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Ornithological Methods

Sex determination of Eastern White-crowned Sparrows (*Zonotrichia leucophrys leucophrys*) using wing chord length

Determinación del sexo en *Zonotrichia leucophrys leucophrys* usando la longitud de la cuerda alar

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ABSTRACT. Determining the sex of individuals in sexually monomorphic bird species outside of the breeding season is difficult. However, many monochromatic species exhibit sex differences in morphometrics, including wing chord length. For example, recent studies found that the Puget Sound (*pugetensis*) and Gambel's (*gambelii*) subspecies of White-crowned Sparrow (*Zonotrichia leucophrys*) can be sexed reliably using wing chord length. However, the Eastern subspecies (*leucophrys*) has not been evaluated. We measured the wing chord length of Eastern White-crowned Sparrows migrating through the Long Point Bird Observatory and specimens from the Fatal Light Awareness Program to determine if this subspecies can be reliably sexed using wing chord length. We combined wing chord length measurements with molecular sexing to test the prediction that males have a significantly longer wing chord length than females. Unfortunately, we only captured one definitive-cycle female and were thus unable to test our prediction in definitive-cycle birds. Males in first-cycle plumage had significantly longer wing chord lengths than females in first-cycle plumage and, outside of an overlapping range (74–76 mm), this subspecies can be reliably sexed using wing chord length. Our results support the use of wing chord length as a tool for bird banders and researchers to determine the sex of Eastern White-crowned Sparrows in first-cycle plumage in the hand outside of their breeding season or in the absence of a cloacal protuberance or brood patch.

RESUMEN. La determinación del sexo en individuos de especies de aves sexualmente monomórficas fuera de su temporada de reproducción es difícil. Sin embargo, muchas especies monocromáticas muestran diferencias entre sexos en morfometría, incluyendo la longitud de la cuerda alar. Por ejemplo, estudios recientes encontraron que las subespecies *pugetensis* y *gambelii* de *Zonotrichia leucphrys* pueden ser sexadas confiablemente usando la longitud de la cuerda alar. Sin embargo, la subespecie *leucophrys* no ha sido evaluada. Medimos la cuerda alar de individuos de Zonotrichia leucophrys leucophrys que migran a través del observatorio de aves de Long Point y especímenes del programa Fatal Light Awareness, para determinar si esta subespecie puede ser sexada confiablemente usando la longitud de la cuerda alar. Combinamos mediciones de la longitud de la cuerda alar con sexado molecular para poner a prueba la predicción de que la longitud de la cuerda alar en los machos es significativamente más larga que en las hembras. Desafortunadamente, solo capturamos una hembra en ciclo definitivo y por lo tanto no pudimos poner a prueba nuestra predicción en aves en ciclo definitivo. Los machos en el plumaje del primer ciclo tuvieron una longitud de la cuerda alar significativamente más larga que las hembras en plumaje de primer ciclo y, fuera del rango de sobreposición (74-76 mm), esta especie puede ser sexada confiablemente utilizando la longitud de la cuerda alar. Nuestros resultados dan soporte al uso de la longitud en la cuerda alar como una herramienta para los anilladores de aves e investigadores para determinar el sexo de Zonotrichia leucophrys leucophrys en plumaje de primer ciclo, en la mano, fuera de su temporada reproductiva o en la ausencia de protuberancia cloacal o parche de incubación.

Key Words: *blood; DNA; feather; molecular sexing; wing chord; Zonotrichia leucophrys*

INTRODUCTION

Sex is an important factor to consider in biological studies. In species with sexually dimorphic plumage (e.g., dichromatism), sex is easily determined by an individual's plumage, either throughout the annual cycle or when in alternate (breeding) plumage. However, in sexually monomorphic species (species with no morphological sex differences), which represent > 50% of extant bird species (Vučićević et al. 2016), it is difficult or even impossible to determine the sex of individuals, even when birds are in the hand, without taking genetic samples. This situation is a challenge that many ornithologists face when conducting ecological studies that rely on accurately determining the sex of individuals to evaluate sex-specific differences in populations or other features of interest. For example, sex differences may be important considerations for studies of molt (Morton 1992*a*), physiology (Moore et al. 1982, Morton 1992*b*), protandry (Seewagen et al. 2013, Schmaljohann et al. 2016), sex ratios (King et al. 1965), social behaviors (Jeans 2022), and stress (Romero et al. 1997, Landys et al. 2004). Many species with monochromatic plumage exhibit sex differences in morphometrics, including wing chord length, but these data are not available for all species (Pyle 1997).

