capture year third	-2.00	1.18	17	-1.70	0.11	





**FIGURE 5.** Relationships among variables that remained significant in explaining spottiness after stepwise multiple regression. Data from (A, C–E) females ( $n = 32$ ) and (B) males ( $n = 55$ ) were analyzed separately. Lines indicate linear associations between the variables, and as the data were not normal, we used Spearman rank correlations.

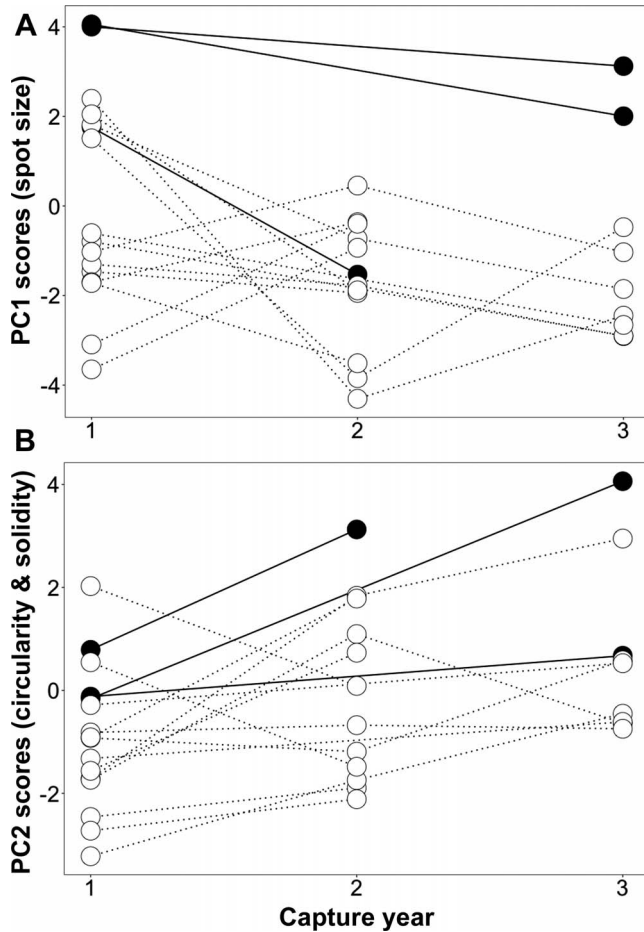
words, spot size decreased from the first to the second capture. Spot circularity and solidity decreased and percent cover increased from the first to the third capture, with the same trend from the first to the second capture (Figure 6).

## DISCUSSION

The spots of female Spotted Sandpipers were larger, covered a greater percentage of plumage, but were less numerous and less regularly shaped than the spots of males. The sexes overlapped in the spottiness metrics, but the dimorphism was great enough that 82% of individuals could be correctly assigned to their sex by linear discriminant analyses. Analysis of spotted pattern by image granularity (Stoddard and Stevens 2010) was

equivalent to the corresponding metrics in our method of pattern quantification.

Mixed factors explained male and female patterns of spottiness. Considering both sexes, larger spots correlated with fewer mites, and this relationship was stronger in females than in males. Since mite load was defined by mites located on the wing feathers, these mites may not directly cause damage to neck or chest plumage, but mites on the wings may correlate with the bird's total ectoparasite load and, more generally, overall body condition. As Spotted Sandpipers molt their breast feathers twice per year and their wing feathers only once, after the breeding season (Pyle 2008), feather mites have accumulated for many months before the next breeding season (Haribal et al. 2011). Consequently, the mites on the wing may affect not only the current health of the



**FIGURE 6.** Individual changes in the (A) first and (B) second principal components for adult Spotted Sandpipers ( $n = 16$ ) captured across multiple years with the sexes analyzed together. Lines connect PC scores of the same individual from year to year (females: black circles and solid lines; males: open circles and dashed lines).

sandpiper, but also the development of the spotted plumage before the next breeding season starts. Although our limited sample size does not allow us to test this prediction, melanized plumage ornamentation may thus be indicators of an individual's condition the prior year (e.g., Johnsen et al. 1996).

