Reliability of morphological criteria for sexing of birds during ringing, assessed using molecular methods - a study of thirteen species of passerines and near-passerines.

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Summary

Understanding the ecology and conservation of bird species often requires accurate sex determination of individuals. Species with sexually dimorphic plumage can usually be sexed in the hand based on consistent and definitive differences in plumage between sexes, but there are often challenges related to (i) how sexual dimorphism develops with age (e.g. juveniles are often impossible to sex based on morphology), (ii) individuals that show intermediate visual morphological traits, or (iii) consistent but subtle trait differences that require considerable experience to identify reliably. Species with sexually monomorphic plumage (e.g. over half of all avian species globally) pose an even greater challenge, and can often not be sexed in the hand. The aim of this study was to use molecular methods to identify definitively the sex of individuals of both monomorphic and dimorphic species caught at a ringing site in south-west Portugal, in order to evaluate the standard morphological sexing techniques for species showing sexual dimorphism in plumage, or in biometric measurements. Blood samples were collected from a range of species during ringing, and DNA was extracted. Molecular methods were successful in identifying the sex of 202 individuals across 13 species of birds (eight species with sexually dimorphic plumage, and five species with sexually monomorphic plumage). Molecular methods were consistent with the morphological sexing in the field for six of the eight species with dimorphic plumage. However, discrepancies between the two methods were identified for Pied Flycatcher and Eurasian Hoopoe. Finally, biometric measurements taken in the field were used to assess whether species with monomorphic plumage could have been correctly sexed based on biometric differences between males and females reported in literature.

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Introduction

The marking and identification of individual birds using metal rings dates back to the 1890's. Over a century later, ringing / banding has become a global scientific method of studying bird species, with over four million birds being ringed every year in Europe alone (EURING 2007). Using bird ringing as a scientific research method is effective when studying many aspects of avian biology, including survival, population change, migration and behavioural ecology (Korner-Wievergelt *et al* 2014).

A standard practice of all ringing schemes is to record, when possible, the sex of the birds ringed. Knowing the sex of an individual is crucial in wide ranging fields of study including ecology, behaviour, genetics, and conservation biology (Çakmak *et al* 2017). The difficulty and uncertainty of sex determination creates a considerable problem in population and conservation studies (Çakmak *et al* 2017). Birds with visually monomorphic plumages pose the greatest problem as they cannot readily be sexed in the hand. 50-60% of bird species have sexually monomorphic plumage in both juvenile and adult stages (Price & Birch 1996, Griffiths *et al* 1998).

Even sexually dimorphic species may pose a problem in some circumstances. In some species, such as the House Sparrow, *Passer domesticus*, in which the adults are clearly dimorphic, the plumage of juvenile birds is very similar to that of females. Therefore, adult males can be more confidently sexed than adult females, or juveniles of either sex. Aging of the bird using plumage characteristics will allow correct identification between adult females and juvenile birds, although juveniles will remain unsexed until they complete their post juvenile moult. Most passerine birds – including otherwise monomorphic species - can be sexed by the presence of an incubation patch in females or cloacal protuberance in males (Jones 1971, Quay 1986). This sexing method which is generally classed as reliable does require caution, as 6% of Marsh Tits, *Poecile palustris* in the British Trust of Ornithology (BTO) were incorrectly sexed using incubation patch and cloacal protuberance (Broughton & Clarke 2017). However, these criteria can only be used during the breeding season.

In the past, researchers have identified the sex of birds with monomorphic plumage by sacrificing individuals for dissection and sex identification based on internal anatomy (Kalchreuter 1971). Berthold (1969) used a small incision into the body cavity of living individuals to observe the gonads. Biometric and molecular techniques offer a more ethical, less invasive set of methods for identifying sex.

It is sometimes possible to sex species using biometric measurements such as wing length, tarsus length, or other measures of structural body size (Svensson 1992). However, sexing methods based on biometric measurements do not always guarantee correct sex identification. Ellrich *et al* (2010) used logistic regression to sex passerines over large geographical ranges using morphological traits and found that sexing of Garden Warblers (*Sylvia borin*) was unreliable, whilst the majority of the European Robin (*Erithacus rubecula*), Eurasian Reed Warbler (*Acrocephalus scirpaceus*), Reed Bunting (*Emberiza schoeniclus)* and Willow Warbler (*Phylloscopus trochilus)* were sexed correctly. However, not all individuals could be sexed due to overlap in morphological traits between males and females.