The most common method of sex determination in monochromatic bird species is by examining the individual for the presence of sex characteristics during the breeding season, typically either a cloacal protuberance (male) or a brood patch (female; Pyle 1997). However, outside of the breeding season, individuals do not exhibit these characteristics, and therefore, researchers cannot reliably determine their sex during the pre-migratory, migratory, and overwintering periods. When breeding

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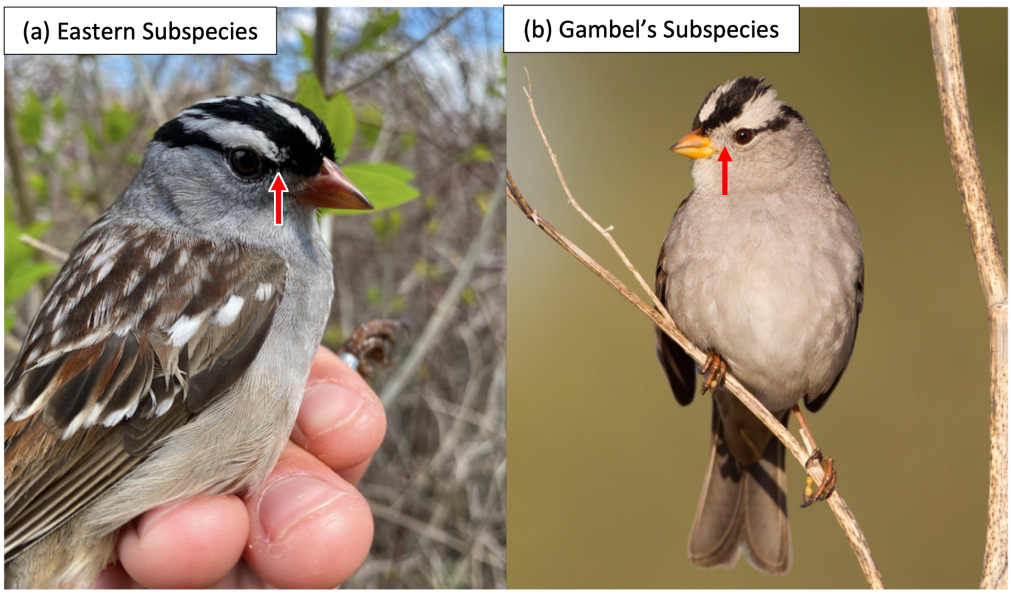
Fig. 1. The Eastern White-crowned Sparrow (a) is the dominant subspecies in eastern North America. It can be easily distinguished from the Gambel's subspecies (b), which occurs rarely in eastern North America, by the presence of black in the lores, indicated by the red arrows. Photos: (a) Ryan Leys, (b) Brian Sullivan (ML 27434901).



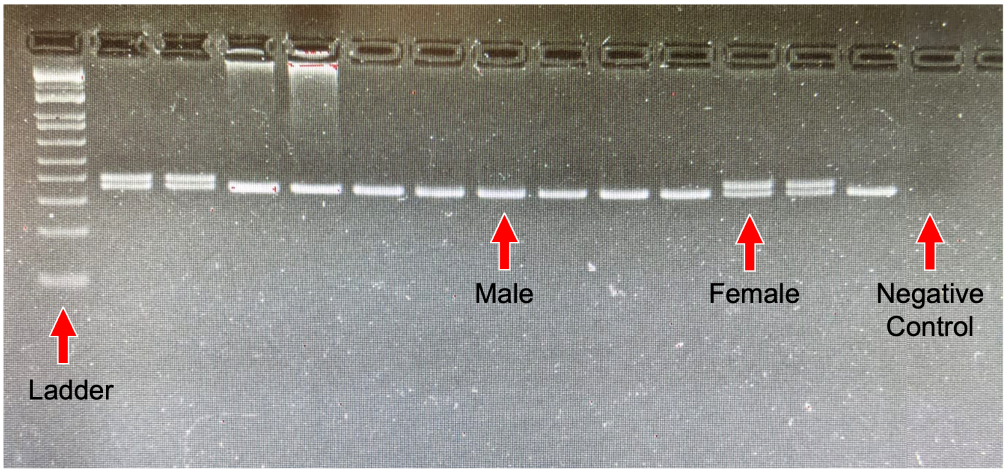
characteristics are not present, the most common method of sex determination is using polymerase chain reaction (PCR) to amplify two conserved chromo-helicase-DNA-binding (CHD) genes that allow for DNA-based sex determination (Griffiths et al. 1996, 1998, Vučićević et al. 2016).

