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Sexing first-calendar-year Carrion Crows *Corvus corone* from biometrics reveals variation between years in post-fledging sex ratio

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ABSTRACT

Sex is an important parameter to consider when studying population dynamics and movement ecology, for example, though sex determination is often difficult in young birds of sexually monomorphic species, with large biometric overlap between sexes. We aimed at determining the sex of first-calendar-year (1cy) Carrion Crows, in order to study temporal trends in sex ratio. We performed molecular sexing of a sample of 48 females and 45 males, creating a statistical framework to confidently discriminate sexes using four morphometric variables. Although biometrics overlapped between sexes, we developed a discriminant function which separated the sexes with high accuracy (88%). Then we applied this discriminant function to biometrics obtained from all 1cy individuals captured, to determine their sex. We found a female-biased overall sex ratio of the captures: female Carrion Crows might therefore be more numerous than males, or more prone to enter traps. There were significant variations in sex ratio between years, but not between months.

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sex ratio; biometrics; Carrion crow; sexing; DNA sexing

Most bird species display sexual dimorphism, males being often larger and more colourful than females. Some species, however, are sexually monomorphic, such as many corvids. Yet, sex is an important parameter in ecological studies, such as demographic, behavioural or movement studies. In monochromatic species, it is sometimes possible to distinguish males and females by specific behaviours (Bavoux *et al* 2006), most obviously during copulation, or using external measurements. As examples, females are on average smaller than males in American Crow *Corvus brachyrhynchos* (Emlen 1936), Jack Snipe *Lymnocryptes minimus* (Sikora & Dubiec 2007), Purple Sandpiper *Calidris maritima* (Hallgrimsson *et al* 2008), and Lesser Black-backed Gull *Larus fuscus* (Hallgrimsson *et al* 2016). Differences between male and female biometrics can be used to sex Carrion Crows *Corvus corone* (Fletcher & Foster 2010), even if such a method sometimes introduces bias. For example, mistakes can be made during data collection, and a local, small population can display atypical size (Broughton & Clark 2017). In addition, morphometrics overlap between sexes (Demongin 2013), so that the accuracy of sexing is often stronger when a combination of variables is used, for example tarsus length, head, bill and wing lengths (Clark *et al* 1991, Fletcher & Foster 2010).

To link sex and biometrics, a classic approach is to develop linear discriminant analyses. This approach requires the initial determination of sex for a sample of individuals, sometimes implicating internal examination of kills (Slagsvold 1982, Fletcher & Foster 2010) or by invasive methods such as laparoscopy (Richner 1989a), but more commonly using non-lethal methods, like molecular sexing (Purwaningrum *et al* 2019) or body examination to record the presence of a brood patch or a cloacal protuberance (Sandoval & Mennill 2013). The discriminant function can further be used to predict the sex of other individuals with available morphometrics. The most frequently used parameters are bill length, bill depth, wing chord and tarsus length, and they generally provide better sexing when used together than alone (Devlin *et al* 2009, Shealer & Cleary 2009, Sandoval & Mennill 2013, Nana *et al* 2014, Jiménez *et al* 2015, Hallgrimsson *et al* 2016). Indeed, even if a unique biometric, such as bill length, is sometimes sufficient to discriminate males and females (Hallgrimsson *et al* 2008), this is hardly generalisable to many species (Hart *et al* 2009).

This method has been used on many bird species, as diverse as Loggerhead Shrike *Lanius ludovicianus*

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(Sustaita *et al* 2014), Western Mountain Greenbul *Andropadus tephrolaemus* (Nana *et al* 2014), terns (*Sterna forsteri,* Bluso *et al* 2006; *S. paradisaea,* Devlin *et al* 2009; *Chlidonias niger*, Shealer & Cleary 2009), sandpipers (*Calidris maritima,* Hallgrimsson *et al* 2008; *C. minutilla* and *C. mauri*, Jiménez *et al* 2015), gulls (*Larus californicus,* Herring *et al* 2010; *L. michahellis,* Hammouda & Selmi 2013; *L. fuscus*, Hallgrimsson *et al* 2016), penguins (*Pygoscelis adeliae,* Kerry *et al* 1992; *P. papua,* Renner *et al* 1998; *Eudyptula minor*, Arnould *et al* 2004), and various corvid species, such as American Crow (Clark *et al* 1991), Carrion Crow, Rook *Corvus frugilegus* and Western Jackdaw *Coloeus monedula* (Fletcher & Foster 2010). For their corvid study, Fletcher & Foster (2010) took measurements of dead crows and couldn't sex all individuals correctly due to the substantial size overlap between sexes, particularly among birds less than a year old. They focused on the biggest males and the smallest females, and had to analyse yearlings and adults separately. Furthermore, initial sexing was through observation of internal organs, which required working on dead individuals and provided no further possibility to study their behaviour and movements.

Sexually monomorphic birds can be sexed using molecular techniques, which require only a sample of DNA, extracted from blood or a feather. Feather sampling provides less DNA but is less invasive for birds and easier to implement in the field than blood sampling (Harvey *et al* 2006). The most common analytical method is to perform a PCR (polymerase chain reaction) of the CHD gene (chromobox-helicase-DNA-binding gene, a gene present in birds; Griffiths *et al* 1998, Cerit & Avanus 2007, Reddy *et al* 2007, Fukui *et al* 2008). PCR is a technique which involves amplifying the DNA of a given gene using primers. These primers are single-strand DNA molecules with the same sequence as a portion of the targeted gene, so that, after DNA denaturing, they hybridise with the DNA of the target gene. They are necessary to initiate DNA replication by the enzyme Taq polymerase. PCR requires two primers, one at the beginning of the gene and one at the end, and they delimit the gene region to be amplified. Three major primers have been developed for birds: P2/P8 (Griffiths *et al* 1998), 2550F/2718R (Fridolfsson & Ellegren 1999) and 1237L/1272H (Kahn *et al* 1998).

Table 1. Annual numbers of male and female Carrion Crows caught during 2016–21.

Year	2016	2017	2018	2019	2020	2021
Females	19	90	19	71	35	105
Males	15	47	24	54	13	50

To sex Carrion Crows, primers 2550F/2718R (Fukui *et al* 2008) and P2/P8 (Baglione *et al* 2002a) have already been used with success. In the present study, we used this technique to determine the sex of a sample of Carrion Crows captured in central Paris for ringing. To consider a homogeneous age class, we focused on individuals in their first calendar year. The males displayed significantly larger morphometric values than females but, to sex Carrion Crows with a good accuracy, we used a linear discriminant analysis to predict the sex of individuals, comprising bill height, bill length, wing chord and tarsus length. We further aimed at studying temporal trends in sex ratio. More precisely, we focused on monthly variations, to determine whether male and female first-calendar-year Carrion Crows show different mortality during the first months of life or if one sex disperses before the other. Dispersal is often biased towards females in birds, and indeed in northern Spain females Carrion Crow disperse before the males do (Baglione *et al* 2005, 2009, Canestrari *et al* 2012). We also studied annual trends in sex ratio. Significant variations between years could reflect sex ratios at hatching, or differential survival of chicks until or after fledging.

Material and methods

We trapped Carrion Crows in the Jardin des Plantes, Paris (48.84°N 2.36°E) from 2016 to 2021. We used a baited cage trap, shaped as a two-metre cube, which crows can enter but not escape through a horizontal ladder on the roof. To ensure a homogeneous sample of individuals, we restricted our study to first-calendar-year individuals. A further reason for investigating young birds is that they were excluded from previous work on sexing corvids from biometrics (Fletcher & Foster 2010). From 2016 to 2021, we captured and measured 542 first-calendar-year crows

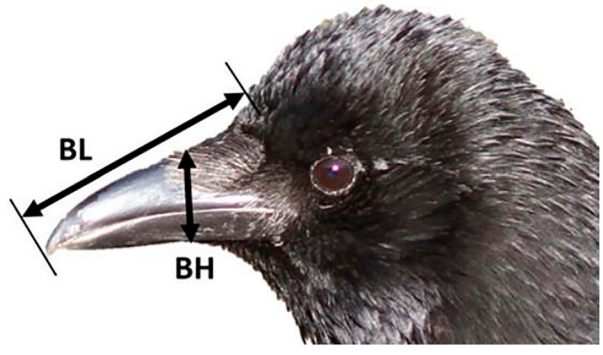

Figure 1. Bill measurements as used in this study: BL = bill length from tip to skull; BH = bill height at distal end of nostril.

during six post-breeding seasons, running from July to December (Table 1). All captures and bird processing were authorised by the French ringing centre (CRBPO) under the reference PP883.