Catry *et al* (2005) used morphometric characteristics such as the bi-modal distribution of wing length in Common Chiffchaff (*Phylloscopus collybita*) to investigate differential distance migration of sexes; males generally have longer wing length but there is a small overlap between the sexes, this means that only birds with extreme wing lengths can be sexed reliably. Using morphometrics, Norman (1983) was able to sex 95% of adult Willow Warblers and 90% of first year birds, showing morphology leaves a small proportion of the population unsexed. Similarly, it is possible to sex a large proportion of Marsh Tit using a threshold of 62/63 mm wing length to distinguish the sexes, which was successful for 92-96% of individuals in a number of studies (King & Muddeman 1995, du Feu & du Feu 2014, Broughton *et al* 2008, Broughton *et al* 2016). A small proportion of birds in these studies were left unsexed. The same sexing criterion was applied to the whole BTO database, identifying that approximately one third of the birds had been incorrectly sexed (du Feu & du Feu 2014, Robinson 2015, Broughton et al 2016). This implies that biometric rules can differ between datasets. Additionally, wing length measurements are not always consistent; in the BTO database, 43% of Marsh Tit wing lengths measured from recaptured individuals differed from their initial measurement (Broughton & Clarke 2017). Where biometric differences between sexes are marginal and/or overlap in measurements is substantial, the percentage of unsexed individuals may be much higher. For example, Madsen (1997) was unable to sex 51% of European Robin as their wing-length was intermediate between the criteria for reliably identifying males and females.

The effectiveness of morphometric sexing criteria may also vary geographically, if there are morphometric differences between populations, or a cline in morphometric measurements (Broughton *et al* 2016a; McCollin *et al* 2015). For example, females of Common Blackbirds, Turdus merula, and males of Song Thrush, Turdus philomelos, exhibit a latitudinal cline in measurements, which larger individuals at higher latitudes in the former and an increase in wing length towards the north of their range in the latter (McCollin *et al* 2015). As a result,

morphometric sexing criteria developed in one part of the species' range may not apply in other locations.

An alternative approach to sexing birds is using molecular methods, based on sex differences in the DNA of male and female birds. DNA can be extracted from faeces, feathers and/or blood. Faecal samples can be time consuming to collect and there is no guarantee of collecting data from every individual. DNA extracted from feathers of birds has been successfully used for molecular sexing (Medeiros *et al* 2012; Çakmak *et al* 2017). However, the amount and quality of the DNA obtained can vary with the number of feathers plucked and the freshness of plumage (Çakmak *et al* 2017). Therefore, more feathers are required to achieve a high quantity and quality of DNA to determine sex, which may be deemed as more traumatic for the bird than a single blood sample (McDonald & Griffiths 2011). Feathers which are not collected freshly are at risk of DNA degradation, therefore they are a less reliable source of DNA (Maurer *et al* 2010; McDonald & Griffiths 2011). Comparatively, blood sampling may be a more invasive methodology and challenging to carry out with passerines due to their relatively small size. Nevertheless, blood sampling has been demonstrated experimentally to be relatively safe when performed by skilled practitioners (McDonald & Griffith 2011) and it is the most reliable and straightforward source of DNA for molecular sexing in the laboratory (Griffiths *et al* 1998).

The sex chromosomes in birds are Z and W, the female is heteromorphic (ZW) and the male is homomorphic (ZZ) (Stevens 1997). The sex-linked CHD gene is used for sex identification. Molecular sex identification methods have been developed using the polymerase chain reaction (PCR) to amplify DNA extracted from samples obtained in the field (Griffiths *et al* 1998, Fridolfsson *et al* 1999, Lee *et al* 2010). Primers specifically anneal to various regions of the DNA and are amplified during PCR (Wang *et al* 2010). The process is followed by gel electrophoresis which enables the bands of primers to be visible under UV light after separation across the gel.

Different primer combinations have been trialled for various bird species. The primer combination P8/P2 was initially designed to target the CHD gene in the domestic chicken (*Gallus gallus domesticus*, Griffiths *et al* 1998). Additional primers have been developed including 2550F/2718R (Fridolfsson *et al* 1999) and P8/M5. Bantock *et al* (2008) used P8/M5 to successfully identify the sex of 90% of Moorhen (*Gallinula chloropus*) specimens from museum collections, using specimens collected across dates ranging from 1855-2001. After a comparison of three primer sets: P8/P2 (Griffiths *et al* 1998), CHD1F/CHD1R (Lee *et al* 2010) and 2550F/2718R (Fridolfsson *et al* 1999), Çakmak *et al* (2017) concluded that all three

primer sets can be used on monomorphic avian species, although success rate varied between avian orders. Success rate of P8/P2 improved after using capillary analysis, which involves running PCR product on a capillary gel with a fluorescent dye, allowing two fragments with a similar length to be identified by peak size. Female bands which could not be separated on the agarose gel could be separated using capillary analysis into two distinguishable peaks. Therefore, capillary analysis is a useful tool when band separation on agarose gel is not possible. The range of primers developed reflects the amount of ongoing research into bird sexing. As numerous species are monomorphic, there is a need for primers suitable for molecular sexing of a wide range of species.