These procedures require taking biological samples, typically blood or feathers, from each individual being studied. Such procedures are minimally invasive but increase bird handling time and require researchers to undergo additional training and permitting. Furthermore, PCR is a somewhat time-consuming and expensive method of sex determination and, most importantly, does not allow researchers to sex individuals immediately in the field. The ability to determine the sex of individuals rapidly in the field is important for many research questions and study designs. Thus, although PCR is an excellent tool for sexing birds, it is not feasible for all researchers or study systems. This situation is an ongoing issue for many bird species because researchers may be unable to balance the sexes during sampling or otherwise examine sex-specific effects when studying wild, monochromatic species, especially in remote areas or in other situations where laboratory facilities are unavailable and DNA-based sex determination methods are not feasible. Therefore, developing tools that allow researchers to determine sex rapidly and accurately in the field is important from both research and accessibility standpoints.

Many bird species exhibit sex differences in wing chord length (Pyle 1997). Compared to DNA-based sexing methods, using wing chord length is a rapid, repeatable, and non-invasive method of sex determination. When standard protocols are followed, wing chord length measurements are highly repeatable, especially compared to other morphometric measurements such as tail length, tarsus length, and bill length (Gill and Vonhof 2006, Warkentin et al. 2016, Carrillo-Ortiz et al. 2021). Accordingly, wing chord length is a standard morphometric measurement taken at most bird banding stations worldwide (Busse and Meissner 2015, Morris et al. 2016, Zhu et al. 2023) and is commonly used by researchers in other contexts. Thus, using wing chord length to determine sex does not increase bird handling time or stress. Reducing handling time is particularly important in cold climates, where handling small species can result in significant body temperature reductions, both during handling and after release (Andreasson et al. 2020). Therefore, when possible, researchers should use wing chord length for sex determination over DNA-based methods, especially for sensitive species or species handled during inclement weather or otherwise challenging climatic conditions.

The White-crowned Sparrow (*Zonotrichia leucophrys*) is sexually monochromatic and has four currently recognized subspecies: Eastern (*leucophrys*), Gambel's (*gambelii*), Nuttall's (*nuttalli*), and Puget Sound (*pugetensis;* Banks 1964). The White-crowned Sparrow is one of the most commonly studied passerine species in North America (Rattenborg et al. 2004, Jones et al. 2008, Lipshutz et al. 2017), so identifying morphometric methods to reliably determine the sex of individuals in the hand would be particularly beneficial for researchers. Previous studies have shown that Puget Sound (*pugetensis*) and Gambel's (*gambelii*) subspecies can be reliably sexed using wing chord length (Fugle and Rothstein 1985, Bell 2020). The Eastern subspecies is the dominant subspecies in eastern North America and can be easily distinguished from the Gambel's subspecies, which occurs rarely in eastern North America, by the presence of black in the lores (Fig. 1). The literature suggests that the Eastern subspecies cannot be reliably sexed using wing chord length; however, after the recent

Fig. 2. P2/P8 sexing PCR results for 13 Eastern White-crowned Sparrows using DNA extracted from blood. Double bands indicate females (Z and W chromosomes), and single bands indicate males (Z chromosome), as shown by the red arrows.



findings by Bell (2020) and personal observations in the field (R. A. C. Leys), we decided to test whether wing chord length could be used to sex the Eastern subspecies. If wing chord length is a reliable measure of sex in Eastern White-crowned Sparrows, wing chord length can be used by researchers and bird banding stations to reliably sex individuals in the field outside of their breeding season.