Sampling and measures

For each bird captured, we measured four biometric parameters (to the nearest 0.1 mm; using a calliper for bill and tarsus and a rule for wing): bill height (BH, measured at the distal end of the nostril) and bill length (BL, from the base of the skull to the bill tip; see Figure 1), wing length (WL, as flattened wing chord) and tarsus length (TL). A single ringer (FJ) measured all individuals.

We performed molecular sexing for a set of 105 first-calendar-year individuals caught between 2019 and 2021. We collected at capture a few body feathers on the flanks or the mantle, favouring growing feathers as they might contain a larger amount of DNA. Feathers were kept in alcohol, to prevent them from drying, which would alter DNA.

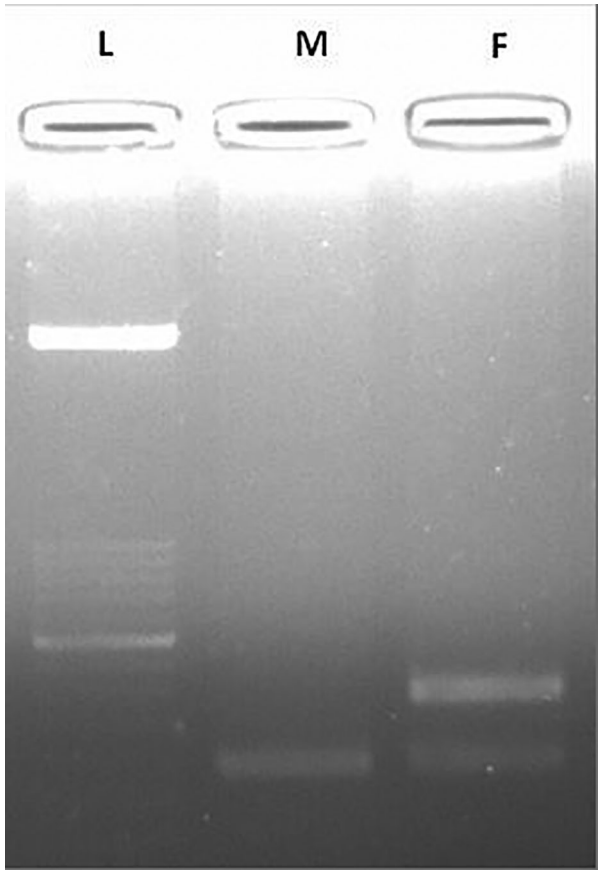

Figure 2. Imagery of the results of a PCR using P2 and P8 primers on DNA extracted from Carrion Crow feathers, followed by a digestion by Hae III: L, ladder, to show the size of the DNA fragment; M, male; F, female.

DNA sexing

Birds were sexed using gene CHD, which has two copies, one on chromosome W (present in females only) and one on chromosome Z (present in both sexes). That is why, after PCR, digestion by enzyme Hae III and gel migration, females display two dark bands on the gel and males only one (Figure 2). For the PCR, primers P2 (5'-TCTGCATCGCTAAATCC TTT-3') and P8 (5'-CTCCCAAGGATGAGRAA YTG-3') were used to initiate the replication of the target gene (Griffiths *et al* 1998).

Lysis

For each bird, we cut the calamus of the sampled feathers into small fragments before putting them in absorbent paper to remove alcohol. We added 180 µL of digestion buffer, 20 µL of proteinase K and 20 µL of dithiothreitol (DTT) to each sample, and we incubated them at 56°C overnight.

DNA extraction

The following day, after one minute's centrifugation at 3000 g, we extracted DNA by using the Eppendorf 5075 epMotion robot. We adjusted DNA binding conditions by adding 200 µL buffer BQ1 and 200 µL 100% ethanol to each sample, on a silica membrane. DNA was bound to the silica membrane by applying vacuum in the tissue binding plate (0.2 bars, five minutes). We washed the silica membrane three times, first with 600 µL buffer BW, and then twice with 900 µL buffer B5. After each wash, we applied vacuum (0.2 bars, five minutes). We washed the silica membrane by applying maximum vacuum (at least 0.6 bars, 10 minutes). We eluted DNA by twice applying 100 µL pre-heated buffer BE onto the membrane, then we incubated it for three minutes at room temperature and applied vacuum (0.4 bars, two minutes).