The present study compares the results of molecular and morphological methods of sex determination at a bird-ringing station in SW Portugal, where a large number of individual passerines and near passerines could not be sexed morphologically. The aims of the project are 1) to confirm the sex-specific characteristics of dimorphic species, allowing an evaluation of morphological sexing criteria, 2) to identify the sex of monomorphic species, and 3) to investigate biometric differences between sexes of monomorphic species sexed through DNA, to compare with differences described using other methodologies.

Methods

Study site

The study was conducted at A Rocha Portugal field centre and bird ringing station, located ~1km from the coast in the Algarve region of southern Portugal (37° 8'40.26"N, 8°36'28.64"W). Ringing at A Rocha field centre started in 1987, making it one of the longest running ringing stations in Portugal, with a database of over 80,000 individual captures. The ringing site is a large well-vegetated garden, surrounded predominately by agricultural fields consisting mainly of livestock pasture and near one of the largest wetlands in the western Algarve. Sampling was carried out on 34 days between 30th September 2017 and 29th March 2018, which included autumn migration and the winter period, but excluded the spring breeding season. Avoiding spring time meant there was no risk of keeping adults away from their nests at a critical time. To minimise impacts on breeding individuals, towards the end of the sampling period when females started to develop a brood patch, sampling of that species was stopped.

Between September 2017 and October 2017 there were frequent ringing sessions (four-five times week), and after that period ringing was carried out weekly until March 2018. Mist nets were open from sunrise until noon, when weather conditions allowed. The nets were checked every hour from dawn, and as the ambient temperature increased later in the morning nets

were checked every half hour. A total of 147 m of mist-nets were used for each ringing session, covering a variety of habitats including next to ponds, *Phragmites* reed beds, a small *Citrus* orchard and under pine trees (*Pinus* spp.) surrounding the A Rocha field centre. Tape lures were used all year round until 25th March 2018, when a constant effort ringing scheme was initiated. The small speaker (5V Audiosonic model SK61523) was used to attract birds that were already present in the garden; the speaker played calls of Willow Warbler, Common Chiffchaff and Eurasian Blackcap (*Sylvia atricapilla*).

Only birds in apparent good health were blood sampled: if the individual was underweight or appeared in bad condition or stressed it was not sampled. Furthermore, no birds were sampled during busy periods when numerous individuals were captured, to ensure the birds were not kept in the holding bags for a long time.

Species and sample size

The sample species were determined by analysis of the ringing database in order to identify species which provide a large enough sample size for the study. The number of individuals of each species caught annually between October and May from 2007 to 2012 was assessed in combination with ensuring the inclusion of monomorphic and dimorphic species. This initial analysis identified thirteen species as suitable for the main study. Of these, five species are sexually monomorphic in terms of plumage: Common Chiffchaff, Willow Warbler, European Robin, Garden Warbler, Iberian Magpie (*Cyanopica cooki*). Three species can be sexed based on subtle differences in colouration: Pied Flycatcher (*Ficedula hypoleuca*), Eurasian Hoopoe (*Upupa epops*) and Common Kingfisher (*Alcedo atthis*). The remaining five species are sexually dimorphic as adults: Common Blackbird, Common Chaffinch (*Fringilla coelebs)*, Eurasian Blackcap, House Sparrow, and European Goldfinch (*Carduelis carduelis*).

Ringing and Biometrics

All captured individuals were identified to species level, ringed, aged, sexed (if possible based on plumage features), and measured following the criteria given in Svensson (1992) and Demongin (2016). Sex was determined for dimorphic species using morphological criteria. Age was determined mainly by feather wear or moult limits within feather tracts. The biometric measurements taken were body mass, wing length, tarsus length, bill depth (measured at the tip of the foremost feathers at the base of the forehead, Svensson 1992, measurement “e” in Demongin 2016) and bill length (bill tip to feathers, Svensson 1992, measurement “c” in Demongin 2016). Measurements of mass were recorded to the nearest 0.1 g using digital scales. Wing length was measured to the nearest 1mm using a stopped wing ruler (British

Trust for Ornithology). Bill depth, tarsus length and bill length were measured to 0.01 mm using an electronic digital calliper (Powerfix).

Blood Sampling

Blood sampling and ringing permits were approved and obtained from the Instituto da Conservação da Natureza e das Florestas (ICNF), Portugal. A small sample of blood was collected onto filter paper from the brachial vein using a small needle prick. Blood was stored on filter paper in a 1.5 ml tube filled with 100% ethanol in a freezer (-20°C). The birds were sampled at the site and released in good condition shortly after capture.

Molecular Analysis

The Chelex extraction method (Walsh, Metzger and Higuchi, 1991) was used to extract DNA from the blood samples. A section of the filter paper containing blood was added to 50 µl of distilled H_2O, to which 20 µl of InstaGene Matrix (BioRad) was then added. The samples were heated to 50°C for 30 minutes, then to 100°C for 8 minutes. The InstaGene Matrix contains a chelex resin, which binds to PCR inhibitors produced in cell lysis as the samples are heated, leaving the DNA as supernatant and ready for use in PCR (BioRad).