We hypothesized that wing chord length varies between male and female Eastern White-crowned Sparrows. Based on wing chord measurements made by R. A. C. Leys over two years of migration monitoring at Long Point Bird Observatory (LPBO), we predicted that males would have a longer mean wing chord length than females, females would have wing chord lengths ≤ 75 mm, males would have wing chord lengths ≥ 78 mm, and individuals with wing chord lengths of 76–77 mm would not be reliably sexed by wing chord length. We predicted these values because R. A. C. Leys noticed that there appeared to be a bimodal distribution of wing chord lengths among the individuals captured at Long Point Bird Observatory, with fewer individuals having wing chord lengths in the 76–77 mm range.

METHODS

Data collection

In small passerines, using blood or feathers for DNA sex determination can be equally effective (Harvey et al. 2006), so we collected blood or feather samples from two groups of Eastern White-crowned Sparrows. The first group (N = 27) consisted of live individuals captured at LPBO's Old Cut Field Station (42° 34'58.5" N, 80°23'54.4" W) near Port Rowan, Ontario, Canada. We captured individuals using mist nets during spring migration (05–20 May 2021; 25 April–17 May 2022) as part of the station's standard, long-term migration monitoring efforts at this location. Each bird was banded, aged, weighed to the nearest 0.1 g, and its wing chord length was measured to the nearest 1 mm. We used an unflattened wing chord length measurement, which is measured from the carpal joint to the tip of the longest primary feather (Pyle 1997, see pages 4–6 for details). We then took either a small (approximately 20 µL) blood sample collected via brachial venipuncture (N = 13 in 2021) or one of the outer rectrices (R6, N = 14 in 2022) from each bird. Blood was blotted onto high wet-strength filter paper saturated with 0.5 M Na-EDTA (pH 8.0) and allowed to air-dry. The second group (N = 11) consisted of deceased birds sampled from the Fatal Light Awareness Program collected during spring (April–May 2021) and fall (August–November 2021) in Toronto, Ontario, Canada. We recorded the age, wing chord length to the nearest 1 mm, and collection date, and removed both R6 feathers from each specimen. All morphometrics were measured by the same researcher (R. A. C. Leys).

Birds were aged using the Wolfe-Ryder-Pyle system (Wolfe et al. 2010). In Eastern White-crowned Sparrows, first-cycle individuals have brown crown stripes, whereas definitive-cycle individuals have black crown stripes (Pyle 1997). From February to June, first-cycle individuals undergo a partial prealternate molt in which they replace some to all of the brown feathers in the crown stripes with black feathers, rendering this criterion unreliable in the spring for birds with black crown stripes (Pyle 1997). However, individuals with black crown stripes in the spring can be conclusively aged as first-cycle using feather shape (pointed in first-cycle vs. round in definitive-cycle) and level of abrasion (abraded in first-cycle vs. fresh in definitive-cycle) in the primary coverts and rectrices (Pyle 1997; see pages 589–591 for details). Following these guidelines, we assigned individuals a molt category of either first-cycle or definitive-cycle (Wolfe et al. 2010). Of the 38 birds we sampled, 28 were first-cycle and 10 were definitive-cycle.

Molecular sexing

We extracted DNA from blood using a standard salt extraction protocol (Miller et al. 1988; details in Appendix 1). DNA was extracted from feathers using a Cell and Tissue DNA Isolation Kit (Norgen) following the manufacturer's recommended protocol for lysate preparation from animal tissues. Prior to beginning this protocol, we used scissors sterilized with 70%

ethanol to remove the feather barbules from the rachis, then cut the rachis into small pieces. Scissors were sterilized between each feather sample to avoid potential contamination. We used an extraction kit for feathers because we expected it to increase the DNA yield and purity compared with the in-house salt extraction protocol we typically use for blood.