PCR

We prepared a premix for each sample with 12 µL water, 1 µL DMSO, 1 µL BSA, 0.5 µL of each primer (P1 and P2), 0.2 µL Taq polymerase and 2 µL Taq buffer $MgCl_2$. We added the mix of dNTP (6.6 µM) and 3 µL of eluates. We centrifuged 20 µL of premix with eluates for 30 seconds. We led PCR using an Applied Biosystems thermocycler (10 minutes at 94°C, 37 cycles, one minute at 52°C, five minutes at 72°C, 10°C).

We migrated eluates on a 2% agarose gel at 135 V during 15 minutes, to ensure that DNA had been amplified. We mixed 5 µL of PCR products with 0.5 µL of enzyme Hae III and 0.6 µL of enzyme buffer and put them in the thermocycler (60 minutes at 37°

C). After PCR gel migration we added 2 µL of bromophenol blue and glycerol to each digested sample, and put 6 µL of the resulting mix on a 2% agarose gel (with BET). A blank was used (4 µL control: mix without DNA + 1.2 µL blue). Samples migrated at 100 V during 30–40 minutes and we observed the migration with ultraviolet light.

Statistical analysis

Molecular sexing was successful for 48 females and 45 males, whose biometrics could be further used to develop the statistical analysis. We tested differences in biometrics between the sexes with two-sample t-tests, first only on birds sexed molecularly, then on the full sample, sexed by discriminant analysis.

We conducted a linear discriminant analysis (LDA) to determine whether BL, BH, WL and LT could predict the sex. We first used TL only, then TL and WL only, and finally all morphological parameters (TL, WL, BL and BH) to compare their accuracy. First, we developed the method on 50% of individuals (23 males and 24 females) and tested it on the remaining 50% (22 males and 24 females) to assess its efficiency in predicting the sex. Then, we implemented the function obtained with all parameters on all 542 individuals, including those which had been molecularly sexed, to obtain the global discriminant function best able to predict the sex of Carrion Crows and we tested its accuracy on all sexed individuals. Finally, we applied the discriminant function on all 1cy crows for which morphometric data were available.

We analysed the annual and monthly variations in sex ratio with two binomial generalised linear mixed models (GLMM), in which the response variable was the sex (1, female; 0, male). In the first model, the explanatory variable was the month, and the random effect was the year. In the second model, the fixed effect was the year and the random effect was the month. The AIC of each model was compared to the AIC of the corresponding null model to determine whether the probability of trapping a female or a male varied between months and years, respectively. The overdispersion was estimated by calculating the sum of squared Pearson residuals and comparing it to the residual degrees of freedom.

Results

Male and female morphological differences

We implemented a linear discriminant analysis on 48 females and 45 males, so prior probabilities were 0.51 for females and 0.49 for males. According to two-sample t-tests, the morphometrics of males were significantly larger than those of the females for the molecularly sexed individuals, but also for the individuals sexed by using the discriminant function (see below). Morphometric means, standard deviations, medians and statistical tests are presented in Table 2 and Figures 3 and 4.

Discriminant function test

The accuracy of the function obtained with WL only was 78%, while the accuracy of the function using WL and LP was 80%, and the accuracy of the function developed with WL, TL, BL and BH was 88%. We used the final function to further predict the sex of all captured individuals.

The discriminant function computes a score: positive for males, negative for females.

The function obtained on 50% of the crows was:

$$Score = -0.006 \times WL + 0.236 \times TL + 1.037 \times BH + 0.019 \times BL - 31$$

Its global accuracy was 85%, with 92% of the females and 77% of the males properly sexed. The confusion matrix is given in Table 3.

The function developed on the 45 males and 48 females was:

$$Score = 0.017 \times WL + 0.116 \times TL + 0.875 \times BH + 0.082 \times BL - 32.2$$

Its global accuracy was 88% (85% for the females and 91% for the males). The confusion matrix is also given in Table 3.

Sex prediction

We used the discriminant function to predict the proportion of males and females captured during 2016–21 (Figure 5, Table 1). In the month model, we obtained a better AIC value for the null model than for the full model (719.3 vs 720.3) and the *P*-value was not significant ($P = 0.087$, $\chi^2 = 8.108$). So the sex-ratio did not vary much across month. For the year model, the AIC of the full model was slightly lower than in the null model (717.5 vs 720.0), and the *P*-value was significant ($P = 0.014$, $\chi^2 = 14.186$, Figure 6). Thus, we find an effect of year on the sex ratio.