Melanized feathers are more resistant to abrasion (Barrowclough and Sibley 1980, Burt 1986, Bonser 1995), and while the spots do not cover the entirety of a feather, the largest of the spots approach the feather's edges (Figure 1A). Melanized feathers are also more resistant to bacterial damage (e.g., due to *Bacillus licheniformis*; Goldstein et al. 2004, Gunderson et al. 2008), and ectoparasite damage (e.g., Møller 1994). In Great Tits (*Parus major*), for instance, flea infestations one year resulted in decreased melanin-based ornament size the following year in both sexes (Fitze and Richner 2002). In Penduline Tits (*Remiz pendulinus*), nest mites were

negatively associated with the size of males' black facial ornaments (Darolová et al. 1997). Therefore, it is possible that the larger spots of Spotted Sandpipers are less likely to be damaged by ectoparasites than smaller spots (but see Bush et al. 2006 regarding feather lice).

In females, spot shape and percent cover were best explained by mass, hematocrit, and day of the season captured, with greater percent cover and shape irregularity correlating with greater mass, higher hematocrit levels, and capture later in the breeding season. In birds, low hematocrit levels indicate anemia, potentially due to bacterial infections, internal parasites (Dein 1986), or mineral deficiencies (Sturkie and Griminger 1986). It is likely that the variable date captured indirectly relates to spot shape and percent cover via weight as Spotted Sandpipers are a migratory species and arrive at the breeding ground with lower body mass (Reed et al. 2013). Pattern regularity, not irregularity, is expected to positively correlate with condition (e.g., Gluckman and Cardoso 2009). However, our measures of spot shape are dependent on the watershed technique, especially for individuals with great spot overlap, and may not reflect the shape of individual spots if analyzed on each feather separately.

Our data indicate that healthier females, as defined by fewer feather mites, greater mass, and higher hematocrit levels, were more spotted than less-healthy females. In males, however, no explanatory variable was significant from the null model in regard to spot shape and percent cover. The question remains why both sexes exhibit a conspicuous spotted pattern in this polyandrous species, especially as the relationships between ornamentation and individual phenotypic metrics differ between the sexes. Although sexual selection models allow for an exaggeration as well as a reduction in traits with respect to mate preference, there is often an inherent bias towards trait exaggeration (Tazzyman et al. 2014). Furthermore, as male Spotted Sandpipers are responsible for almost all incubation and parental care (Maxson and Oring 1980), natural selection is likely selecting for less conspicuous males (see Martin and Badyaev 1996, Götmark et al. 1997 for natural selection selecting for crypsis of the parenting sex). Males' spots may be a genetic by-product of selection on females, or females may be choosing males signaling quality of parental care. Additionally, the spotted patterns could potentially be used for individual identity within a breeding season, such as the facial markings of Ruddy Turnstones (*Arenaria interpres*), another shorebird species (Whitfield 1986).

From the data on recaptured birds, changes in an individual's spot pattern among years related to capture year. These results suggest that pattern varies within an individual's life: spot size tended to decrease and spot shape became more irregular with age (Figure 6). Additionally, as this analysis was limited in sample size, especially for

females, we were unable to draw strong conclusions about female consistency among years. That being said, the population as a whole noticeably changed in spottiness among years based on the 11 spottiness metrics alone. Linear discriminant analyses correctly assigned a significant proportion of individuals to the correct capture year, as well as the correct year and sex class simultaneously.