Primer sets have been previously designed to bind to the sex specific CHD-W gene present on the W chromosome and CHD-Z present on the Z chromosome. The primers then amplify different sequence lengths, allowing sex identification at the later stage of gel electrophoresis. Primer combinations were trialled on the samples in order to find the best primer for each species. The primers used were P8/P2 (Griffiths *et al* 1998), 2550F/2718R (Fridolfsson *et al* 1999) and P8/M5 (Bantock *et al* 2008). The chosen primers which were most effective for the range of passerines and near passerines in the present study were P8/P2 (Griffiths *et al* 1998) as they provided a distinct band separation. All PCRs were carried out in a 5 µl reaction volume containing 1x QIAGEN Multiplex PCR master mix, 0.2 µM of each primer, 0.1 µM of Bovine serum albumin (BSA), and 1 µl template DNA. The PCR machine (Applied Biosystems) was programmed to run for 15 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 90 seconds at the primer-specific annealing temperature of 50°C, 90 seconds at 72°C, and ending with 10 minutes at 72°C. Positive and negative controls were used in the PCR to ensure there was no contamination or any problems with the PCR. Extraction negatives were also tested to ensure there was no contamination during the extraction process.

After adding 4 µl of gel loading dye (Biolabs) the samples were run on a 3% agarose gel with SYBR safe (Thermofisher) for 90 minutes. Gel electrophoresis separated the DNA into bands: two bands indicated female and one band indicating male (Appendix 1). All individuals initially

identified as male were retested to ensure there was no error in band amplification. For European Robin and Eurasian Hoopoe, the bands did not separate well on the agarose gel and so Qiaxel (QIAGEN) capillary electrophoresis was used instead. Capillary electrophoresis was also used to confirm any other samples for which bands were not clearly separated on the agarose gel.

Data Analysis

Statistical analysis was undertaken using the statistical software R (R version 3.3.3, R Core Team 2017). Female sex ratio for each bird species was calculated from the molecular sex data, and deviations from the expected 50:50 ratio were tested for statistical significance with a chi-squared test.

Biometric analysis of monomorphic species (European Robin, Garden Warbler, Willow Warbler and Common Chiffchaff) was dependent on sample size: no meaningful analysis could be completed for Garden Warbler and for Willow Warbler. For European Robin, individual t-tests were used to assess biometric differences between males and females, while for Common Chiffchaffs, due to a larger sample size, it was possible to carry out a logistic regression to "explain" sex (the binomial dependent variable) and consider all biometrics (the independent variables) in combination. To do this, a generalised linear model (GLM) with binomial error family and logit link function was fitted to the data (dependent variable = male/female; predictors = wing length, tarsus, bill length and bill depth). The model contained the independent variables of wing length, tarsus, bill depth and bill length. The model was refined by backwards stepwise deletion. The threshold for significance was $P<0.05$ for all statistical tests.

Results

A total of 454 individuals of the 13 species of interest were caught during the sampling period. During the study a total of 202 of these birds had blood samples taken and were sexed by molecular methods. Recapture of birds which had been blood-sampled (33 recaptures, involving 26 individuals of eight different species) allowed the health of the bird to be monitored – all such birds appeared healthy on recapture, with the small needle-wound healed.

Molecular sexing

The P8/P2 primers (Griffiths *et al* 1998) successfully identified the sex of all 202 individuals. In total, 182 birds were sexed using the agarose gel with the two Z and W bands clearly separating on the gel for females of all species apart from only European Robin and Eurasian Hoopoe (Appendix 1). These two species were therefore sexed using the Qiaxel machine with

the same P8/P2 primers, which allows differences as small as 20 base pairs between DNA bands to be detected. The differences in base pairs between the Z and W band varied between 36 bp and 92 bp (Appendix 2). Figure 1 shows the sex ratios found across the 13 species through molecular sexing (actual values are presented in Appendix 3).

The most extreme sex bias was found in the Common Chaffinch and Common Kingfisher where 100% of individuals were identified as female ($X^2 = 6$, d.f. = 1, $p = 0.014$ and $X^2 = 4$, d.f. = 1, $p = 0.046$, respectively), followed by 72.2% for Willow Warbler ($X^2 = 3.6$, d.f. = 1, $p = 0.059$) and 66.7% for Common Chiffchaff ($X^2 = 4$, d.f. = 1, $p = 0.0456$). Garden Warbler, European Robin, Iberian Magpie and Eurasian Hoopoe showed male biased sex ratios ranging from 67% to 75% but these were not significant (all p-values ≥ 0.132, Appendix 4). All other species had sex ratios very close to 50:50.

Morphological sexing using plumage features

Out of the 202 birds which were sampled, only 116 individuals (57.4%) could be sexed using morphological criteria based on sex differences in plumage. For 112 of these 116 individuals (96.6%), the molecular sexing result agreed with the morphological criteria. The four individuals for which the morphological sexing differed from the molecular sexing were three Pied Flycatcher and one Eurasian Hoopoe.