There was no other dispersion in binomial models ($P > 0.4$, ratio = 1.0, $\chi^2 = 540.6$ in the month model, $P > 0.4$, ratio = 1.01, $\chi^2 = 542.0$ in the year model).

Table 2. Tarsus length, wing length, bill length and bill height of crows sexed either by DNA sexing or by linear discriminant analysis, and values from the literature (Demongin, 2013). Values are given in mm; *P*, t and df were obtained with a two-sample t-test to compare biometrics of males and females.

	Tarsus length						Wing length					
	Mean (± SD)	min	max	*P*	t	df	Mean (± SD)	min	max	*P*	t	df
DNA sexing												
Female	55.1 ± 3.0	48.8	62.6	<0.001	5.8	89.7	304.0 ± 10.7	277	330	<0.001	7.6	100.9
Male	58.4 ± 2.5	50.1	62.4				318.6 ± 9.6	290	337			
Discriminant function												
Female	54.7 ± 2.9	44.7	60.0	<0.001	17.6	478.5	304.79 ± 9.5	271	328	<0.001	19.5	448.0
Male	58.8 ± 2.5	50.1	63.8				320.5 ± 9.0	286	339			
Literature												
Female	-	52.8	60.0				-	283	335			
Male	-	58.0	65.4				-	298	345			
	Bill length						**Bill height**					
	Mean (± SD)	min	max	*P*	t	df	Mean (± SD)	min	max	*P*	t	df
DNA sexing												
Female	55.6 ± 2.7	50.6	62.9	<0.001	6.5	91.8	17.1 ± 0.8	15	18.7	<0.001	8.5	91.9
Male	59.0 ± 2.4	53.1	63.3				18.5 ± 0.7	16.9	19.9			
Discriminant function												
Female	55.2 ± 2.2	50.4	61.2	<0.001	20.4	439.1	16.9 ± 0.7	14.3	18.7	<0.001	25.2	455.8
Male	59.1 ± 2.1	54.0	65.7				18.5 ± 0.7	17.1	20.1			
Literature												
Female	-	49.5	58.5				-	17.0	18.5			
Male	-	52.0	63.0				-	17.9	20.5			

Discussion

We developed a discriminant function allowing us to determine the sex of Carrion Crows, a monomorphic bird species, using a combination of four morphometric parameters (bill height, bill length, wing chord and tarsus length), with a high accuracy (88%), similar to accuracies reached in other studies and species, which ranged from 73% to 99% (Devlin *et al* 2009, Sandoval & Mennill 2013, Nana *et al* 2014, Hallgrimsson *et al* 2016). The highest result has been obtained for species which display a slight dimorphism, for example Western Sandpiper *Calidris mauri* (Jiménez *et al* 2015).

The sex ratio might be biased and not strictly mirror the real value for Carrion Crows visiting the capture site,