After extraction, we used a nanodrop spectrophotometer (ND-1000) to measure the concentration of DNA extracted from blood and feathers. The mean concentration of DNA extracted from blood was 171 ng/μL (range = 26–296 ng/μL), with 260/280 ratios around 1.8. The mean concentration of DNA extracted from feathers was 39 ng/μL (range = 7–143.5 ng/μL), with 260/280 ratios around 1.4. One feather sample with relatively high concentration of DNA (143.5 ng/μL) had some blood on the feather shaft, which likely explains the higher DNA yield. Removing this sample from our calculation, the mean DNA concentration of feathers was 28.1 ng/μL. A 260/280 ratio of approximately 1.8 is usually considered "pure" for DNA, whereas lower ratios may indicate the presence of protein, alcohol, or other contaminants that absorb strongly at 280 nm; thus, we obtained higher quality DNA from blood than feathers, despite using a kit designed for extracting DNA from animal tissues for the feather samples. We diluted the DNA extracted from blood to 20 ng/μL; given the generally lower concentration of DNA extracted from feathers, we left those samples undiluted.

We then used the P2/P8 sexing protocol described by Griffiths et al. (1998) to determine the sex of Eastern White-crowned Sparrows, conducting PCR reactions in a total volume of 15 μL, including 20 ng total genomic DNA as template for blood or mean = 39 ng (range = 7–143.5 ng) total genomic DNA for feathers, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1X buffer, 0.2 μM of each primer, and 0.5 units *Taq* DNA polymerase (Invitrogen). For feather samples that did not amplify after our first PCR attempt, we attempted PCR up to three more times, adding 0.8–1.5 mg/mL bovine serum albumen to improve amplification. All PCR reactions included a negative control using ddH_2O instead of template, and two positive controls that used 20 ng total genomic DNA from a male and female Eastern White-crowned Sparrow of known sex as determined by PCR amplification from blood (Fig. 2). Thermocycling conditions included an initial denaturation step of 94°C for 90 s; 30 cycles of 94°C for 30 s, 48° C for 45 s, and 72°C for 45 s; and a final extension step of 72°C for 5 min.

Statistical analysis

All analyses were conducted using R statistical software version 1.4.1717 (R Core Team 2021). Given the unequal sample sizes we obtained for sex within molt category (details in *Results*), we excluded definitive-cycle birds (N = 7) from further statistical analysis. Given our relatively small sample size for first-cycle individuals (N = 20, details in *Results*), we wanted to pool the data to increase statistical power. Before pooling our data, we used a Welch's t-test in base R to evaluate whether there were significant differences in wing chord length of first-cycle individuals between live birds and specimens from the Fatal Light Awareness Program collection. Using these 20 first-cycle birds, we then ran a Welch's t-test to test for a significant difference in wing chord length between the sexes. To control the false discovery rate associated with multiple testing, we calculated adjusted P values for each of the two t-tests with 'p.adjust' in base R using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

Next, we determined the wing chord length ranges in first-cycle individuals for each sex using the method outlined by Pyle (1997): we calculated the mean wing chord length for each sex, then added and subtracted two standard deviations (SD) from each mean. We used these ranges because they represent approximately 95% of the individuals in the population, which is the current acceptable standard from the Bird Banding Laboratory and the Canadian Wildlife Service (Pyle 1997). We then used long-term banding data to calculate the proportion of Eastern White-crowned Sparrows that can be reliably sexed using wing chord length. We counted the number of individuals that fell outside the overlapping range of wing chord lengths where sex determination is not reliable (based on our data set of molecularly sexed birds) and divided this number by the total number of individuals banded at LPBO between 2015 and 2022.

RESULTS

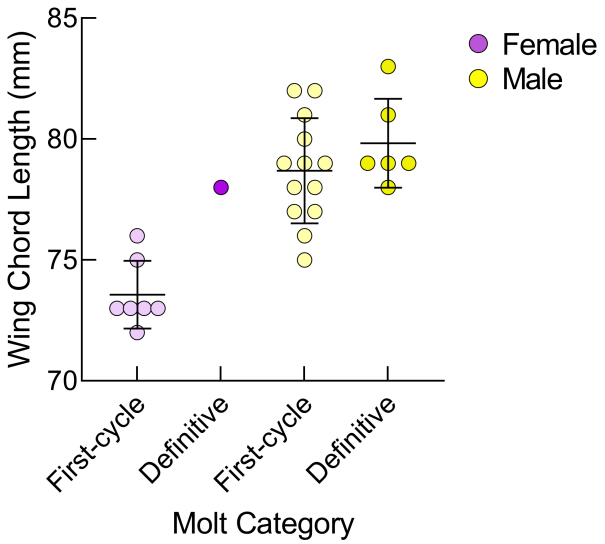
Harvey et al. (2006) reported that using blood or feathers was equally effective for DNA sex determination in small passerines. However, we found that DNA extraction from blood was more effective than feathers. We obtained usable DNA from all 13 blood samples but were only able to amplify DNA from 56% (14/25) of feather samples. After molecular sexing, we determined that we had sampled 19 males (13 first-cycle, 6 definitive-cycle) and 8 females (7 first-cycle, 1 definitive-cycle).