A total of 17 individual Pied Flycatcher were sampled, but only seven individuals could be sexed based on plumage features. Out of these seven individuals, three (42.9%) were found to have been sexed incorrectly using plumage criteria. One individual, aged as juvenile, was sexed as male with the molecular method but sexed as a female using the plumage criteria. The other two individuals were sexed as females with the molecular method but sexed as males using the plumage criteria; one of these individuals was aged as a juvenile and the second as an adult.

A total of four Eurasian Hoopoe were sampled, with only three of these individuals sexed using morphological criteria. After applying the molecular method, one bird was found to have been sexed incorrectly using plumage criteria. It was sexed as a female and aged as a juvenile in the field, but was male according to molecular method.

Sexing using Biometric measurements

Differences in biometrics between males and females which are monomorphic or sexed using subtle differences were compared statistically, with the exception of Common Kingfisher, Iberian Magpie and Eurasian Hoopoe for which sample sizes were too small (n < 5 individuals).

These results are summarized in Table 1. In our sample there was a significant difference between males and females in wing length for Willow Warbler and Common Chiffchaff; there were no other significant differences for other biometrics for these species or for any biometrics of the other species tested (Table 1). Our results indicate that male Willow Warbler have wings 3.6 mm longer on average than females (range: 64-71 mm for 5 males and 62-68 mm for 13 females), while male Common Chiffchaffs have wings 4.7 mm longer on average than females (range 59-64 mm for 12 males and 53-61 mm for 24 females, with one female presenting an atypically long wing length of 65mm).

Literature presenting differences in biometrics between males and females is available for Common Chiffchaff (Svensson 1992, Demongin 2016), Willow Warbler (Svensson 1992, Demongin 2016), European Robin (Svensson 1992, Madsen 1997, Demongin 2016), Pied Flycatcher (Demongin 2016), Common Kingfisher (Baker 2016) and Eurasian Hoopoe (Demongin 2016, Baker 2016). Criteria provided for juvenile Eurasian Hoopoe did not allow sexing due to overlap of the female and male wing lengths. Table 2 summarizes the success rate in sexing these birds in our sample based on biometric differences from available literature. Willow Warblers showed the highest proportion of birds that would be sexed correctly based on biometrics (72%), while less than 60% of Common Chiffchaff and 55% of European Robin (at best) would be sexed correctly based on biometric differences (Table 2). Incorrectly sexed birds through morphometric sexing included, two male and two female Willow Warbler and one female Common Chiffchaff classified as the opposite sex. For the European Robin, either two or five individuals were wrongly sexed depending on the morphometric criteria used (Table 2).

Discussion

Comparison of morphological sexing with molecular sexing

Molecular sexing was successful for all 13 species in this study using the primers P8/P2 (Griffiths *et al* 1998). For seven out of the nine species in the present study that have a degree of sexual dimorphism there was 100% agreement between molecular sexing and the morphological criteria, based on plumage differences between the sexes. This provides confidence in the sexing techniques used in the field but also highlights the difficulty found for two of the species, namely the Pied Flycatcher and Eurasian Hoopoe. Both species are normally sexed by the colouring of the plumage of the two sexes, rather than biometric measurements, which show substantial overlap between the sexes. Plumage colouring can change substantially as the feathers become worn, sun-bleached or damaged, which increases the difficulty of identifying differences in colour for each sex. Light levels at the time

of sexing (e.g. direct sunlight or shade) can also affect perception of the plumage colouration. In addition, different ringers may have different eyesight performance, meaning that their colour perceptions may differ.

Morphological sexing based on plumage colouration is likely to be even more challenging for juvenile birds due to feather wear; for example, in Collared Flycatcher (*Ficedula albicollis*) females and young birds have more worn feathers compared to males at the end of the breeding season (Merilä & Hemborg 2000). Indeed, three of the four incorrectly sexed birds in the present study (two Pied Flycatcher and one Eurasian Hoopoe) were aged as juveniles. Sexing of Eurasian Hoopoe is the same all year round, with males having a pink chin and breast and a pinkish mantle, whereas females have a cinnamon chin and breast with only a pinkish tinge in the mantle. The females show more striped feathers on the sides of the belly and breast compared to the males (Demongin 2016). These differences are easier to perceive when there is a direct comparison of male and female next to each other is possible. Juveniles are even more difficult to sex and can only be sexed with confidence when there is distinct male-type or female-type colouration -but many show intermediate colouration.

