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EVALUATION OF METHODS FOR GENDER DETERMINATION OF LESSER KESTREL NESTLINGS

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ABSTRACT.—The traditional method of determining gender of Lesser Kestrel (*Falco naumanni*) nestlings by visual assessment was tested for accuracy by using data from birds banded as nestlings and recaptured as adults. Concordance between gender assignment by different observers, and between visual and molecular gender determination was also evaluated. We tested whether color measurement of rumps and tails could improve gender determination. Based on recaptured kestrels, gender determination by eye had a 9.7% error, and was significantly greater for males than for females. Observers mostly relied on rump and tail color to assign gender to nestlings. Assessment of head, shoulders, tail, and rump patterns did not provide additional information that could improve gender determination in nestlings at the time of banding. Gender assignment based on color measurement on digital photos of rumps and tails did not improve determination by eye, but color measurement from a scanned rump feather approached 100% accuracy. We provide a discriminant function equation based on red, green, and blue brightness values (RGB) of a scanned rump feather and propose this as an efficient and effective method for gender determination in Lesser Kestrel nestlings.

KEY WORDS: *Lesser Kestrel*; Falco naumanni; *digital image analysis*; *gender determination*; *nestlings*; *RGB values.*

EVALUACIÓN DE MÉTODOS PARA LA DETERMINACIÓN DEL SEXO EN POLLOS DE *FALCO NAUMANNI*

RESUMEN.—Evaluamos la forma tradicional de determinar visualmente el sexo de los pollos de *Falco naumanni* mediante las recapturas de individuos adultos anillados, cuyo sexo había sido determinado en la etapa de pollos. Se calculó la concordancia en la determinación del sexo entre diferentes observadores, así como entre la determinación del sexo de modo molecular y visual. Además, se investigó si medidas del color de la cola y la rabadilla determinadas a partir de fotografías digitales o de plumas de la rabadilla escaneadas aumentaban el porcentaje de acierto en la determinación del sexo de los pollos. El porcentaje de error en la determinación visual del sexo fue de 9.7%, y fue significativamente mayor en el caso de los machos. Los observadores se basaron mayormente en el color de la cola y la rabadilla para asignar el sexo a los pollos. Aunque fue dimórfico, el patrón de manchas de la cabeza, los hombros, la cola y la rabadilla no aportó información adicional para mejorar la determinación del sexo de los pollos en el momento del anillado. La determinación del sexo a partir de las medidas de color tomadas de fotos digitales de la cola y la rabadilla ofreció peores resultados que la determinación visual tradicional. Sin embargo, la medida de color de la pluma de la rabadilla escaneada ofreció un porcentaje de acierto en la determinación del sexo cercano al 100%. Se ofrece una función discriminante, basada en los valores de brillo del rojo, verde y azul de las plumas de la rabadilla escaneadas, como un método eficaz para determinar más confiablemente el sexo de los pollos de *Falco naumanni.*

[Traducción del equipo editorial]

The Lesser Kestrel (*Falco naumanni*) is a small colonial falcon that exhibits a dichromatic plumage. Adult males have an unspotted chestnut back, a mostly blue-gray inner wing, and a blue-gray hood. Adult females are brownish with dark bars on the head, back, and tail (Cramp and Simmons 1980). Juvenile plumage of both sexes resembles that of adult females, but shows dichromatism in the rump and tail (blue-grayish in males versus brownish in females; Bijlsma et al. 1988, Negro and Hiraldo 1992, Tella et al. 1996b, Palumbo 1997). Tail and rump color can be determined when feathers start growing, and this happens when chicks are 2 wk old (pers. observ.). These characters have been used traditionally to determine gender in Lesser Kestrel nestlings at the time of band-

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Table 1. Recognized pattern categories and colors of the four characters used to assign gender of Lesser Kestrel nestlings in southwestern Spain.

HEAD PLUMAGE	SHOULDER PLUMAGE	RUMP PLUMAGE	TAIL	COLORS
Down	Unspotted	Unstriped	Unstriped with thin subterminal bar	Brownish
Unstriped	Thinly spotted	Striped	Unstriped with thick subterminal bar	Non-uniform gray
Thinly striped	Heavily spotted		Thinly striped	——
			Striped	
Heavily striped			Heavily striped	Uniform gray

ing, and it has been assumed that this visual gender assignment is accurate (Negro and Hiraldo 1992). However, based on our own experience of 13 yr working with the species, there were nestlings with intermediate coloration that were difficult to assign gender. Observers may differ in their assignment, and male features in adult females (Tella et al. 1997) and mosaic plumages have both been described for this species (Tella et al. 1996a). All this suggests that errors in gender assignment of nestlings have been underestimated.

There are characters in addition to rump and tail color (e.g., marking pattern of head, shoulders, rump, and tail) that show variability among nestlings, and these seem to be associated with nestling gender. Not all banders seem to be aware of these differences, and it is unclear if consideration of other characters could improve gender determination in the field.

Molecular techniques could be used as a 100% accurate standard for other techniques (Ellegren and Sheldon 1997, but see Dawson et al. 2001), but require access to a genetics lab and have an economic cost. Ideally, methods of gender determination in wildlife species should be inexpensive, produce an immediate result, and require a minimal amount of handling stress on birds. These techniques should also be accurate for all age groups and populations (Eason et al. 2001). For these reasons, field methods, which are based on differences in size or color between sexes (e.g., Borras et al. 1993, Martín et al. 2000, Balbontín et al. 2001), are advantageous. Nonetheless, methods based on plumage features need some development, and they have not been adequate for determining the sex ratio at the time of hatching.

The goal of this study is to increase the accuracy of gender determination in Lesser Kestrel nestlings. Our objectives are: (1) to test the accuracy of the traditional visual gender determination employed for Lesser Kestrel nestlings at the time of banding and (2) to evaluate alternative gender determination methods. For this purpose, we first tested if visual gender determination by banders is accurate by using data on birds banded as nestlings and recaptured as adults. Second, we determined gender in a sample of nestlings with molecular methods and considered this the reference gender assignment to test the accuracy of visual assignment by three observers. By using categorized color and plumage pattern in key areas of bird physiognomy, we compared the discrimination ability of each of these characters. Finally, we tried to improve the traditional visual gender determination by building discriminant function models using color measurements of rump and tail from digital photos taken in the field and from color measurements of rump feathers in the lab.

METHODS

Gender Determination by Banders. From 1988 to date, Lesser Kestrel nestlings have been banded in several colonies in southwestern Spain. Gender determination was done by different banders following a visual assessment based on published differences in plumage (Bijlsma et al. 1988, Negro and Hiraldo 1992, Tella et al. 1996b, Palumbo 1997). We recaptured 476 nestlings as adults, which allowed us to evaluate the accuracy of the gender determination by banders.

During the 2000 breeding season (early June to mid-July), 62 Lesser Kestrel nestlings from 18–33 d old were visually assigned to gender by three banders. Nestlings were classified according to marking pattern and color of four areas: head, shoulders, rump, and tail (Table 1) These features were evaluated independently, and a final gender determination was made by each observer considering all the characteristics together.

Capturing Images with a Digital Camera. We used a Kodak DC40 (Rochester, NY U.S.A.) flash enabled digital camera (DC) with a 756 × 504 pixel matrix and 24-bit color. The color value of each pixel is characterized by

Table 2. Contingency table where the influence of family on gender determination was evaluated. Where P is the probability of assigning gender correctly, $(1-P)$ was the complementary probability, and N was the number of pairs (Sokal and Rohlf 1995).

OBSERVED	EXPECTED
Number of pairs with both siblings assigned to gender correctly or incorrectly.	$N\,(P^2 + (1-P)^2)$
Number of pairs with one sibling assigned to gender correctly and the other incorrectly.	$N\,(2P\,(1-P))$

brightness values of red, green, and blue (RGB) scaled in a range from 0–255.

To reduce environmental variability, we photographed nestlings on a copy stand baseboard with flash illumination. Moreover, as the same individual may still show some variation in its RGB values from photograph to photograph (Villafuerte and Negro 1998), we used two control chips (standards), provided by a gray scale card (Smithe 1975) along with the object to be photographed to further standardize the images. Photos were taken at similar distances to objects from directly overhead and using oblique views to provide further analysis of color from critical gender-determination areas.

From each nestling, a rump feather was removed and later scanned with a desktop scanner (Hewlett-Packard Scanjet 5200c, Palo Alto, CA U.S.A.), setting the resolution at 150 dpi. Analysis of color was made from the digital image created using the same procedure as with the digital photos.

The Software. Portions of the image to be analyzed (e.g., portion of tail between dark stripes) and portions of standard chips were respectively selected with the "lasso" and "Rectangle marquee" tools of Adobe Photoshop® (San Jose, CA U.S.A.) for Windows® (Redmond, WA U.S.A.). Following the procedure used by Villafuerte and Negro (1998) to analyze digital images, color from each rump and each tail was separated into RGB values. The theoretical and observed values of the standard chips were used to calculate linear regressions for each primary color. Observed red, green, and blue values of the standard gray chips were used to correct the observed values in the rump and in the tail. This procedure makes RGB values from photos made under different illumination conditions comparable (Villafuerte and Negro 1998). We did not follow this procedure with scanned feathers because the distance from the lens and the illumination source were always the same and, therefore, images could be compared directly.

Molecular Gender Determination. A drop of blood was taken by venipuncture of the brachial vein and stored in 1-ml ethanol. Crude DNA extracts were prepared by boiling 5 μl of the blood in 100 μl of a 100 mM NaOH solution for 10 min, then 0.5 μl of the supernatant was used directly as the template for PCR.

The CHD1W and CHD1Z genes were amplified using primers 2917F and 3088R (Ellegren 1996). Sexes can be discriminated in an agarose electrophorectic gel, as males display a single PCR product of around 550 bp, while females display also an additional product of 450 bp. PCR was performed in a final volume of 25 μl containing 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP, 0.2 μM each primer, and 0.04 U/μl of Taq DNA polymerase. The thermal profile comprised an initial denaturation step of 94°C for 2 min, followed by a single cycle of 2 min at 94°C, 30 sec at 55°C, and 1 min at 72°C, and 34 cycles of 30 sec at 92°C, 30 sec at 50°C, 45 sec at 72°C. A final extension step of 72°C for 5 min was added after the last cycle. The same cycling parameters were used with all primer sets. Twenty μl of the PCR reaction were analyzed by electrophoresis in a 2% agarose gel containing 0.3 μg/ml ethidium bromide. Known male and female blood samples were used as positive controls. PCR products were visualized and photographed under UV light.

Statistical Analysis. Because the probability of assigning gender correctly could covary among brood mates, their presence in the data set of recaptured birds can be considered a source of pseudoreplication. Therefore, the probability of correctly assigning gender for a nestling was not independent from the gender assignment of his brood mate. For this reason, we tested whether this effect could influence our results. We selected pairs of nestlings of the same gender (25 pairs of male siblings and 19 pairs of female siblings) from the data set of resighted birds We subdivided these pairs into two groups: the first group comprised pairs in which gender determination for both siblings was either correct or incorrect (20 pairs of male siblings and 17 pairs of female siblings), and the second comprised those in which the gender of one member was assigned correctly while the other was assigned incorrectly (five pairs of male siblings and two pairs of female siblings). We compared the distribution of these cases versus that expected by chance considering the probability (P) of making a correct assignment (Table 2). In the few nests with more than two siblings of the same gender, two birds were selected at random.

We also tested if siblings were more similar in color by testing for a brood effect on color values of scanned rump feathers with a generalized linear model (GLM, McCullagh and Nelder 1983).

We used a GLM to test if different factors like age at the time of banding, nestling body condition (see Rodríguez and Bustamante 2003), and true gender could influence the probability of determining the gender of a nestling successfully. The response variable in the model was correct gender determination (true/false), using a binomial error and a logit link. The statistical significance of each predictor (factor or continuous variable) was tested by sequentially removing all predictors from the complete model, starting from the one producing the smaller increase in the model deviance (Crawley 1993). Models were fitted using the GLM procedure of S-plus 2000 (Professional, Release 2. 1988–99 MathSoft, Inc., Seattle, WA U.S.A.).

Table 3. General linear model built to test the explanatory ability of age, nestling body condition, and sex on the probability of assigning gender correctly to the nestlings. Each row represents the change in degrees of freedom and deviance when the variable was removed from the model. Chi and P values are also shown. The null deviance = 278.5746 with 446 df and residual deviance = 269.1751 with 443 df.

Explanatory Variables	Δ	Chi	P	Percent Total Deviance
Body condition	1	−1.33	0.18	0.6
Age	1	1.71	0.09	1
Sex	1	2.32	0.02	2

To test the concordance between molecular and visual gender determination (both character by character and the final gender evaluation for each observer), we calculated the Kappa value (percent of agreement corrected for chance agreement; Titus et al. 1984), then we tested the concordance between observers calculating the Kappa value from a contingency table in which each row represented an individual classified as male, female, or unknown (the three categories of the columns). Cell entries were the number of observers agreeing on each category (Siegel and Castellan 1988).

Finally, we built several discriminant functions through a forward stepwise variable selection procedure (F to enter = 3.0, F to remove = 2.0, Tolerance = 0.01) in Statistica 99 (StatSoft 1999). We built a discriminant function for each set of predictive variables: (1) color and pattern recorded visually by each observer, (2) color from rump and tail measured on digital photos, and (3) color of scanned rump feathers. For the first one, we used as possible predictors the recorded category of color and marking pattern of rump and tail, and the category of the marking pattern of head and shoulders obtaining a discriminant function for each one of the observers. For the last two, mean, minimum, maximum, and standard deviation values of red, green, and blue brightness values were used as potential predictors in the analyses.

Results

Brood Effect. The probability of correctly assigning gender for a bird within a brood was independent from the probability of success in the gender determination of his brood mate, both for males (P = 0.35, Fisher's exact test) and for females (P = 0.33). Brood did not explain the variability in the brightness values of red ($F_{1,55}$ = 0.85, P = 0.36), green ($F_{1,55}$ = 1.5, P = 0.23), and blue ($F_{1,55}$ = 0.64, P = 0.43), which allowed us to use nestlings as independent sample units even when more than one bird from the same brood was present in the sample.

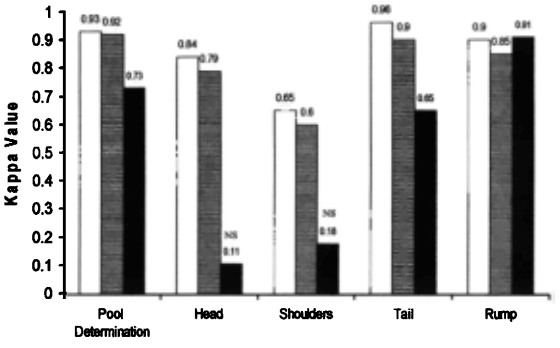

Figure 1. Kappa values for the concordance between molecular and visual gender determination by both single characters and the pooled evaluation inferred from all the characters. Bar colors represent the three different observers (empty bars for observer 1, shaded bars for observer 2, and black bars for observer 3). Non-significant concordances were denoted as NS.

Success of Gender Determination by Banders. On average, 90.3% of kestrels recaptured as adults (N = 476) were assigned correctly to gender at the time of banding (κ = 0.81, Z = 17.99, $P < 0.01$). This indicated that the method was in general adequate, but the error in gender determination was significantly greater than 0 (95% Confidence Interval [CI] = 8.0–14.0%). A significantly greater fraction of males than females were determined incorrectly (31/243 versus 15/233, respectively; P = 0.008, Fisher's exact test). Mean error rate in gender determination according to recaptures is 14.6% for males (95% CI 10.0–20.0%) and 6.4% for females (95% CI 4.0–10.0%).

According to the GLM model, the success in gender determination was only related to the gender of the bird (Table 3), which indicated a higher probability of assigning gender correctly for females. The body condition of the nestling had no explanatory ability on its gender determination, and although there was a slight trend for increasing determination success with nestling age, this trend was not significant (Table 3).

Visual Gender Determination Characters. All characters evaluated to classify gender in Lesser Kestrel nestlings visually showed some degree of sexual dimorphism. For two of the observers, head and shoulder patterns were used with high accuracy in classification of gender when the pattern was clear (Fig. 1), but many of the birds were un-

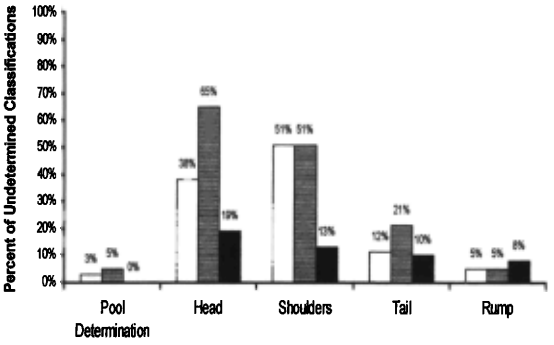

Figure 2. Percent of birds for which gender could not be determined. Bar colors represent different observers (empty bars for observer 1, shaded bars for observer 2, and black bars for observer 3). Exact values are provided above bars.

determined based on this character (Fig. 2). The contrary pattern was found for the remaining observer (black bars in Figs. 1, 2), who classified more birds, but made more errors. Considering undetermined birds as assigned gender incorrectly, none of the observers achieved a significant agreement between molecular gender determination and head pattern ($\kappa = 0.14$, $\kappa = -0.37$, and $\kappa = -0.11$ for the three observers, respectively) or shoulder pattern ($\kappa = -0.2$, $\kappa = -0.2$, and $\kappa = 0.03$, respectively). Both tail and rump characters showed high agreement between molecular and visual determinations for the three observers with a low number of unknown individuals. Among birds classified erroneously, observers were not consistent in agreement with their gender assignment ($\kappa = -0.17$, $Z = -1.072$, $P = 0.142$), suggesting that these were individuals with intermediate characteristics.

Differences Between Observers. Of 62 nestlings, 47 were evaluated by all three observers. Each observer evaluated 61, 57, and 48 nestlings, respectively (Table 4). There was a high agreement between observers whether we considered undetermined birds as a third category ($\kappa = 0.77$, $Z = 4.5$, $P < 0.01$) or as errors ($\kappa = 0.8$, $Z = 8.9$, $P < 0.01$). The gender assignment by the three observers had a high and significant agreement with molecular gender determination. Percentages of correct gender determination for each observer were: 97% ($\kappa = 0.93$, $P < 0.01$), 96% ($\kappa = 0.92$, $P < 0.01$), and 87% ($\kappa = 0.73$, $P < 0.01$). The observers did not determine gender for 3%, 5%, and 0% of the nestlings, respectively. Including the undetermined birds as errors, the accuracy level of the observers was similar to results from the recaptures (error rate = 7, 9, and 13% for each observer, respectively). The small sample size did not allow us to test if males were misclassified more frequently than females.

Discriminant Analyses. By building a discriminant function of the color and pattern categories (Table 1) recorded by each observer, we obtained a different discriminant function for each observer. For the first observer the discriminant function included only rump color and resulted in an error frequency of 7%. For the second observer, the discriminant function included two variables: rump and tail color, and also had a 7% error. The discriminant function for the third observer used the shoulder pattern (plus tail and rump color), and produced a classification with 8% error.

By using RGB values from digital photos of individuals to build a discriminant function, we had an error frequency of 21% when using only rump color values, a 19% error when using only tail color values, and a 17% error using both tail and rump values. The best discriminant function included standard deviation of blue from tail, and standard deviation of red from rump (83% correct classification, $N = 53$ nestlings). Kappa value from the classification matrix of this discriminant function indicated an agreement with molecular gender determination significantly greater than chance ($\kappa = 0.66$, $Z = 4.74$, $P < 0.01$).

The color of scanned rump feathers that isolated the red, green, and blue brightness (RGB) com-

Table 4. Number of birds for which we assigned gender by molecular and visual determination. The number of misclassifications is indicated between parentheses.

GENDER	MOLECULAR TECHNIQUE	OBSERVER 1	OBSERVER 2	OBSERVER 3
Male	28	27 (1)	24 (3)	20 (3)
Female	34	34 (3)	33 (1)	28 (1)
Non-evaluated	0	1	5	14

ponents resulted in the method with greatest accuracy. Males and females could be separated by mean blue (B) value, mean green (G) value, and standard deviation of green value. This discriminant function correctly classified 98.2% of the individuals (1.8% error, $N = 57$). The agreement between the discriminant function classification and molecular gender determination was significantly greater than chance ($\kappa = 0.96$, $Z = 7.1$, $P < 0.01$). The method provides a lower error rate than gender determination by banders according to recaptures (Yates corrected Chi-square = 3.04, one-tailed $P = 0.04$), an error rate similar to those obtained by two of the banders, but lower than that obtained by the remaining one ($P = 0.034$, Fisher exact test). The discriminant function equation to separate males (positive values) from females (negative values) based on RGB values of rump's scanned feather was:

$$D = 25.2931 + 1.0002(\bar{x}B) - 1.046(\bar{x}G) - 0.2298(\text{SD of G}).$$

DISCUSSION

Previous works (Negro and Hiraldo 1992, Aparicio and Cordero 2001) with a limited sample of birds ($N = 45$ and $N = 14$, respectively) suggested that visual gender assignment based on plumage characteristics in Lesser Kestrel nestlings was 100% accurate. Our analysis involving a larger sample indicated that 9.7% of nestlings were incorrectly assigned to gender by banders and those errors in males were twice as frequent as in females. Although all the visual characters evaluated showed a certain sexual dimorphism, the color of rump and tail were clearly the characters most useful in the gender determination of Lesser Kestrel nestlings (Fig. 1). Marking patterns of rump and tail did not provide any useful extra information for gender determination. On the other hand, rump and tail color can be evaluated as soon as the rump and tail feathers start growing, while the marking patterns require a more developed feather before an accurate assessment can be made. The head and shoulder patterns showed a certain amount of dimorphism, visible only when the nestlings were close to fledging and the down had disappeared. For this reason, these characters tend to give a high percentage of birds classified as unknown. The discriminant functions built with the color and marking pattern categories as recorded by each observer supported this conclusion. Rump color was entered into the best discriminant function of all three observers, while shoulder pattern was entered only in the function of one of them. The color of the rump measured on a scanned rump feather was able to correctly determine the gender of 98.2% of the birds. This result was similar to the visual determination. The fact that color measurement on digital photos taken in the field performed worse than visual assignment suggests that our standardization of photographs was not adequate, and that differing illumination conditions had a strong influence on the result. This also indicated that a higher resolution camera should be used for this kind of analysis. In addition, field observers can compare nestlings of the same or different broods. This seems to be a useful advantage (Bijlsma et al. 1988) in distinguishing between males and females with intermediate characters.

The discriminant function built from scanned rump feather color offers an inexpensive, relatively efficient, and objective way to classify gender of Lesser Kestrel nestlings, although a scanner resolution of 300 dpi is recommended (S. Talbot pers. comm.). It is measurably more accurate and objective than the traditional visual method for observers with variable level of experience (1.8% error rate versus 13% for the observer with less experience) and circumvents potential biases due to variance within humans regarding the perception of color (McMahon et al. 2004). To remove a feather from a nestling at the time of banding is a simple and relatively nonintrusive task. Because access to a genetic laboratory is not available to all researchers, the utility of this method to improve gender determination accuracy for field biologists is obvious, especially when errors related to visual gender assignment are likely skewed toward one gender. Nonetheless, different questions require different levels of accuracy in terms of gender determination, and studies that need the maximum accuracy or focus on the primary sex ratio should always use molecular techniques. Also, feathers plucked for gender determination may be used to address other behavioral and reproductive genetic questions (e.g., Alcaide et al. in press).

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