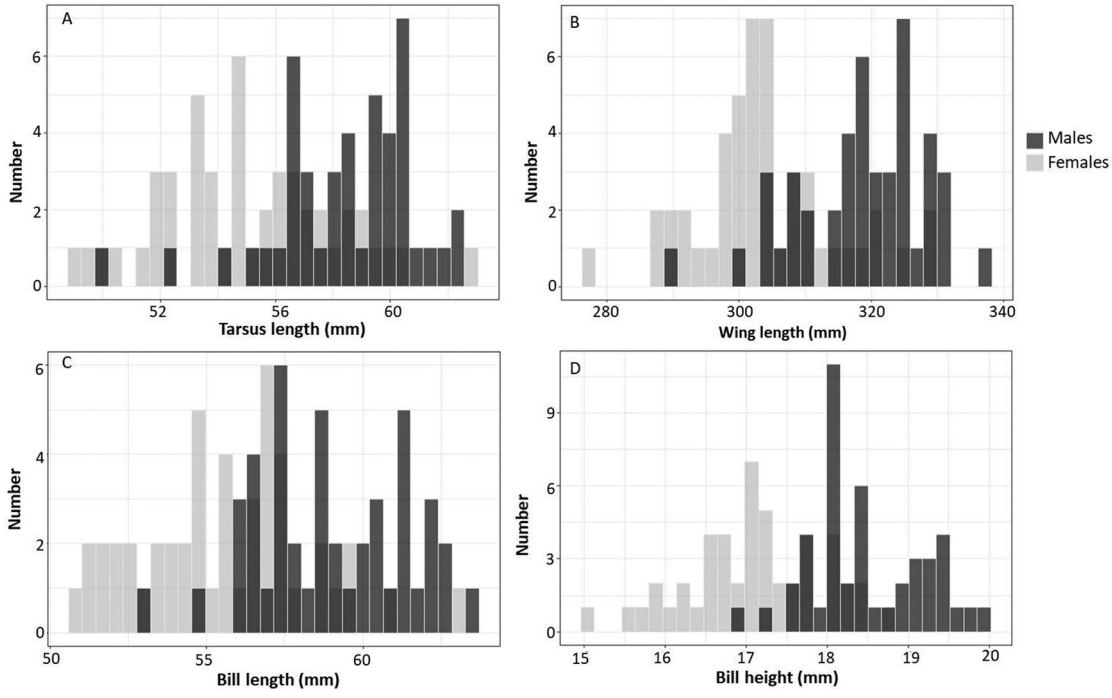

Figure 3. Frequency distribution of morphometric values of molecularly sexed Carrion Crows: (a) tarsus length, (b) wing length, (c) bill length, (d) bill height; 48 females, pale bars; 45 males, dark bars.

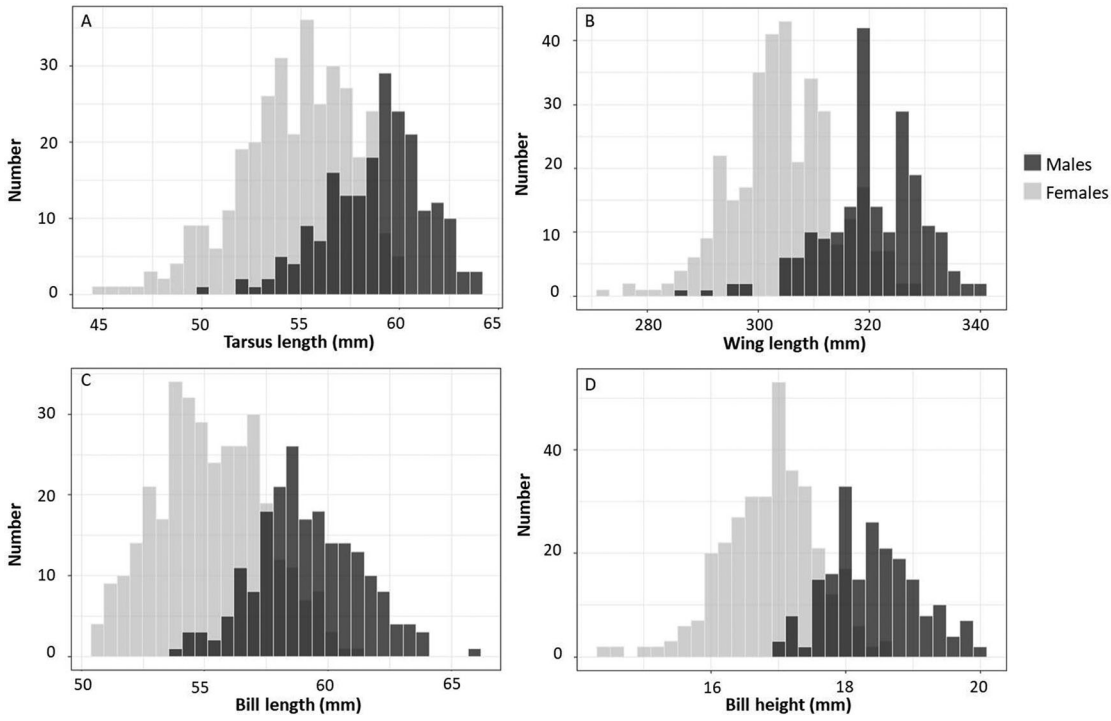

Figure 4. Frequency distribution of morphometric values of 542 Carrion Crows sexed by discriminant analysis: (a) tarsus length, (b) wing length, (c) bill length, (d) bill height; females, pale bars; males, dark bars.