The Pied Flycatcher sampled in this study were sexed according to the plumage criteria in Demongin (2016). By the time they reach SW Portugal in autumn, the adult Pied Flycatcher have undergone their post breeding moult. At this time, adult males have black central tail feathers and upper tail coverts, whereas adult females have brownish central tail feathers and upper tail coverts. Juveniles can only be sexed after their post juvenile moult, after which males have black central tail feathers and upper tail coverts, whereas these feathers are brown in females. However, it is not always possible to sex individual juveniles with intermediate coloured tail feathers. Additional plumage features include the pattern of colouration of tail feathers five and six; males show a squared edge of white colouration, whereas females show a diffused edge. These small differences in sexing criteria can be difficult to interpret in the hand, especially for juveniles, for which there is extensive overlap between males and females in these features (Demongin 2016).

The Pied Flycatcher is a migratory species; therefore, birds arrive in Portugal from a range of habitats in northern Europe where they are exposed to different environmental factors which can change the feather wear of the individual. Furthermore, variability in coloration exists among males, some having a darker upperparts than others, with implications for sexual selection (Sætre *et al* 1994). Therefore, some individuals may be easier to sex than others. Differing dorsal colouring of males may lead to only the blacker individuals being sexed, leaving the duller individuals unsexed or incorrectly sexed as females. Selective sexing may be a

reason for apparent sex ratio biases in ringing databases as a result of only sexing individuals that show extreme male or female characteristics, when one sex is easier to sex morphologically than the other.

Comparison of biometric sexing with molecular sexing

Sexing using biometrics alone was also shown to be problematic, either because individuals with extreme measurements for their sex can be sexed incorrectly, or because many individuals have intermediate measurements and so cannot be sexed. The biometric measurements for the species with monomorphic plumage show there is a broad range of measurements which overlap for male and female. The range of origins of migratory species may influence the wing length as the differences can be related to geographical differences in biometrics, as well as dietary and habitat differences (Herrera 1978). Among the European Robin, individuals with shorter tarsi and longer bills feed on a greater variety of prey (Herrera 1978). For example, Copete *et al* (1999) and Marchetti *et al* (1995) showed that more migratory subspecies of Reed Bunting (*Emberiza schoeniclus*) and *Phylloscopus* warblers, respectively, have a longer wing length than short-distance migrants and resident subspecies. A further consideration is the age category of the individuals, as first year passerines have shorter wings on average than adult birds of the same population (Alatalo *et al* 1984). It has been identified in the Marsh Tit, where juvenile males can have similar wing length to adult females. Broughton *et al* (2016b) identified a wing length division for each sex and each age category, female adult Marsh Tit was ≤63 mm and juvenile males criteria was ≥ 63 mm.

Analysis of sex ratios in the field site

The results provided strong evidence for a female bias in the Common Chaffinch population and some evidence for a female bias in Common Chiffchaffs, Willow Warblers and Common Kingfisher. In Portugal, Common Kingfisher are partial migrants and most dispersal occurs in juveniles or females, whereas adult males generally remain on territory (Cramp 1985, Arizaga *et al* 2010). As females are likely to be more dispersive, the high ratio of females captured at this non-breeding site is in line with expectations, even though the sample size is too small to draw firm conclusions.

Sex segregation during migration has been described for many passerine species (Campos *et al* 2011) and can explain the female bias found for the other species. Specifically, Catry *et al* (2005) also found a female sex-bias for Common Chiffchaffs in southern Portugal in specific habitats, including wetlands, scrub and orchards. Likewise, Gordo (2016) found a 2:1 female to male sex ratio in Common Chiffchaffs in southern Spain. The present study suggests similar sex-specific differences in migration or wintering habitat selection for Willow Warbler and

Common Chaffinch in Portugal, although to our knowledge no other studies have reported this before.

Conclusions

This study was successful in molecular sexing of a wide range of species, using a primer pair which successfully gave results for all species while highlighting problems of sexing birds using morphological and biometrical approaches. It can be confirmed that most individuals of most species sexed by morphology using plumage-based criteria are correctly sexed, but caution should be applied particularly to species sexed based on colouration (e.g. Eurasian Hoopoe and Pied Flycatcher), as sexing of birds using morphological criteria can be dependent on many factors including the condition of the plumage and the age of the bird. In some cases, only individuals which show extreme male or female characteristics can be sexed using morphological criteria, which can create an apparent but spurious sex ratio bias in bird ringing data sets. In addition, birds which are classed as young birds may be more difficult to sex using morphological criteria if they have not yet completed their moult into adult plumage. Therefore, the age of an individual can influence the likelihood of it being correctly sexed, highlighting the importance of considering age when sexing birds.

