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Sex Determination of Eurasian Woodcock *Scolopax rusticola*: a molecular and morphological approach

Arianna Aradis[1]*, Giuseppe Landucci[2], Marcello Tagliavia[3], Massimiliano Bultrini[1]

[1]*ISPRA, Institute for Environmental Protection and Research - Via Vitaliano Brancati 60, 00166 Roma, Italy*
[2]*Tenuta Presidenziale di Castelporziano - Via Pontina 690, 00128 Roma, Italy*
[3]*IAMC-CNR UOS Capo Granitola - Mazara del Vallo (Trapani), Italy*
**Corresponding author: arianna.aradis@isprambiente.it*

Abstract – We used molecular sexing and morphological analysis to characterize sexual size dimorphism (SSD) in Eurasian Woodcock (*Scolopax rusticola*) wintering in central Italy. We analyzed SSD in 259 individuals (125 males and 134 females) sexed molecularly based on size differences in CHD-linked sequences from W- and Z- chromosomes. Females were significantly larger than males in bill measurements, tarsus and length of tarsus plus middle toe, while males had longer wing chord and ninth primary length. A discriminant function analysis was applied to a set of morphometric traits to study whether parameters of body size may be used to reliably sex individuals of this species in the field. We formed two equations, one for adults, which was 78.7% accurate, and one for juveniles, which was 76% accurate. Discriminant analysis showed that the length of the ninth primary, tarsus and nalospi was the most useful trait in sexing adult Eurasian Woodcock. Wing chord, nalospi and tarsus were helpful in sexing juvenile birds. Combining the results of DNA molecular sexing and several biometrics, would enable the development of easier sexing techniques. Our results will aid future studies looking at gender differences in the field.

Key-words: biometrics, CHD2550F and CHD2718R, discriminant function analysis, field measurements, gender determination, sexual dimorphism.

INTRODUCTION

Many birds are monomorphic or show little dimorphism between sexes. The shorebirds (Charadriiformes: suborders Charadrii and Scolopaci) display a considerable degree of size and plumage dimorphism that almost encompasses the range of variation found in the Class Aves (Jehl & Murray 1986). They include species with normal dimorphism as males being the larger sex, others with slight sexual size differences, while others have a reverse dimorphism with females being the larger sex (Jehl & Murray 1986, Szekely *et al.* 2004).

The Eurasian Woodcock *Scolopax rusticola* is a wader with a wide breeding range in central, northern and eastern forests of Europe and winters primarily in France, Britain, Ireland, northern Spain, Italy and areas fringing the Mediterranean (Cramp & Simmons 1983, Piersma 1996). Similar to other Charadrii waders, the Eurasian Woodcock displays sexual monomorphism in plumage and size with slight differences between the sexes (Clausager 1973, Cramp & Simmons 1983, Ferrand & Gossman 2009).

Several studies have attempted to identify biometrics that could be used to distinguish males and females (MacCabe & Brackbill 1973, Stronach *et al.* 1974, Rockford & Wilson 1982). Those previous studies focused on differences between morphological measurements taken from museum skins or birds shot during the hunting season neither of which is ideal for field situations. Measurements from museum specimens could be imprecise due to shrinkage or sex mislabeling of the specimens (Summers 1976, Lee & Griffiths 2003, Wilson & McCracken 2008). In addition, some measurements from specimens are not repeatable on live birds (e.g. body length).

Differences in biometric measurements to infer gender have been investigated using various statistical analyses such as linear models, principal component analysis and discriminant function analysis (Remisiewicz & Wennerberg 2006, Schroeder *et al.* 2008, Brady *et al.* 2009). The discriminant function analysis is the most popular of these statistical methods and its use has increased in recent decades (Dechaume-Moncharmont *et al.* 2011). Effectiveness of discriminant functions must be estimated before choosing the best-suited morphological variables to sex individuals.

Currently verification of sex is easily done through molecular techniques using feathers or a small drop of blood as the source of DNA. Various methods have been proposed for sexing of non-ratite birds (Griffiths *et al.* 1998, Fridolfsson & Ellegren 1999), and molecular sexing of Eurasian Woodcock has been reported by Väli & Elts (2002) and Vučićević *et al.* (2012).

There is, to our knowledge, no available biometric work to assess sex determination on live birds from the wintering Woodcock population in Europe and our data from captured and released birds is especially relevant. In this study, we investigated whether several morphometric measurements taken during ringing activity were useful in sexing Woodcock caught during the wintering period in central Italy, focusing on those measurements that are easy to collect in the field.

MATERIALS AND METHODS

Woodcocks were captured as part of a long term study to monitor the wintering ecology of the species in central Italy, ongoing since 1993. The study area is inside the Presidential Estate of Castelporziano, a protected area of approximately 6000 ha located 20km south of Rome (41°44'N-12°24'E). The vegetation consists of broad-leaf forest dominated by Holm Oak *Quercus ilex*, Turkey Oak *Q.cerris*, Pedunculate Oak *Q. robur*, Cork Oak *Q. suber* and Hungarian Oak *Q. farnetto*, as well as Mediterranean scrub, Stone pine *Pinus pinea*, large grazing areas and fields cultivated for growing oats.

During the 1994-2012 ringing seasons, between October and February, we caught 1290 Woodcocks, including 878 juveniles and 412 adults.

Woodcocks were caught in grazed areas and other open areas using night-lighting method modified from Glasgow (1958). Captured birds were fitted with aluminum leg bands and classified as adults (more than one calendar year old) or juveniles (hatched the preceding summer) according to plumage characteristics and moult status (Clausager 1973, Ferrand & Gossman 2009). The following body size measurements were recorded: wing chord length (WING: maximum flattened chord), primary 9th (P9), reduced primary or outermost primary length (RP), head plus bill length (HEAD-L: from the tip of the bill to the back of the skull), bill length (BILL1: from the tip of the bill to the feathering), nalospi (BILL2: length of bill from the tip to the proximal edge of the nostrils), tarsus length (TARSUS: from the rear of the tibia to the last completed scale), tarsus + toe length (TT: tarsus plus mid-toe length without nail ±1 mm), tail (TAIL: length of the central pair of rectrices) and WEIGHT. Wing was measured to the nearest 1 mm with a zero-stop ruler and other linear measurements to the nearest 0.1 mm with a Vernier caliper. The P9 and the RP were measured with a wing rule where the end but was replaced by a small vertical blunt pin. The tail was measured with a square ended flat ruler. Birds were weighed using a Sartorius electronic balance (precision: ±0.1 g). All measurements were taken by the same ringer (GL) to avoid bias between data collectors.

During 2005-2007, we collected blood and feathers sample from 259 Woodcocks. A small drop of blood (0.1ml) was obtained from the leg vein and absorbed on a filter paper. Blood spot specimens were dried over an open non absorbent surface at 15–22°C and then stored in low gas-permeable zip-closure bags at room temperature until they were analyzed.

Molecular sexing of birds was performed by direct-PCR, targeting CHD-linked (chromodomain helicase DNA binding protein gene) sequences from Z- and W-chromosomes (Fridolfsson & Ellegren 1998, Vučićević 2012). PCR were carried out in a final volume of 20 µl, containing 20 ng DNA, 0.2 mM dNTP, 0.5 µM of each primer (CHD2550F and CHD2718R), 0.2 µl of Phire Hot-Start II DNA Polymerase (Thermo Scientific) and 1x reaction buffer. Reaction conditions were: initial denaturation at 98°C for 20 sec followed by 40 cycles composed of denaturation at 98°C for 10 s, annealing at 50°C for 10 s, extension at 72°C for 15 s, and a final extension at 72°C for 2 min. PCR products were fractionated by electrophoresis, with a voltage gradient of 5V/cm, onto agarose gel pre-stained with GelRed (Biotium). Gels were visualized under UV light.

Dimorphism index (DI) was calculated for each morphometric character following Weidinger & Franeker (1998): SSD % = 100* [(male mean size/female mean size) – 1]. For all variables, coefficients of variation (CV = (SD/mean) × 100) were calculated for each sex to indicate the degree of variability of each measurement (Sokal & Rohlf 1995). We used Pearson correlations to examine relationships among the morphometric characters. We examined data for assumptions of normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene test respectively.

Because adult birds might differ from juveniles in measurements, a two-way factorial ANOVA was run on class age with age and sex as independent factors and with interaction term sex × age. Inter-sexual differences in morphological traits were tested with a two-sample *t*-test in each age class.

Forward stepwise discriminant analyses was performed on biometrics to obtain combinations of character-

istics (discriminant functions), that best distinguished the sexes, by using their dis assumption of homogeneity of the variance–covariance matrices was checked with Box's M test.

Following Sikora & Dubiek (2007), the cut-off point used for classifying cases was obtained as the weighed average of the values at the group centroids. If the discriminant score was above the cut-off point the case was classified as male and if below as female.

As suggested by Dechaume-Moncharmont *et al*. (2011), the classification success rate was assessed with a jackknifed cross-validation procedure in which each case is classified using a discriminant function based on all cases except the given case. Some variables were not included in the discriminant analysis: weight because this measure can vary greatly according to several factors, and tail length for the high rate of missing data. Deviation of the sex-ratio from parity was tested with the G-test.

Unless otherwise stated, metrics are reported as mean ± 1 SD, and differences were considered statistically significant at $P < 0.05$.

All statistical analyses were carried out using the R software (R Development Core Team 2011).

RESULTS

Of the 259 birds that were sexed by DNA analysis, 125 were identified as males and 134 as females. The sample analyzed with molecular methods showed the expected pattern of two bands in females and one band in males. The set of 2550F/2718R primers (Fridolfsson & Ellegren 1999) proved to be successful in sex identification of Eurasian Woodcock. The sex-ratio was not different from parity in either age group ($G_{1,91} = 2.82$, $P = 0.093$ for adults; $G_{1,168} = 0.292$, $P = 0.589$ for juveniles). Adult Eurasian Woodcock differed from juvenile birds in WING ($F_{1,255} = 5.31$ $P<0.05$; = 204.5 ± 5.5 mm for adults and = 203.2 ± 5 mm for juveniles), P9 ($F_{1,254} = 3.98$ $P < 0.01$; = 134.3 ± 4 mm for adults and = 133.6 ± 4.1 mm for juveniles) and RP ($F_{1,245} = 9.59$, $P < 0.05$; = 23.3 ± 1.9 mm for adults and = 22.1 ± 1.9 mm for juveniles), so adult and juvenile birds were treated separately in all further analyses (Table 1).

Morphological measurements from the molecularly sexed Woodcock revealed intersexual differences in seven measurements in both age classes (Tables 2 and 3). There were no differences in WEIGHT and RP, which were excluded from further analysis as being unlikely to contribute to sex recognition. WEIGHT and RP showed the highest within-sex variation in both age groups. The degree of sexual dimorphism differed among variables, BILL2 was the most dimorphic variable in adults and TARSUS in juveniles. The variables with sexually dimorphic traits were highly correlated: WING and P9 (r = 0.82 juveniles; r = 0.86 adults), BILL1 and HEAD-L (r = 0.76 juveniles; r = 0.68 adults), BILL1 and BILL2 (r = 0.91 juveniles; r = 0.87 adults), TARSUS and TT (r = 0.75 juveniles; r = 0.74 adults).

For adult birds, the discriminant analysis selected BILL2 (Wilks' Lambda: 0.87, $P < 0.001$), P9 (Wilks' Lambda: 0.72, $P < 0.001$), TARSUS (Wilks' Lambda: 0.68, $P < 0.001$) and produced the following equation: Z_a = 0.02251524* P9 – 0.02522944*BILL2 – 0.03901201 *TARSUS. The cut-off point was Z_a = -0.2701418 (Fig. 1A). If $Z_a \leq$ -0.2701418 the bird is a female; $Z_a >$ -0.2701418 then it is a male. This equation accurately assigned sex to 78.7% of the adult Woodcock (79.6% of the females, 77.1% of the males; Fig. 1A) whose sex was determined by PCR. Jack-knifed cross-validation produced the same classification success rate (95% CI: 68.4%–86.3%).

Table 1. Two-way ANOVA (sex, age) in Eurasian Woodcock in central Italy *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$; ns: not significant.