because of potential heterogeneities in capture probabilities between sexes, age classes, or resident and visiting individuals. It could reflect the proportion of males and females that enter the trap, which can be influenced by potential differences in behaviour, food foraging, and propensity to join a captive flock of conspecifics. For example, females may be less fearful or more active in food foraging than males. Proportions of males and females trapped can also depend on food availability and competition. Individuals that are less competitive and aggressive, maybe females, could be more prone to access supplementary food and enter the trap. On the contrary, dominant males could be the first to enter the trap, according to a group hierarchy to access food resources. Indeed, a study showed that dominant males were the first to make contact with novel food (Chiarati *et al* 2012). More generally, dominance–subordination relationships influence access to resources and rates of survival (Richner 1989b). This effect could increase in case of low 'natural' food availability, and it could be interesting to study whether the probability to enter the trap is condition-dependent. Although body mass was not used in the present analysis, it could be informative to estimate and compare the body condition of trapped males and females.

Table 3. Confusion matrix of the discriminant functions: (a) developed on 50% of the molecularly sexed crows and tested on the remaining 50%; (b) developed and applied on the 93 molecularly sexed individuals (45 males and 48 females).

Predictions	Females	Males
(a)		
Females	22	5
Males	2	17
Accuracy	91%	77%
(b)		
Females	41	4
Males	7	41
Accuracy	85%	91%

The sex ratio of crows caught during 2016–21 seemed to vary significantly across years. However, it didn't vary across months, so the survival of 1cy individuals after fledging does not seem to be sex dependent, and one sex does not appear to disperse before the other. This is not the case in Carrion Crow populations in northern Spain, where females disperse before the males, which are more prone to delay their dispersal (Baglione *et al* 2009). Indeed, they stay with their parents the following year more often than females, and help their parents rear the chicks (Baglione *et al* 2002b, 2009, Canestrari *et al* 2012). This delayed dispersal is not very common, and occurs mainly in northern Spain and in Italy, whereas young usually disperse during the first autumn or winter after fledging (Baglione *et al* 2005).

The variation between years could rather be driven by changes in sex ratio before the chicks leave the nest. First, the sex ratio of eggs could vary depending on environmental conditions. Then, the nest survival

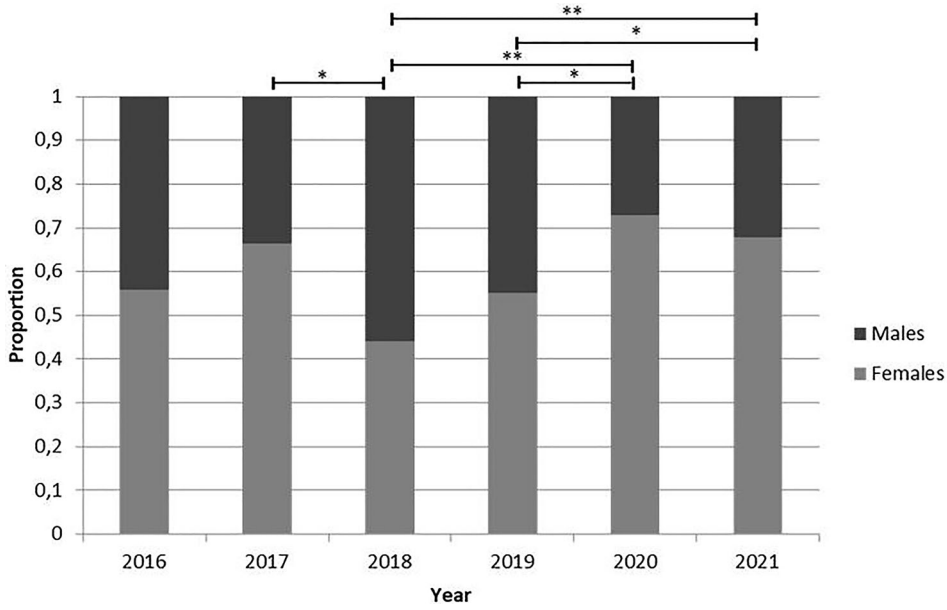

Figure 5. Annual proportions of males and females within 542 first-calendar-year Carrion Crows caught during 2016–21 and sexed by discriminant analysis. Significant differences between years are indicated: * $P < 0.05$; ** $P < 0.01$; two-sample t-tests.

of male and female chicks is susceptible to vary differentially depending on resource availability. For example, young males may be more dominant than females, as they are bigger, and more prone to access food in the nest (Richner 1989b, Canestrari *et al* 2012). Sex could also impact the ability to survive with low resources: the biggest chicks, probably males, might be less affected by food restriction, because they can survive on their reserves, or, on the contrary, they may need more food than smaller chicks, and so be more sensitive to starvation. Finally, the differential mortality could occur after fledging. For instance, road accidents are a major source of mortality for Carrion Crows fledging in urban areas, as they leave the nest before being able to fly well: traffic density is likely to vary from year to year, especially since the Covid pandemic that began in 2020, which increased working from home and reduced traffic density. Yet, one sex may be more prone to accident than the other. This study was conducted on urban crows only, and it would be interesting to perform the same analysis on rural crows, in order to compare the results.

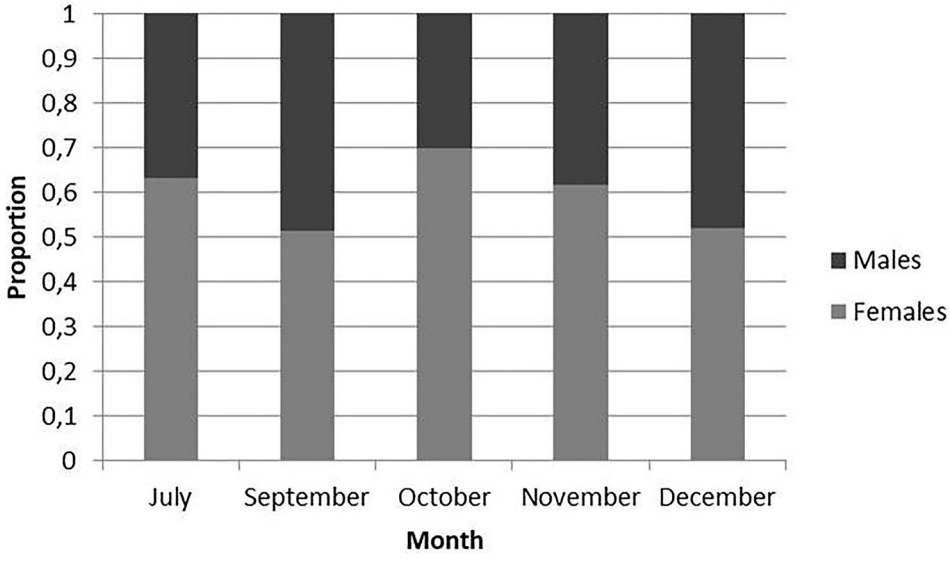

Figure 6. Monthly proportions of males and females within 542 first-calendar-year Carrion Crows caught during 2016–21 and sexed by discriminant analysis.

Our morphometric values were similar to those found in the literature (Fletcher & Foster 2010, Demongin 2013), even if the difference between maxima was higher in our study (Table 2). Morphometric values of male and female Carrion Crows do overlap, but a linear discriminant analysis allowed the sexing of 1cy crows, using DNA sexing as a non-lethal method. Some errors could occur during sexing, as molecular sexing is not always totally reliable. For example, because of allelic dropout, females can be mistyped as males (as they display only one band on the gel instead of two) (Casey *et al* 2009), whereas CHD-Z polymorphisms can lead a male to be sexed as a female (Dawson *et al* 2001). Indeed, polymorphisms lead to two different CHD alleles on each Z chromosome of the males. If mutations cause deletions, the fragments amplified by PCR can have different sizes and result in two bands on the gel, instead of one. The DNA fragments can also interact during PCR and create heteroduplex, which also leads to mistakes (Casey *et al* 2009). The region amplified by primers P2/P8 is particularly prone to deletions, which is why some species (ratites for instance; Casey *et al* 2009) cannot be sexed with these primers. Nevertheless, this set of primers is suitable for Carrion Crows (Baglione *et al* 2002a).

To increase the reliability of this technique, some improvements have been made. For example, new primers have been developed to increase the accuracy of molecular sexing, some of them permitting the sexing of museum specimens (Bantock *et al* 2008). A specific marker of W chromosome made it possible to sex a species which cannot be sexed by traditional methods (Shizuka & Lyon 2008). Finally, other molecular PCR-based techniques can be used to sex birds, such as single-strand conformation polymorphism (SSCP) or microsatellites (Morinha *et al* 2012).

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