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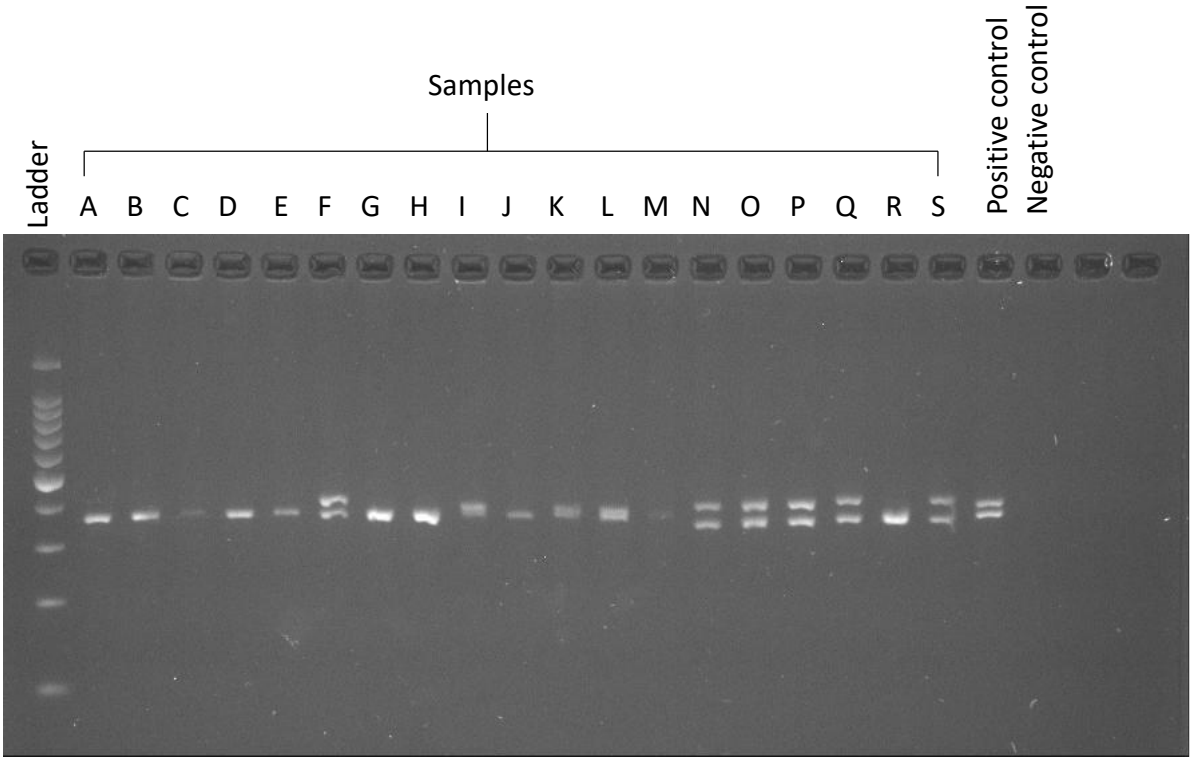
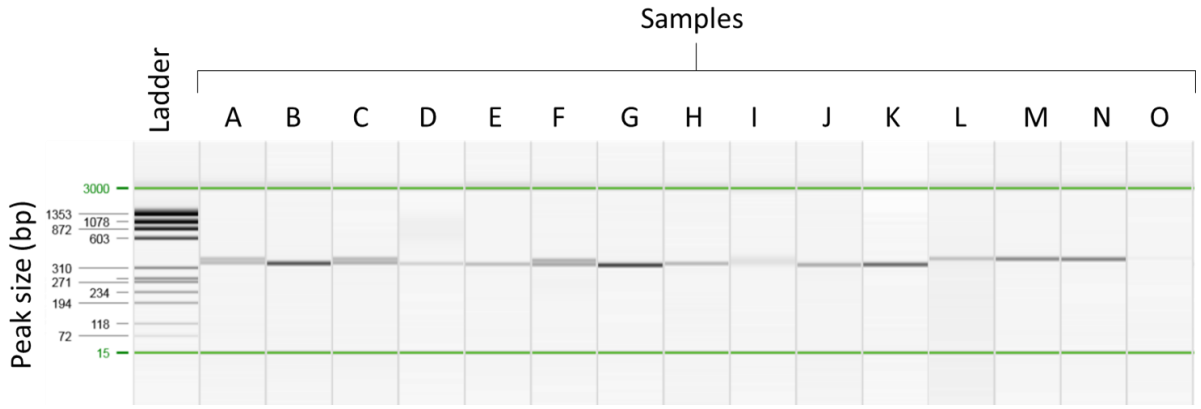
Appendices

Appendix 1. Example of gel image where two bands indicate female and one band indicates male. The gel is 3% agarose and the image includes a positive female control and a negative control.



Appendix 2. Section of the Qiexel (QIAGEN) report where band separation can be seen for (A-K) European Robin, *Erithacus rubecula* and (L-O) Eurasian Hoopoe, *Upupa epops*. Two bands indicate female and one band indicates male, the band separation varies between 36bp and 49 bp.



Appendix 3. Male and female totals for each species sampled, identified using molecular sexing, with the percentage female calculated.