Factors such as wintering ground conditions could influence the development of the spot-bearing breeding plumage, resulting in the changes observed at the breeding ground across years at both the population and the individual level. For instance, in migratory species (e.g., American Redstarts, *Setophaga ruticilla*), temperature and humidity during the winter affect feather chroma and brightness of breeding plumage (Reudink et al. 2015) and regrown feathers lost during the winter have lower chroma (Tonra et al. 2014). Molting into breeding plumage farther from the wintering grounds can result in lighter melanin-based plumage (Barn Swallows, *Hirundo rustica*; Norris et al. 2009). Also, the speed of feather growth, a potential proxy for body condition, can cause varying effects on the size of plumage ornaments (e.g., Barn Swallows and House Sparrows, *Passer domesticus*; Vágási et al. 2012, Saino et al. 2015)

It is likely that the spotted plumage pattern in Spotted Sandpipers is under sexual selection given the sexual dimorphism in spot size, percent cover, and spot shape. Since the spotted pattern is absent in the sister species (the socially monogamous Common Sandpiper, *A. hypoleucos*) and in all related species in the Scolopaci suborder (see Gibson and Baker 2012 for phylogeny), it is likely that the plumage pattern is relatively recent in the evolutionary history of sandpipers. Furthermore, evolutionary increases in melanization across Charadriiformes (i.e. shorebirds) strongly correlate with increases in testosterone and sexual dimorphism of plumage, although species exhibiting sex-role reversal were removed and female testosterone levels were not included (Bókonyi et al. 2008).

As more studies address mutual mate choice and male mate choice, the importance of female ornamentation is being recognized (Amundsen 2000, LeBas 2006, Kraaijeveld et al. 2007, Clutton-Brock 2009, Rubenstein and Lovette 2009, Edward and Chapman 2011, Muck and Goymann 2011, Wiebe and Vitousek 2015, Argüelles-Ticó et al. 2016). In systems with sex-role reversal, female competition for mates outweighs that of males, and one would expect a corresponding reversal in the more ornamented sex. By investigating a morphological trait that is evident in both sexes and varies across individuals, we can determine what factors may be affecting the expression between and within the sexes. In the case of Spotted Sandpipers, their spotted pattern is likely a recently evolved trait that is more strongly expressed in females in terms of percent cover and spot size. Beyond the sexual dimorphism in plumage pattern, female

spottiness positively correlates with several metrics associated with health and male spottiness positively correlates with a subset of those health metrics. Determining the predictive variables for expression not only indicates its role as a potential signal to conspecifics, whether male or female, but may also reveal the different selective pressures each sex experiences.

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## APPENDIX

**APPENDIX TABLE 5.** Pairwise Spearman rank correlations of the plumage spottiness metrics with the sexes combined. Correlations in bold indicate  $P < 0.05$  with Bonferroni corrections applied.

	Area	Perimeter	Major axis	Minor axis	Percent cover	Spot count	Circularity	Aspect ratio	Roundness	Solidity
Perimeter	<b>0.98</b>									
Major axis	<b>0.98</b>	<b>0.98</b>								
Minor axis	<b>0.98</b>	<b>0.95</b>	<b>0.96</b>							
Percent cover	<b>0.80</b>	<b>0.85</b>	<b>0.78</b>	<b>0.76</b>						
Spot count	−0.40	−0.31	−0.41	−0.43	0.12					
Circularity	0.03	−0.13	0.00	0.11	−0.30	−0.45				
Aspect ratio	−0.58	−0.50	−0.53	−0.68	−0.33	<b>0.40</b>	−0.57			
Roundness	<b>0.56</b>	<b>0.49</b>	<b>0.50</b>	<b>0.67</b>	0.35	−0.35	<b>0.50</b>	−0.97		
Solidity	0.15	0.04	0.13	0.23	−0.09	−0.39	<b>0.70</b>	−0.54	<b>0.50</b>	
Angle	0.26	0.26	0.24	0.25	0.26	0.04	−0.14	−0.14	0.17	−0.05

**APPENDIX TABLE 6.** Pairwise Spearman rank correlations of the plumage spottiness metrics, analyzed with the sexes separated. Correlations in bold indicate  $P < 0.05$  with Bonferroni corrections applied.