	Sex			Age		Sex x Age	
	df	**F**	**P**	**F**	**P**	**F**	**P**
WEIGHT	1,252	0.01	ns	0.40	ns	1.13	ns
WING	1,255	11.41	***	5.31	*	0.03	ns
RP	1,245	0.05	ns	9.59	**	0.81	ns
P9	1,254	18.43	***	3.98	*	0.33	ns
HEAD-L	1,252	16.46	***	0.28	ns	1.00	ns
BILL1	1,255	21.49	***	0.21	ns	0.29	ns
BILL2	1,254	24.75	***	2.44	ns	0.00	ns
TARSUS	1,254	28.74	***	0.63	ns	1.28	ns
TT	1,255	15.30	***	1.48	ns	0.80	ns

Table 2. Male and female body measurements [(a)] (mean ± SD), range (min-max), coefficients of variation (CV) and sexual size dimorphism (SSD) of adults Eurasian Woodcock wintering in central Italy. All measurements are given in mm, except weight in g. The difference in the measurements between the sexes was tested with a *t*-test.

	Female (n = 54)		**Males** (n = 37)				
	Mean (± SD)	**Range**	**Mean** (± SD)	**Range**	***P***	**CV** (%)	**SSD** (%)
WEIGHT	317 ± 31.1	230.6 – 386.6	314 ± 26.7	220.3 – 366.9	0.4898	9	1.38
WING	203.5 ± 4.9	192.0 – 215.0	204.2 ± 5.4	194.0 – 220.0	0.0419	3	1.16
RP	23.2 ± 1.8	18.0 – 27.0	22.5 ± 2	17.5 – 26.5	0.4888	8	1.23
P9	133.3 ± 3.8	126.0 – 144.0	134.5 ± 4.2	128.0 – 145.0	0.0037	3	1.92
HEAD-L	112.5 ± 3	107.1 – 119.2	111 ± 3.5	105.8 – 117.7	0.0366	3	1.17
BILL1	75.4 ± 3.0	69.5 – 82.2	73.7 ± 3.2	66.7 – 79.0	0.0061	4	2.21
BILL2	63.8 ± 2.7	58.2 – 71.0	62.5 ± 3.1	56.7 – 66.4	0.0007	3	2.88
TARSUS	38.1 ± 1.2	34.3 – 41.7	37.1 ± 1.3	34.3 – 39.7	0.0101	3	1.88
TT	80.8 ± 2.4	75.0 – 87.0	79.1 ± 2.6	73.0 – 86.0	0.0594	3	1.24

[(a)] WING: maximum flattened chord; P9: ninth primary; RP: reduced primary; HEAD-L: head plus bill length; BILL1: bill length from the tip of the bill to the feathering; BILL2: nalospi, length of bill from the tip to the proximal edge of the nostrils; TARSUS: tarsus length; TT: tarsus plus mid-toe length without nail.

For juveniles, BILL2 (Wilks' Lambda: 0.74, $P < 0.001$), TARSUS (Wilks' Lambda: 0.84, $P < 0.001$), WING (Wilks' Lambda: 0.76, $P < 0.001$), contributed to the sexing procedures and produced the following equation: $Z_j = 0.01331001*WING - 0.01238357*BILL2 - 0.05974008*TARSUS$. The cut-off point was $Z_j = -3.305091$ (Fig. 1B). If $Z_j \leq -3.305091$ the bird is a female; $Z_j > -3.305091$ then it is a male. This equation accurately assigned sex to 76.0% of the juveniles Woodcock (78.8% of the females, 73.6% of the males; Fig. 1B) whose sex was determined by PCR. Jack-knifed cross-validation classified 70.7% of the juveniles (95% CI: 63.0% – 77.3%).

DISCUSSION

Our equations correctly sexed 78.7 % of adults (79.6% of females and 77.1% of males) and 76% of juveniles (78.8% females and 73.6% males). We found that in adults the best discriminant variables in the Eurasian Woodcock wintering in central Italy were ninth primary, nalospi and tarsus length while in juveniles were wing chord, nalospi and tarsus.

The measure of nalospi could be a more precise discriminant among bill measurements to separate females. When in our equations we evaluated the performance of the

Table 3. Male and female body measurements[(a)] (mean ± SD), range(min-max), coefficients of variation (CV) and sexual size dimorphism (SSD) of juveniles Eurasian Woodcock wintering in central Italy. Presentation as in Table 2.

	Female (n = 80)		**Males** (n = 88)				
	Mean (± SD)	**Range**	**Mean** (± SD)	**Range**	***P***	**CV** (%)	**SSD** (%)
WEIGHT	310 ± 28.2	209.7 – 375.0	314 ± 26.7	252.2 – 370.5	0.4078	9	1.14
WING	202 ± 4.3	190.0 – 214.5	204.2 ± 5.4	187.0 – 220.0	0.0046	2	1.06
RP	22.6 ± 1.8	18.0 – 28.5	22.5 ± 2	16.5 – 27.0	0.7651	8	0.74
P9	132.6 ± 3.7	121.5 – 142.5	134.5 ± 4.2	119.0 – 146.5	0.0016	3	1.43
HEAD-L	113.2 ± 3.4	103.0 – 120.1	111.0 ± 3.5	100.0 – 119.5	0.0000	3	1.93
BILL1	75.8 ± 3.3	65.5 – 82.4	73.7 ± 3.2	65.4 – 81.5	0.0000	5	2.77
BILL2	64.4 ± 3	54.2 – 71.2	62.5 ± 3.1	55.5 - 73.0	0.0000	5	2.93
TARSUS	38.2 ± 1.3	35.3 – 43.0	37.1 ± 1.3	34.1 – 41.0	0.0000	4	2.88
TT	80.7 ± 2.5	75.0 – 87.0	79.1 ± 2.6	72.0 – 86.0	0.0000	3	1.98

[(a)] WING: maximum flattened chord; P9: ninth primary; RP: reduced primary; HEAD-L: head plus bill length; BILL1: bill length from the tip of the bill to the feathering; BILL2: nalospi, length of bill from the tip to the proximal edge of the nostrils; TARSUS: tarsus length; TT: tarsus plus mid-toe length without nail.

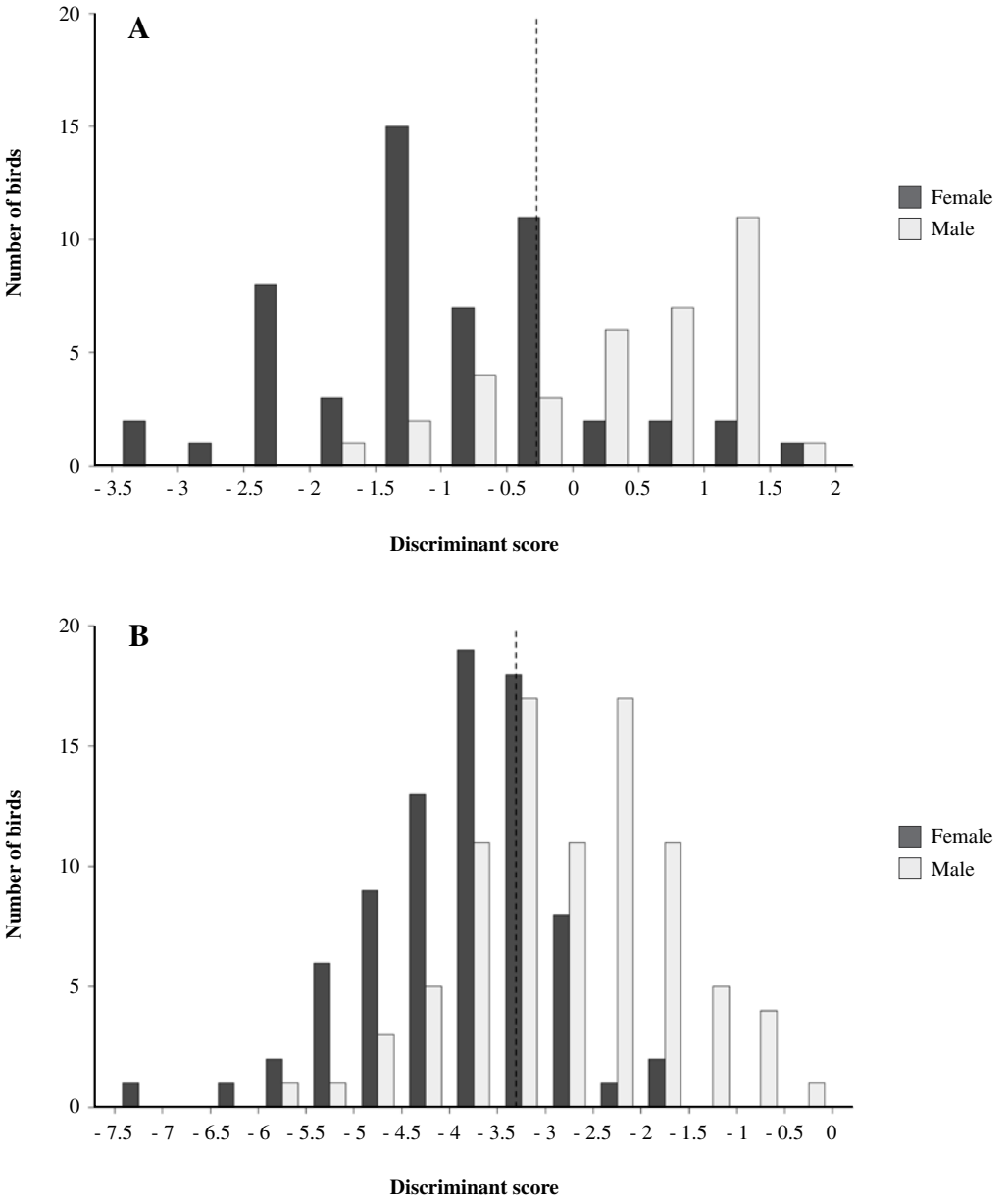

Figure 1. Distribution of male and female Eurasian Woodcock based on discriminant scores. The dotted line represents the cut-off score. (A) Adults, (B) Juveniles.

bill measure from the tip of the bill to the feathering, including ninth primary and tarsus length as discriminatory variables, the proportion of birds of known sex that were classified correctly was lower (75.3%) and consistent with the percentage found in previous study (Stronach *et al.* 1974, Rochford & Wilson 1980, Hoodless 1994, Fadat 1995).

The length of the ninth primary feather (P9) has never been used to distinguish females from males in this species. Fadat (1995) found that the first longest primary (P10) is longer on males than in females of 2.5 mm in both age groups, nonetheless with an high range of variation. In autumn and winter, the study of primaries remiges, as predictor of sex, deserves more examination mainly in adult class because they have generally completed their large feathers moult by the end of september (primaries, secondaries, tertials and rectrices) (Clausager 1973, Ferrand & Gossman 2009).

In juveniles this measure could be less helpful because

their feathers are more worn as they don't moult their primaries during the first year (Ferrand & Gossman 2009).

In the American Woodcock *Scolopax minor*, females and males are separated on the basis of differences in the width of the first three primaries (Martin 1964, Artmann & Schroeder 1976) and the maximum wing chord measurement.

In the breeding period, Hoodless (1994) produced a discriminant equation with 94% of cases sexed on the basis of the three measurements bill, tail and weight. During the breeding season, the weight is a reliable measure to insert in a discriminant analysis because females are heavier prior to egg laying and after incubation (Hoodless 1994). However, outside this season, the weight is not a suitable variable because it fluctuates depending on season, prey availability, migration moult strategy (Fadat 1995, Gossmann & Ferrand 1998).

In our sample, we did not find any differences in weight between males and females in either age class as reported in a previous study (Fadat 1995). From our experience, tail length is not an easy trait to measure on live woodcock. The main capture technique used with this species in winter (dazzling) can cause accidental loss of some rectrices preventing accurate measurement of the tail.

The morphological characters used to sex Eurasian Woodcock in the past have to be revised with a view to their utility for sexing birds during ringing activities. The differences between age group and the season in which the measures are taken have to be stressed.

A potential problem with the discriminant function equations for sexing birds is the variation among the different bird-ringers in taking measurements. It is important to take standard measurements so that results are comparable among studies and data can be replicated by other workers as advised by Winker (1998).

The effective use of the discriminant analysis depends on the high accuracy rates of correctly assigned sex. For example, Sikora & Dubiek (2007) in Jack Snipe *Lymnocryptes minimus*, found a discriminant function, based on four morphological traits, reliable to sexing of 99% of individuals. De Marchi *et al*. (2012) produced a discriminant function that correctly classified 97.4% of Crab Plovers *Dromas ardeola* providing an efficient tool for sexing this species in the hand. Such high successes in sex identification may support this approach when DNA analysis is not be possible and non-invasive sampling is required.

Identifying morphological characteristics to improve the percentage of the Eurasian Woodcock sexed in the field during ringing activity could improve understanding of demographic issues and also yield valuable insights into their management and conservation.

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