Species	Male	Female	Total	Female (%)
Common Chiffchaff	12	24	36	66.67
Willow Warbler	5	13	18	72.22
Garden Warbler	4	2	6	33.33
Eurasian Blackcap	19	21	40	52.50
European Robin	8	3	11	27.27
Pied Flycatcher	7	10	17	58.82
House Sparrow	19	13	32	40.63
Common Chaffinch	0	6	6	100
European Goldfinch	3	2	5	40
Common Blackbird	10	10	20	50
Iberian Magpie	2	1	3	33.33
Common Kingfisher	0	4	4	100
Eurasian Hoopoe	3	1	4	25

Appendix 4. Table of chi-squared test results for sex ratio bias in all species sampled in this study.

Species	**Sample size**	**X^2**	**DF**	**P-value**
Common Chiffchaff	36	4	1	0.046
Willow Warbler	18	3.556	1	0.059
Garden Warbler	6	0.667	1	0.414
Eurasian Blackcap	40	0.100	1	0.752
European Robin	11	2.273	1	0.132
Pied Flycatcher	17	0.529	1	0.467
House Sparrow	32	1.13	1	0.289
Common Chaffinch	6	6	1	0.143
European Goldfinch	5	0.2	1	0.655
Common Blackbird	20	0	1	1
Iberian Magpie	3	0.332	1	0.564
Common Kingfisher	4	4	1	0.046
Eurasian Hoopoe	4	1	1	0.317

638 **Tables**

639 **Table 1: Biometric comparisons between male and female of passerine species.** Results in bold indicate significant differences at
640 α=0.05.

Species	**Sample size**	**Wing**			**Tarsus**			**Bill Length**			**Bill Depth**		
		t	**df**	**p-value**	**t**	**df**	**p-value**	**t**	**df**	**p-value**	**t**	**df**	**p-value**
Willow Warbler (*Phylloscopus trochilus)*	18	2.709	16	**0.016**	0.184	16	0.856	ND	ND	ND	0.874	16	0.395
European Robin (*Erithacus rubecula)*	11	0.414	9	0.689	1.078	9	0.309	0.109	3	0.920	0.349	9	0.735
Garden Warbler (*Sylvia borin*)	6	0.634	4	0.561	0.945	4	0.398	0.501	3	0.651	0.298	4	0.787
Common Chiffchaff (*Phylloscopus collybita)*	6	5.212	33	**<0.0001**	1.384	33	0.176	0.047	33	0.963	0.293	33	0.772
Pied Flycatcher (*Ficedula hypoleuca*)	17	1.030	15	0.319	0.073	15	0.943	1.043	7	0.332	0.302	15	0.767

641 **Table 2: The success rate of sexing passerines caught in Portugal based on wing length differences from available studies.**

Species	Correct sex	Incorrect sex	Impossible to sex	Source(s)	Wing length thresholds (mm)
Common Chiffchaff (*Phylloscopus collybita)*	22	1	14	Svensson (1992)	≤56=F, ≥62=M
	18	1	18	Demongin (2016)	≤55=F, ≥62=M
Willow Warbler (*Phylloscopus trochilus)*	13	4	1	Svensson (1992)	≤65=F, ≥67=M
	7	2	9	Demongin (2016)	≤63=F, ≥68=M
European Robin *(Erithacus ubecula)*	2	2	7	Svensson (1992)	Ads. <72= F, >75=M Juv. <71= F; >74=M
	6	5	0	Madsen (1997)	<71=F, ≥71=M
	2	1	8	Demongin (2016)	≤68=F, ≥75=M
Eurasian Hoopoe (*Upupa epops)*	0	1	3	Demongin (2016)	Ads. ≤146=F, ≥152=M Juv. ≤140=F, ≥150=M
	0	1	3	Baker (2016)	Ads. ≤146=F, ≥152=M Juv. 142-151=F, 141-152=M
Pied Flycatcher (*Ficedula hypoleuca*)	3	2	12	Demongin (2016)	≤74=F, ≥81=M
Common Kingfisher (*Alcedo atthis*)	1	0	3	Baker (2016)	≤74=M, ≥80=F

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Figure Legends

Figure 1: The percentage of females for 13 passerine species calculated from molecular sexing from A Rocha Portugal study site in the Western Algarve in 2017/18. The species are as follows; Chiff = Common Chiffchaff (*Phylloscopus collybita)*, WW = Willow Warbler (*Phylloscopus trochilus)*, GW = Garden Warbler (*Sylvia borin*), BC = Eurasian Blackcap (*Sylvia atricapilla*), R = European Robin (*Erithacus rubecula)*, PF = Pied Flycatcher (*Ficedula hypoleuca*), HS = House Sparrow (*Passer domesticus*),, Chaff = Common Chaffinch (*Fringilla coelebs)*, GF = European Goldfinch (*Carduelis carduelis*), BB = Common Blackbird (*Turdus merula)*, IM= Iberian Magpie (*Cyanopica cooki)*, KF = Common Kingfisher (*Alcedo atthis)* and HP = Eurasian Hoopoe (*Upupa epops)*. The numbers in brackets indicate the sample size. The horizontal line indicates 50%.

Figures

Figure 1: