

SEX DETERMINATION OF DUPONT'S LARK *CHERSOPHILUS DUPONTI* USING MOLECULAR SEXING AND DISCRIMINANT FUNCTIONS

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SUMMARY.—*Sex determination of Dupont's lark Chersophilus duponti using molecular sexing and discriminant functions.*

Aims: To test for sexual size dimorphism in external measurements of the Dupont's lark *Chersophilus duponti*, using a sample of live birds previously sexed by molecular techniques, and to obtain discriminant functions to easily sex birds in hand.

Location: Birds were captured in different populations throughout Spain and Morocco. Most birds were trapped in the Ebro Valley (north-eastern Spain).

Methods: A total of 317 adult and 42 yearlings were captured, banded, weighted and measured. A drop of blood was extracted for molecular sexing. After testing for sex differences in body size, discriminant function analyses were performed to identify the best traits for sexing both juveniles and adults.

Results: All the measured parameters differed significantly between sexes in adult Dupont's larks. The best discriminant function accurately assigned sex to 99.0 % of the adults. The parameter which gave the best single factor correlation with sex was wing length, and the discriminant function with only this variable classified correctly 97.5 % of all the adults. An adult would be a male if wing length > 97 mm and a female if wing length < 97 mm. Sex had a significant effect on all parameters of juvenile individuals as well, except for bill depth. The best discriminant function, using wing length and cranium size, predicted correctly the sex of 97.6 % of the juveniles.

Conclusions: Dupont's lark showed clear size dimorphism, males being heavier and larger than females in nearly all measured traits. From an evolutionary perspective, this difference could be explained by processes of intra- and inter-sexual competition, and even by potential costs linked to song flight in males. In any case, the discriminant functions produced using morphometry of individuals previously sexed by molecular procedures provided a highly accurate, inexpensive and fast method for sexing this threatened species in hand, which can help to interpret and understand many questions about its behavioural and population ecology.

Key words: sexual size dimorphism, biometry, discriminant function analysis, molecular sexing, Dupont's lark, *Chersophilus duponti*.

RESUMEN.—*Determinación del sexo en la alondra de Dupont Chersophilus duponti utilizando técnicas moleculares y funciones discriminantes.*

Objetivos: Examinar el dimorfismo sexual en medidas corporales de la alondra de Dupont *Chersophilus duponti*, utilizando una muestra de aves vivas cuyo sexo se determinó con técnicas moleculares, y obtener funciones discriminantes para sexar las aves fácilmente en mano.

Localidad: Las aves se capturaron en varias poblaciones tanto en España como en Marruecos. La mayoría de las aves fue capturada en el Valle del Ebro (NE de España).

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Métodos: 317 aves adultas y 42 juveniles fueron capturadas, anilladas, pesadas y medidas. Se extrajo una gota de sangre para el sexado molecular. Tras examinar si existían diferencias entre sexos en medidas corporales, se aplicaron análisis de funciones discriminantes para establecer las mejores medidas capaces de determinar el sexo de las alondras de Dupont, tanto juveniles como adultas.

Resultados: Todos los parámetros medidos difirieron significativamente entre los sexos de las alondras adultas. La mejor función discriminante asignó el sexo correctamente al 99,0 % de estas aves. La longitud del ala proporcionó la mejor correlación de un único parámetro con el sexo de los adultos, clasificando correctamente el 97,5 % de los mismos. De acuerdo con la función resultante, las alondras con un ala > 97 mm serían machos y con ala < 97 mm hembras. En aves juveniles, el efecto del sexo también fue significativo en casi todos los parámetros, con la excepción de la altura del pico. La mejor función discriminante determinó correctamente el sexo en el 97,6 % de los juveniles.