	Area	Perimeter	Major axis	Minor axis	Percent cover	Spot count	Circularity	Aspect ratio	Roundness	Solidity
<b>Females</b>										
Perimeter	<b>0.97</b>									
Major axis	<b>0.96</b>	<b>0.96</b>								
Minor axis	<b>0.97</b>	<b>0.95</b>	<b>0.96</b>							
Percent cover	<b>0.75</b>	<b>0.73</b>	<b>0.67</b>	<b>0.72</b>						
Spot count	−0.35	−0.33	−0.40	−0.32	0.22					
Circularity	0.16	0.01	0.16	0.22	−0.04	−0.24				
Aspect ratio	−0.54	−0.49	−0.49	−0.66	−0.31	0.16	−0.58			
Roundness	0.56	0.51	0.49	<b>0.68</b>	0.35	−0.15	<b>0.53</b>	−0.97		
Solidity	0.21	0.10	0.22	0.26	−0.02	−0.34	<b>0.86</b>	−0.51	0.45	
Angle	0.24	0.23	0.12	0.21	0.32	0.05	−0.02	−0.16	0.25	−0.02
<b>Males</b>										
Perimeter	<b>0.95</b>									
Major axis	<b>0.97</b>	<b>0.96</b>								
Minor axis	<b>0.96</b>	<b>0.88</b>	<b>0.91</b>							
Percent cover	<b>0.59</b>	<b>0.69</b>	<b>0.57</b>	<b>0.49</b>						
Spot count	−0.33	−0.17	−0.34	−0.41	<b>0.47</b>					
Circularity	0.28	0.04	0.22	0.41	−0.27	−0.65				
Aspect ratio	−0.57	−0.41	−0.47	−0.72	−0.15	<b>0.44</b>	−0.73			
Roundness	<b>0.54</b>	0.40	0.42	<b>0.71</b>	0.18	−0.37	<b>0.65</b>	−0.97		
Solidity	0.26	0.10	0.21	0.39	−0.12	−0.42	<b>0.68</b>	−0.61	<b>0.57</b>	
Angle	0.09	0.08	0.10	0.07	0.11	0.12	−0.14	−0.01	0.02	−0.05

**APPENDIX TABLE 7.** Spearman rank correlation matrix of predictor variables used in the linear regression models. Correlations in bold indicate  $P < 0.05$  with Bonferroni corrections applied.

	log(Mass)	Tarsus	Mites	Hematocrit
Tarsus	0.19			
Mites	−0.06	0.27		
Hematocrit	−0.12	−0.14	−0.13	
Day of the season	−0.40	0.24	<b>0.33</b>	−0.28

**APPENDIX TABLE 8.** Variable loadings of the first 3 principal components (PC) calculated from spottiness metrics of each sex separately. Text in bold indicates the most heavily loaded metrics for each corresponding PC ( $r_s < -0.55$  or  $r_s > 0.55$ ).

	PC 1		PC 2		PC 3	
	Loading	$r_s$	Loading	$r_s$	Loading	$r_s$
<b>Females</b>						
Minor axis	<b>0.41</b>	<b>0.97</b>	0.11	0.15	-0.07	-0.07
Area	<b>0.38</b>	<b>0.91</b>	0.21	0.28	-0.10	-0.10
Major axis	<b>0.37</b>	<b>0.89</b>	0.20	0.27	-0.22	-0.24
Perimeter	<b>0.37</b>	<b>0.87</b>	0.27	0.38	-0.15	-0.14
Aspect ratio	- <b>0.33</b>	- <b>0.77</b>	0.29	0.48	-0.24	-0.19
Roundness	<b>0.32</b>	<b>0.76</b>	-0.25	-0.41	0.31	0.27
Percent cover	<b>0.26</b>	<b>0.62</b>	<b>0.39</b>	<b>0.55</b>	0.17	0.24
Spot count	-0.22	-0.39	0.27	0.31	0.42	0.47
Solidity	0.18	0.42	- <b>0.47</b>	- <b>0.75</b>	-0.04	-0.10
Circularity	0.22	0.43	- <b>0.47</b>	- <b>0.80</b>	0.05	0.05
Angle	0.10	0.26	0.12	0.23	<b>0.74</b>	<b>0.75</b>
Variance explained	52%		24%		10%	
<b>Males</b>						
Minor axis	<b>0.41</b>	<b>0.98</b>	-0.11	0.16	0.03	0.02
Area	<b>0.39</b>	<b>0.92</b>	-0.20	0.31	0.11	0.10
Major axis	<b>0.37</b>	<b>0.85</b>	-0.22	0.36	0.21	0.19
Perimeter	<b>0.35</b>	<b>0.80</b>	-0.32	0.51	0.12	0.12
Aspect ratio	- <b>0.34</b>	- <b>0.81</b>	-0.24	0.31	0.24	0.23
Roundness	<b>0.33</b>	<b>0.77</b>	0.19	-0.28	-0.29	-0.26
Percent cover	0.19	0.41	- <b>0.49</b>	<b>0.80</b>	-0.25	-0.21
Spot count	-0.20	-0.44	- <b>0.37</b>	<b>0.60</b>	-0.46	-0.35
Solidity	0.24	0.54	0.35	-0.53	-0.20	-0.23
Circularity	0.25	0.59	<b>0.44</b>	- <b>0.65</b>	0.01	0.16
Angle	0.02	0.00	0.00	0.03	<b>0.69</b>	<b>0.76</b>
Variance explained	51%		24%		10%	

**APPENDIX TABLE 9.** Results of stepwise linear regression models for predicting the first and second principal components of plumage spottiness. The sexes were analyzed separately, and all variables were scaled in the models. For each PC model, every step is additive to the one immediately above it, indicated by (+) and the subsequent changes in  $\Delta AIC_c$ , but the significance of each explanatory factor is analyzed independently from the others in the same model.  $K$  is the number of parameters in the model. Models listed in bold are those that fit the data best.

		Step	Estimate	SE	$t$	$P$	$K$	$\Delta AIC_c$	
Females ( $n = 32$ )	PC 1:	Full					7	8.12	
		Null	0.13	0.36	0.36	0.76	2	8.76	
		+ <b>Mites</b>	<b>-1.29</b>	<b>0.36</b>	<b>-3.55</b>	<b>0.001</b>	**	<b>3</b>	<b>0<sup>a</sup></b>
	PC 2:	Full					7	5.64	
		Null	-0.18	0.20	-0.87	0.39	2	12.85	
		+ <b>Mass</b>	<b>1.05</b>	<b>0.26</b>	<b>3.95</b>	<b>&lt;0.001</b>	***	3	10.95
+ <b>Hematocrit</b>		<b>1.04</b>	<b>0.27</b>	<b>3.91</b>	<b>&lt;0.001</b>	***	4	3.46	
	+ <b>Day</b>	<b>0.53</b>	<b>0.22</b>	<b>2.47</b>	<b>0.02</b>	*	<b>5</b>	<b>0<sup>b</sup></b>	
Males ( $n = 55$ )	PC 1:	Full					7	8.62	
		Null	0.37	0.31	1.20	0.24	2	2.74	
		+ <b>Mites</b>	<b>-0.70</b>	<b>0.31</b>	<b>-2.24</b>	<b>0.03</b>	*	<b>3</b>	<b>0<sup>c</sup></b>
	PC 2:	Full					7	4.92	
		<b>Null</b>	<b>0.04</b>	<b>0.22</b>	<b>0.17</b>	<b>0.86</b>		<b>2</b>	<b>1.83</b>
		+ Tarsus	0.54	0.23	2.33	0.02	*	3	0.33
	+ Day	-0.37	0.23	-1.60	0.11		4	0 <sup>d</sup>	

Lowest  $AIC_c$  value for models: (a) 140.72, (b) 107.65, (c) 252.50, and (d) 215.09.