There was no significant difference in wing chord length between live and dead birds (t = −0.14, adjusted P = 0.891, df = 17.9). Male Eastern White-crowned Sparrows in first-cycle plumage had significantly longer wing chord lengths than first-cycle females (male: mean = 78.7 mm ± 2.2 SD, female: mean = 73.6 mm ± 1.4 SD; t = −6.4, adjusted P < 0.0001, df = 17.2; Fig. 3). First-cycle males had a wing chord length range of 74.3–83.0 mm, whereas first-cycle females had a wing chord length range of 70.8–76.4 mm. These results mean that there is an overlapping range of 74–76 mm for which sex cannot be reliably determined by wing chord length for Eastern White-crowned Sparrows in first-cycle plumage. Thus, in our full data set, 71.4% (20/28) of first-cycle birds could be reliably sexed by wing chord length. From the 2015–2022 LPBO data set, the number of individuals in first-cycle plumage that could be reliably sexed by wing chord length was 63.8% (4376/6854).

DISCUSSION

We show that males of the Eastern subspecies of the White-crowned Sparrow in first-cycle plumage have significantly longer wing chord lengths than females. While our sample size was small (20 first-cycle birds), our results do suggest that the Eastern subspecies can be reliably sexed using wing chord length outside an overlapping range of 74–76 mm in first-cycle individuals. Individuals within the overlapping range cannot be reliably sexed by wing chord length and should be denoted as having an unknown sex. Finding an overlapping range in wing chord length for which sex determination is not reliable is common in passerines, but when the range of overlap is small enough, this measurement can still be used to reliably sex most individuals in a population (Pyle 1997). According to our calculations for first-

Fig. 3. Eastern White-crowned Sparrow wing chord lengths (mm) by molt category and sex. Points represent the individual measurements for all 27 birds that were molecularly sexed, and lines represent the mean ± standard deviation for each category.



cycle birds, the 2 mm overlapping range in wing chord length for males and females would allow nearly two-thirds of birds in first-cycle plumage to be reliably sexed in the hand. Therefore, we recommend that banders and other researchers use wing chord length to determine the sex of individuals in first-cycle plumage, and only use PCR when individuals fall within the overlapping range. When conducting DNA-based sexing, we recommend using blood samples because DNA extraction from blood was 100% effective, whereas DNA extraction from feathers was only 56% effective.

Other methods have been developed to sex monochromatic bird species, such as performing discriminant function analysis of multiple morphometric measurements (including tarsus length, tibia length, bill length, and tail length; Desrochers 1990, Analla et al. 2022). These measurements, combined with wing chord length, could be used to reduce the overlapping range (i.e., birds that cannot be sexed) in monochromatic first-cycle Eastern White-crowned Sparrows; however, these data are time-consuming to collect and may not be practical for all researchers. Nevertheless, future studies that incorporate additional morphometric measurements could evaluate the utility of discriminant function analysis for sex determination of Eastern White-crowned Sparrows regardless of molt cycle. Future research is also needed to test whether other sexually monochromatic bird species can be reliably sexed by wing chord length. Based on personal observations (R. A. C. Leys), candidate species to test are the American Tree Sparrow (*Spizelloides arborea*) and the Brown Creeper (*Certhia americana*).