Conclusiones: Las alondras de Dupont examinadas mostraron un claro dimorfismo sexual, los machos siendo más pesados y grandes que las hembras en casi todos los parámetros analizados. Estas diferencias pueden ser explicadas por procesos de competencia intra- e intersexual, e incluso por cuestiones de balance energético ligadas al canto en vuelo de los machos. En cualquier caso, las funciones discriminantes obtenidas usando variables morfométricas de individuos sexados con técnicas moleculares proporcionaron una herramienta precisa, económica y rápida para determinar el sexo en mano, lo cual puede ayudar a interpretar y entender muchas incógnitas sobre la ecología del comportamiento y de poblaciones en esta especie.

Palabras clave: dimorfismo sexual, biometría, análisis de función discriminante, sexado molecular, alondra de Dupont, *Chersophilus duponti*.

INTRODUCTION

Sexual size dimorphism is common among birds (Price, 1984). Several specific groups, e.g. seabirds and raptors - many of them with reversed sexual size dimorphism - received much attention by research studies about sexual dimorphism in body size (see e.g. González-Solis, 2004; Krüger, 2005) whereas data from Passeriformes is scarce or focused on selected species of a few families like Paridae or Fringillidae (e.g. Blondel *et al.*, 2002; Badyaev, 2005).

Larks (family Alaudidae) are rather small oscine passerines occurring mainly in open, arid or semi-arid habitats and sexual size dimorphism seems to be a frequent pattern among species that are apparently monomorphic in plumage, with males being larger than females (Cramp, 1988). Nevertheless, little information exists about these morphometric differences and the few data available is based on museum specimens, whereas studies with live birds lack molecular sexing (Cramp, 1988; Svensson, 1992).

Dupont's lark *Chersophilus duponti* is restricted to steppe areas with natural vegetation of Spain and North Africa (Cramp, 1988; Garza *et al.*, 2003b). Loss and fragmentation of suitable habitat have reduced dramatically its populations, which are nowadays roughly estimated in 1300-1900 breeding pairs for Spain and 5500-10000 breeding pairs for North Africa (Garza *et al.*, 2003a; Tella *et al.*, 2005; BirdLife, 2005). These low population numbers and negative population trends have resulted in uplisting Dupont's lark to "Endangered" in the Spanish Red List (Garza *et al.*, 2003b) and to "Near Threatened" in the IUCN Red List respectively (BirdLife, 2005). Although Dupont's lark is subject of current research studies, mainly focused on conservation issues (Garza *et al.*, 2005; Laiolo and Tella, 2005, 2006a, 2006b, 2007; Laiolo *et al.*, *in press*; Seoane *et al.*, 2006; Suárez *et al.*, 2006), many aspects of the species' biology are still poorly understood or totally unknown due to its secretive behaviour. For example, reliable sex determination is crucial for the understanding of individual-

based ecological and behavioural data, but straightforward and inexpensive sexing techniques such as discriminant functions have not been developed to date.

Therefore we test here for sexual size dimorphism in the Dupont's lark using a large sample of live birds previously sexed by molecular techniques. To our knowledge, this is the first of such studies carried out with a member of the family Alaudidae. As yearlings can be easily differentiated from adults attending to plumage features until their autumnal complete moult (Svensson, 1992), discriminant functions were developed separately for each age-class.

MATERIAL AND METHODS

Biometrics

A total of 359 Dupont's larks was trapped during five years (2002: $n = 28$, 2003: $n = 3$, 2004: $n = 146$, 2005: $n = 150$, 2006: $n = 32$) in different populations throughout Spain and Morocco. Most birds ($n = 300$) were captured in the Ebro Valley (North-eastern Spain: provinces of Navarra, Zaragoza, Huesca and Teruel), 30 specimens in the High Moulouya/High Plateau region (Eastern Morocco) and another 29 distributed among several small populations of Spain (provinces of Segovia, Palencia, Zamora, Murcia and Almería). Most birds were captured between February and July ($n = 242$; 67.4 %) and during September/October ($n = 112$; 31.2 %), whereas captures in other months were scarce ($n = 5$; 1.4 %). Territorial birds were located aurally during surveys carried out before dawn, and trap groups (3 - 4 spring loaded traps baited with yellow mealworms) were later placed in each localised territory. A playback equipment (CD player and loudspeaker) forecasting conspecific song and calls was used to attract the birds. 43 birds were captured without using the playback equipment.