Our findings are important for banding stations and researchers. Banding stations play a critical role in population trend monitoring for migratory species (Hussell and Ralph 2005, Miller-Rushing et al. 2008). They are also crucial for researchers because they provide access to birds for research projects and information that is vital for the conservation of migratory bird species, the latter of which is highlighted by the fact that the 2022 State of the World's Birds report included data that were made possible by banding stations (BirdLife International 2022). The tool that we have created for sexing Eastern White-crowned Sparrows in first-cycle plumage will allow banding stations in eastern North America to improve their data by recording the sex of first-cycle individuals in this subspecies. Providing a reliable sex determination range for first-cycle birds is particularly important because the proportion of first-cycle birds captured at banding stations can be as high as 87% in autumn and 66.4% in spring (Mills 2016). Further, collecting wing chord length data is part of the standard banding protocol for most banding stations, so the tool we have provided can be seamlessly implemented into each banding station's operations without adding additional steps to the process or causing the birds additional stress. For other researchers, our study provides a simple tool for sexing first-cycle Eastern White-crowned Sparrows in the hand. Wing chord length measurements are quick, inexpensive, non-invasive, and repeatable, and they allow researchers to select individuals for their study based on sex and to test for differential responses between the sexes when considering aspects of this subspecies' biology.

Our tool is particularly important in the pre-migratory, migratory, and overwintering periods when breeding characteristics are not present. One limitation of our study is that, due to our small sample size, we were only able to statistically determine that first-cycle birds can be reliably sexed by wing chord length. Future research should focus on evaluating the use of wing chord length as a tool for sexing definitive-cycle Eastern White-crowned Sparrows. We encourage researchers working on Eastern White-crowned Sparrows during the breeding season to compare the wing chord lengths of individuals with breeding characteristics (i.e., individuals of known sex) with the wing chord length ranges we present here. We expect that doing so will further validate this method and increase researcher confidence in the wing chord length ranges we have identified as reliable for sex determination in first-cycle Eastern White-crowned Sparrows. Relatedly, if there are researchers with existing DNA libraries for Eastern White-crowned Sparrows, we encourage them to validate our method using DNA-based sex determination.

Our findings open avenues for future research on this subspecies; for example, researchers may be able to use sex, based on wing chord length, to test for evidence of protandry in first-cycle Eastern White-crowned Sparrows or to evaluate temporal changes in the population sex ratio of first-cycle birds. Protandry is the phenomenon of males arriving earlier to breeding locations than females (Coppack and Pulido 2009). Climate change is increasing the degree of protandry in many species, including Barn Swallows (*Hirundo rustica;* Møller 2004) and Willow Warblers (*Phylloscopus trochilus;* Hedlund et al. 2022), but so far seems to have no effect on other passerine species (Rainio et al. 2007). Protandry has risks, i.e., in years with adverse conditions at breeding sites, early arriving males experience increased mortality (Møller 1994), but also has benefits, i.e., early arrival leads to increased fitness in years with favorable conditions (Kokko et al. 2006). Our study provides a critical tool for testing

whether first-cycle Eastern White-crowned Sparrows exhibit protandry, and if the degree of protandry has shifted due to climate change. Long-term banding stations such as LPBO have decades of historical migration banding data, including information on wing chord length and capture date, which can be used to determine whether protandry exists and how the degree of protandry may be shifting due to climate change.

Population sex ratios can be described in two main ways: focusing on offspring or adult sex ratios. Offspring sex ratio, the ratio of male to female offspring produced by breeding adults, has been the subject of many studies (e.g., Svensson and Nilsson 1996, Whittingham and Dunn 2000, Bonier et al. 2007). Adult sex ratio, however, has received less attention and remains poorly studied (Donald 2007). Contrary to mammals, wild bird populations tend to be male biased, with males outnumbering females by 33% on average (Donald 2007). This information is consistent with our finding, as only 29.6% (8/27) of the individuals in our study were female. Male bias in bird populations is even more pronounced in globally threatened species (Donald 2007), which has profound implications for the management of threatened species and illustrates the importance of quantifying sex ratios in bird populations. The tool we provide here will allow researchers to calculate the sex ratio of first-cycle Eastern White-crowned Sparrows.

Author Contributions:

R. A. C. L. designed the study in consultation with L. A. G. Both R. A. C. L. and L. A. G. collected and analyzed the data. R. A. C. L. wrote the manuscript with input from L. A. G. Both coauthors approved the final manuscript.

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Data Availability:

The data/code that support the findings of this study are openly available in Open Science Framework at https://osf.io/rhaex/. Ethical approval for this research study was granted by the Canadian Wildlife Service and Environment and Climate Change Canada: permits 10169 CM, SC-OR02022-00089, and 10169E,F. All animal procedures were approved by the McMaster University Animal Research Ethics Board (AUP 18-05-20) and the University of Waterloo Animal Care Committee (43009).

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Sex determination of Eastern White-crowned Sparrows (*Zonotrichia leucophrys leucophrys*) using wing chord length

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Extracting DNA from Avian Blood

1. When ready to extract DNA: label an autoclaved 1.5 mL tube for each sample to be extracted. Set aside 24 tubes for each set of extractions (23 individuals, and 1 blank, to ensure you do not contaminate your samples along the way). Add all the chemicals as you would to real samples to the blank (except the blood).

2. Add a small amount (approx. 100 – 200 µL) of the blood/lysis buffer sample (making sure to take a small amount of the aggregate or clot of blood, as that is where the DNA is concentrated) to 400 µL of Cell Lysis Buffer and 3 µL of 20 mg/mL of Proteinase K (stored at -20 ^{o}C). If you hold the pipette firmly and support it with your other hand, you should be able to add the lysis buffer with one tip without touching the blood, thus using less tips. If you cannot do this, and you do expose the tip to the blood, then use a new pipette tip each time. Use new tips for the Proteinase K in each sample.

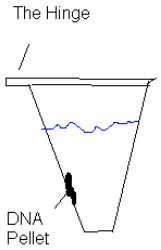
3. Flick each tube and vortex briefly to mix, then spin them down very briefly in the centrifuge (hold "short" on the centrifuge, until it reaches 10 000 RPM, then release the button).

4. Incubate at 60 ^{o}C for 2 h. Invert the tubes a little every 15 min or so, to speed along the digestion, or use the shaking heat block to do the same thing (set to 300 – 400 rpm). Alternatively, samples can be incubated overnight to ensure full digestion.

5. Once the digestion is finished, spin down the tubes briefly to get the condensed liquid to the bottom. After, add 200 µL of 7.5 M ammonium acetate solution to precipitate the proteins out of the samples. Vortex each sample for 15 sec until well mixed. Centrifuge on max for 7 min. The proteins (reddish-brown) should be collected at the bottom. Carefully pour off the top aqueous phase into a new-labelled tube. Place the old tubes with the blots and proteins into the biohazard waste.

6. Add 900 µL of room temperature 100% isopropanol (2-proponal) and quickly invert the samples a few times. This should bring the DNA out of solution since it is not soluble in alcohol. It will have a white stringy appearance. The more DNA present, the larger the blob. If the blood sample was small or very dilute, you may not see anything, and that is fine.

7. Spin the tubes down on max in the centrifuge for 7 min. Orient the hinge of the tube is oriented at the top when placed into the centrifuge so DNA will pellet on the side where the hinge is.



8. Decant the solution into a beaker by pouring the liquid out slowly so you do not disturb the pellet. Keep an eye on the pellet as you pour to ensure it is still there. Blot the mouth of each tube on a clean piece of paper towel, then invert the samples and let dry for 30 sec.

9. Add 200 µL ice-cold 70% ethanol to each sample. Rock the samples side to side so the ethanol washes the inside of the vial—this will help dissolve any of the remaining ammonium acetate. Try not to let the pellet dislodge.

10. Centrifuge the tubes on max for 10 min.

11. Decant the 70% ethanol, the same way as the isopropanol. Blot the mouth of each tube on a clean piece of paper towel, then invert the samples and let dry for approx. 20 – 30 min.

12. Add 100 – 200 µL 1X TE to each tube. Adjust volume added based on the size of the pellet.

13. Flick the tubes vigorously to dissolve the pellets. Tap the tubes down to get most of the liquid down. Ensure pellets are fully dislodged from the side of the tube and fully dissolved in the TE.

14. Congratulations, your DNA is extracted! You can now Nanodrop the stock solution and calculate what dilution is needed to make a 20 ng/ µL working solution.

Notes

Protocol modified from: Elizabeth A. MacDougall-Shackleton Lab, Western University, London, ON, Canada

Protocol originally modified from: Miller S. A., D. D. Dykes, and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research, 16(3), 1215. https://doi.org/10.1093/nar/16.3.1215