The birds were banded and measured following a standardized protocol by seven different persons. They were weighted on an electronic balance to the nearest 0.1 g. Wing, eighth primary and tail length were measured with ruler to the nearest 0.5 mm. Tarsus, hind claw, cranium size (tip of the bill to posterior pole of head), and three different bill variables (tip of the bill to skull, tip of the bill to distal edge of nostrils and bill depth) were measured with a digital calliper to the nearest 0.01 mm. See Svensson (1992) for details of measurements. A drop of blood was extracted from the brachial or the jugular vein for molecular sexing and stored in pure ethanol.

Molecular sexing

Intron size differences between Z and W copies of CHD1 gene are often found and have been exploited for PCR-based molecular sexing of birds (Griffiths *et al.*, 1996, 1998; Fridolfsson and Ellegren, 1999). PCR products spanning the targeted intron are generated from both CHD1-Z and CHD1-W using primers targeting conserved flanking exon sequences, and these are later distinguished by electrophoresis on a 2 % agarose gel.

DNA was extracted from blood samples (Gemmell and Akiyama, 1996), with two steps of chlorophorm extraction. We determined Dupont's larks' sex by PCR amplification of CHD1 introns using primers P2 (5'TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTC-CCAAGGATGATRAAYTG-3). PCR was carried out in a total volume of 25 microlitres, with the following final reaction conditions: 3.5 mM MgCl, 0.2 mM dNTPs, 1x Reaction Buffer, 0.01 % gelatine, 0.2 microM of each primer (P2 and P8), and 0.5 U of Taq polymerase (Bioline), and using 60-100 ng of genomic DNA as template. PCR amplifications were performed in an MJ Research model PTC-200 thermocycler under the following thermal cycling: an initial denaturing step at 94 °C for

2 min followed by 55 °C for 30 seconds, 72 °C for 1 min and 33 cycles of denaturation at 92 °C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72 °C for 45 seconds, ending with a final extension at 72 °C for 5 minutes. Ten microlitres of the reaction was analysed by electrophoresis in 2 % agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) in the presence of SyBr (Molecular Probes). PCR products were subsequently visualized under UV light and photographed with a digital image system (Eastman Kodak Company). Sex identification was confirmed by using control individuals with known sex showing a single band with an estimated size of 295bp for males, and two bands of 295 and 340bp for females respectively. Birds used as controls were 3 females showing active brood patch when captured and 33 males individually colour-banded, which were singing, defending territories and monitored by us between 6 and 16 months after first capture (Laio-lo *et al.*, *in press*). All of them resulted correctly sexed though molecular procedures.

Statistical analysis

After sexing all birds by molecular procedures, we applied two One-way ANOVAs to detect sex differences in body size. Adults and juveniles were analysed separately, since the former were significantly larger and heavier (results from One-way ANOVAs not shown). Then, forward stepwise discriminant function analyses were performed to determine the best measurements which identify the sex of both juveniles and adults. We applied a jackknife procedure to validate the data (Amat, 1993; Bertellotti *et al.*, 2002). In this way, each individual in the sample was classified using a discriminant function derived after excluding the individual being classified from the whole sample. This procedure chooses the discriminant function with the lowest percentage of misclassification. Sexual size dimorphism

(SSD, in %) was calculated as an index $100 * [(mean\ value\ of\ the\ male\ character / mean\ value\ of\ the\ female\ character) - 1]$ (Weidinger and van Franeker, 1998, see also Greenwood, 2003). All the analyses were performed with SPSS 13 (2004).

RESULTS

All the measured parameters differed significantly between sexes in adult individuals (Table 1). The parameter which gave the best single factor correlation with sex of adults was wing length (canonical correlation 0.766) and the discriminant function with only this variable classified correctly 97.5 % of all the adult individuals ($n = 314$). The following function was generated:

$$D_{ad1} = 0.397 * (\text{wing length}) - 38.526$$

An adult individual would be a male if wing length > 97 mm and a female if wing length < 97 mm. 97.2 % of the 286 adult males and 100 % of the 28 adult females were classified correctly.

The stepwise forward discriminant function analysis selected both wing length and cranium size (canonical correlation 0.770) for distinguishing between the sexes in adult birds ($n = 311$), and we obtained the following equation:

$$D_{ad2} = 0.344 * (\text{wing length}) + 0.387 * (\text{cranium size}) - 50.72$$

where an adult would be male if $D_{ad2} > 0$, and a female if $D_{ad2} < 0$. This function accurately assigned sex to 99.0 % of the adult individuals (98.9 % of 283 males and 100 % of 28 females).

Sex had a significant effect on all parameters of juvenile birds except for bill depth (Table 2). The parameters which gave the best single factor correlation with sex in juveniles were cranium size and wing length (canonical cor-

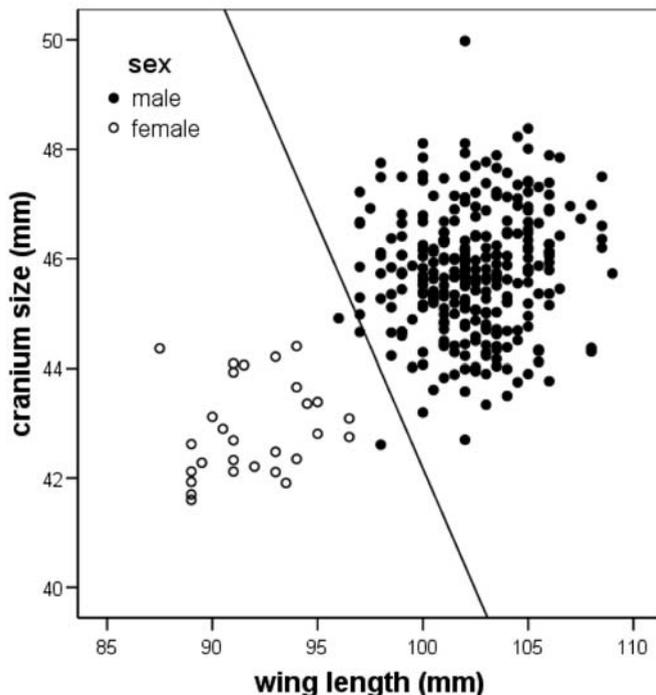


FIG. 1.—The discriminant function using wing length and cranium size to sex adult male (above line) and female (below line) Dupont's larks.

The line is defined as $D_{ad2} = 0.344 * (\text{wing length}) + 0.387 * (\text{cranium size}) - 50.72$.

[Función discriminante utilizando la longitud del ala y el tamaño del cráneo para sexar los machos adultos (encima de la línea) y las hembras adultas de alondra de Dupont.

La línea fue definida como $D_{ad2} = 0,344 * (\text{longitud de ala}) + 0,387 * (\text{tamaño de cráneo}) - 50,72$.]

relation 0.760 and 0.699). The discriminant functions obtained for these two characters separately classified correctly 92.9 % (cranium size) and 85.7 % (wing length) of the cases. The best discriminant function for juveniles (canonical correlation 0.776) included also wing length and cranium size, and the following discriminant function was produced:

$D_{juv} = 0.296 * (\text{wing length}) + 0.787 * (\text{cranium size}) - 60.17$

A juvenile individual would be a male if $D_{juv} > 0$, and a female if $D_{juv} < 0$. This discriminant function predicted correctly the sex of 97.6 % of the juvenile individuals ($n = 42$, 96.8 % of 31 males and 100 % of 11 females).

DISCUSSION

The sexes of Dupont's lark are indistinguishable in coloration, but showed clear size dimorphism, adult males being heavier and larger than females in all measured traits. The discriminant functions produced through morphometric variables of individuals previously sexed by molecular procedures provided a highly accurate, inexpensive and fast method for sexing this species in hand. In fact, the exceptional high accuracy of the obtained discriminant functions was not affected neither by the sample including birds captured during different periods of the year nor by the numerous

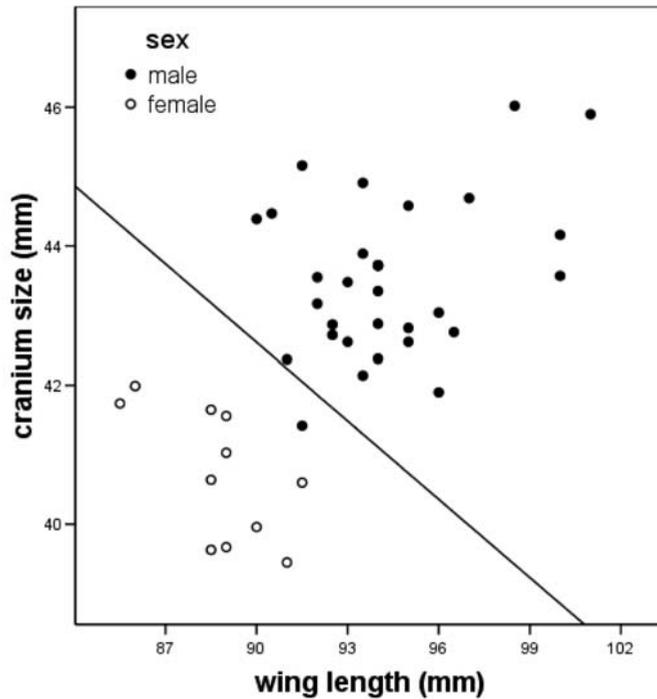


FIG. 2.—The discriminant function using wing length and cranium size to sex juvenile male (above line) and female (below line) Dupont's larks.

The line is defined as $D_{\text{juv}} = 0.296 * (\text{wing length}) + 0.787 * (\text{cranium size}) - 60.17$.

[Función discriminante utilizando la longitud del ala y el tamaño del cráneo para sexar los machos juveniles (encima de la línea) y las hembras juveniles de Alondra de Dupont.

La línea fue definida como $D_{\text{juv}} = 0,296 * (\text{longitud de ala}) + 0,787 * (\text{tamaño del cráneo}) - 60,17$.]

banders taking the measurements. Sex determination relying on morphometrics is independent of temporally limited external characters such as those developed during the breeding period (i.e., brood patch, cloacal protuberance), so it is applicable across the whole year. Indeed, only less than 1 % of the whole sample could not have been sexed with the discriminant equations generated here because of moulting the longest wing feathers, despite 24 % of the birds ($n = 87$) were moulting when they were captured.

Two different functions were calculated for adults because of the high discriminant power of wing length alone, besides no need of tak-

ing a calliper measurement. Sexual size dimorphism was even visible in juvenile Dupont's larks. Although differences are less evident than in adult individuals, and even non-significant for bill depth, our discriminant functions correctly classified an unusually very high percentage of individuals. Our results on sex determination are applicable to both European and North African populations of the nominate subspecies *C. d. duponti* as the sampled birds cover a large part of its breeding range. Northeastern Africa's subspecies *C. d. margaritae* seems to have a similar morphometric pattern, although significant differences between the two subspecies have been reported and data are

TABLE 1

Mean (\pm standard error) measurements of adult Dupont's larks. Sex differences according to one-way ANOVA and sexual size dimorphism (SSD) were also shown.

[Media (\pm error estándar) de alondras de Dupont adultas. Se muestran las diferencias entre sexos (ANOVA de una vía) y el dimorfismo sexual (SSD).]

Variable	Males	Females	ANOVA	SSD
Body mass (g)	39.7 \pm 2.2 <i>n</i> = 285	35.0 \pm 2.2 <i>n</i> = 27	<i>F</i> = 108.0 <i>P</i> < 0.001	13.4 %
Wing length (mm)	102.4 \pm 2.5 <i>n</i> = 286	91.9 \pm 2.5 <i>n</i> = 28	<i>F</i> = 441.9 <i>P</i> < 0.001	11.4 %
Eighth primary length (mm)	77.0 \pm 2.4 <i>n</i> = 276	69.4 \pm 2.8 <i>n</i> = 24	<i>F</i> = 215.7 <i>P</i> < 0.001	11.0 %
Tail length (mm)	65.6 \pm 2.7 <i>n</i> = 283	58.0 \pm 2.9 <i>n</i> = 28	<i>F</i> = 203.8 <i>P</i> < 0.001	13.1 %
Cranium size (mm)	45.8 \pm 1.1 <i>n</i> = 286	42.9 \pm 0.9 <i>n</i> = 28	<i>F</i> = 172.7 <i>P</i> < 0.001	6.8 %
Bill to skull (mm)	24.3 \pm 1.1 <i>n</i> = 279	22.3 \pm 1.4 <i>n</i> = 26	<i>F</i> = 76.10 <i>P</i> < 0.001	9.0 %
Bill to nostrils (mm)	15.0 \pm 0.9 <i>n</i> = 288	13.7 \pm 0.9 <i>n</i> = 28	<i>F</i> = 58.72 <i>P</i> < 0.001	9.5 %
Bill depth (mm)	6.1 \pm 0.4 <i>n</i> = 288	5.8 \pm 0.5 <i>n</i> = 28	<i>F</i> = 15.00 <i>P</i> < 0.001	5.2 %
Tarsus length (mm)	23.8 \pm 0.8 <i>n</i> = 289	23.0 \pm 0.7 <i>n</i> = 28	<i>F</i> = 26.75 <i>P</i> < 0.001	3.5 %
Hind claw length (mm)	10.7 \pm 1.4 <i>n</i> = 288	10.0 \pm 1.4 <i>n</i> = 26	<i>F</i> = 5.225 <i>P</i> < 0.05	7.0 %

very scarce (Cramp, 1988). More morphometric data is required to develop discriminant functions for in-hand sexing of the other subspecies of Dupont's lark. These discriminant functions provide an easy tool for sexing Dupont's lark in hand which can help to understand and interpret ecological and behavioural processes of this threatened species.

Three main hypotheses have been proposed to explain the evolution of sexual size dimorphism (see Hedrick and Temeles, 1989 for a review). The sexual selection hypothesis suggests that differences in size evolved through intrasexual competition for mates and mate choice by the opposite sex which result in variance in mating success (Darwin, 1871). The

reproductive role division or dimorphic niche hypothesis proposes that intrinsic differences between the reproductive role of males and females produce sexual size dimorphism (Ralls, 1976). The intersexual food niche differentiation hypothesis predicts that dimorphism enables each sex to occupy a different niche and thus lessen food competition between sexes (Selander, 1966, 1972).

In alaudid species such as spike-heeled lark (*Chersomanes albofasciata*) and long-billed larks (genus *Certhilauda*), males have a relatively larger bill than do females. This sexual size dimorphism may be related to feeding habits, as it is more evident in the most insectivore species rather than in those with more

TABLE 2

Mean (\pm standard error) measurements of juvenile Dupont's larks. Sex differences according to One-way ANOVA and sexual size dimorphism (SSD) were also shown.

[Media (\pm error estándar) de alondras de Dupont juveniles. Se muestran las diferencias entre sexos (ANOVA de una vía) y el dimorfismo sexual (SSD).]

Variable	Males	Females	ANOVA	SSD
Body mass (g)	37.1 \pm 2.6 <i>n</i> = 31	33.0 \pm 1.8 <i>n</i> = 11	<i>F</i> = 23.03 <i>P</i> < 0.001	12.4 %
Wing length (mm)	94.3 \pm 2.7 <i>n</i> = 31	88.8 \pm 1.8 <i>n</i> = 11	<i>F</i> = 38.19 <i>P</i> < 0.001	6.2 %
Eighth primary length (mm)	70.4 \pm 2.9 <i>n</i> = 29	66.7 \pm 1.2 <i>n</i> = 11	<i>F</i> = 15.98 <i>P</i> < 0.001	5.5 %
Tail length (mm)	56.1 \pm 2.5 <i>n</i> = 30	52.0 \pm 1.6 <i>n</i> = 11	<i>F</i> = 23.94 <i>P</i> < 0.001	7.9 %
Cranium size (mm)	43.5 \pm 1.1 <i>n</i> = 31	40.7 \pm 0.9 <i>n</i> = 11	<i>F</i> = 54.85 <i>P</i> < 0.001	6.9 %
Bill to skull (mm)	22.1 \pm 1.0 <i>n</i> = 26	20.3 \pm 1.5 <i>n</i> = 11	<i>F</i> = 19.59 <i>P</i> < 0.001	8.9 %
Bill to nostrils (mm)	13.0 \pm 0.7 <i>n</i> = 31	11.6 \pm 0.7 <i>n</i> = 11	<i>F</i> = 32.84 <i>P</i> < 0.001	12.1 %
Bill depth (mm)	6.0 \pm 0.4 <i>n</i> = 31	5.9 \pm 0.5 <i>n</i> = 11	<i>F</i> = 0.385 <i>n.s.</i>	1.7 %
Tarsus length (mm)	23.7 \pm 0.8 <i>n</i> = 31	22.9 \pm 0.6 <i>n</i> = 11	<i>F</i> = 7.387 <i>P</i> < 0.05	3.5 %
Hind claw length (mm)	9.7 \pm 0.6 <i>n</i> = 31	9.0 \pm 0.6 <i>n</i> = 11	<i>F</i> = 11.15 <i>P</i> < 0.01	7.8 %

granivorous feeding habits (De Juana *et al.*, 2004). Extreme differences in bill size between sexes are reported as well from raso lark (*Alauda razae*), with bill of males averaging 23.1 % longer than those of females (Donald *et al.*, 2003). This difference might represent an adaptation that reduces competition for food between the sexes in an arid environment with limited resources. Dupont's lark is mainly insectivorous, but feeding on seeds is reported as well (Cramp, 1988). The species' pronounced arid habitat characteristics and its strict territorial behaviour may have similar consequences on sexual differences in foraging strategies which could cause sexual size dimorphism in Dupont's lark.

Another two mechanisms might play a role in sexual size dimorphism of Dupont's lark: First, males often exhibit song flights with long duration (Cramp, 1988, authors' *pers. obs.*), similar to the skylark (*Alauda arvensis*), in which case the intensity and rate of this sexual display appeared to be enhanced by cost-reducing traits, i.e. wing area or tail length (Møller, 1991). Thus, the large differences in these variables between sexes (SSD in wing length 11.4 % and tail length 13.1 %, respectively) could be rather a consequence of sexual selection. Second, the strict monoteritorial behaviour of Dupont's lark may lead to sexual size dimorphism, which is more distinct in monoteritorial passerines due to high-

er intrasexual competition between males for resources (e.g., territorial quality) than in polyterritorial species (Møller, 1986). In fact, intrasexual competition in males is expected to be even stronger in a scenario of a highly skewed sex ratio towards males, as it has been described for this and other lark species (Tella *et al.*, 2004). Although we captured much more males than females by using playback records of male vocalisations (91% males vs. 9 females), adult sex ratio also resulted highly skewed (79 % males vs. 21 % females) among birds captured in trapping journeys when playbacks were not used to attract birds to the traps). Additional studies should be carried out to test these hypotheses for the causes of sexual size dimorphism in Dupont's lark.

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