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METHODOLOGY

Determining sex in golden eagle (*Aquila chrysaetos*) nestlings

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Abstract
Incorporating sex ratios of nestlings into population viability studies increases knowledge of overall health of endangered populations. Currently, a reliable non-invasive method to identify the sex of golden eagle nestlings is not available; however, claims are commonly made based on morphology. Ten biometric measurements from 43 Scottish golden eagles aged 2–7.5 weeks were assessed to see if sex could actually be determined using this non-invasive methodology. Sex was confirmed via molecular analysis of blood samples. Discrete and principal component analyses of the different biometrics could not correctly determine individual nestling sex. Therefore, despite being more invasive, molecular sexing remains the recommended tool of choice for accurate sex identification of Scottish golden eagle nestlings younger than 7.5 weeks of age. This has important implications for golden eagle field studies where empirical morphological measurements are frequently and typically taken, but we have shown are not reliable in determining the sex of such young nestlings.

Keywords Biometric · Sexing · Golden eagle · Non-invasive · Nestling

Introduction

The ability to assess changes in sex ratio between generations or geographic regions is important for assessing the health of any species, as well as population viability analysis, behavioural or ecological studies and the management of populations (Newton and Gammie 1979; Blood and Studdert 1988; Ferrer and Hiraldo 1992; Reynolds et al. 2007; Donald 2007; Watson 2010; Morinha et al. 2012).

Golden eagles (*Aquila chrysaetos*) are difficult to monitor, meaning knowledge of the natural hatching, fledging and adult sex ratios of a healthy population may be unavailable. Determination of sex soon after hatching provides a population baseline at a key stage, facilitating earlier discovery of demographic trends (Arnold et al. 2007; Székely et al. 2014).

Currently, no reliable field method exists for sexing golden eagle nestlings. However, the reverse dimorphism, where adult females are larger than males (Riley 2012), is currently used by raptor field workers as a guide for estimating sex in nestlings from hatching until fledging age at nine or 10 weeks. Weight, relative size and a number of biometrics from the nestling are used. Whilst simple to do, the current method does not take account of differences between hatching times, variable growth rates or competition within the nest (Watson 2010), making this determination highly subjective and likely to be inaccurate. Previous growth curves published by Ellis (1979) were developed before the development of accurate genetic techniques for sexing and relied heavily on unverified methodologies based on foot size or nestling weight.

Accurate sexing of golden eagle nestlings via biometrics would provide information for golden eagle population health assessment without the associated stress of invasive sampling. It would require specific guidelines and training to ensure repeatability and minimise stress but present far less risk than DNA sexing via blood/oral sampling. In addition, morphometric sexing can provide immediate results as opposed to the delay, costs, and training/licensing of

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molecular methods. Personnel undertaking morphometric measurement of nestlings would likely already be experienced in raptor handling.

In this study, univariate and principal component analyses of biometrics were used to determine if the sex of 43 Scottish golden eagle nestlings aged between 2 and 7.5 weeks of age could be identified via this method.

Materials and methods

Sampling strategy and ethical approval

This animal study was reviewed and approved by the University of Edinburgh. All procedures were conducted in accordance with Natural England/British Trust for Ornithology license authority and Home Office license authority (Project license PB8A1D5C7, local AWERB review identifier PL10-17).

Blood samples and biometrics were obtained from 48 golden eagle nestlings in 37 nests distributed across Scotland's Highlands and Islands undergoing handling for leg ringing during 2018–2019 (Peniche et al. 2022). Nestlings ranged from 2 to 7.5 weeks of age. Age was estimated using a protocol created from descriptions of weekly feather development of American and Scottish golden eagle nestlings (Driscoll 2010; Watson 2010).

Biometrics measured

Eleven biometric measurements were attempted per nestling: ten linear measurements (bill depth (mm), two different measurements of culmen length (mm), hallux (mm), length of wing (mm), foot pad (mm), length of tibiotarsus (mm), length of tarsometatarsus (mm), tarsus width (mm) and tarsus depth (mm)) and body weight (g) (Supplementary Fig. 1). Head, wing and leg biometrics were adapted from Hardey (2006), with the exception of the metatarsal measurement which was an addition. One person measured all biometrics with the same equipment. Body weight was obtained with a spring balance with the nestling suspended in a cloth bag (Salter Brecknell™ Super Samson). Wing, metatarsus, and tarsometatarsus were measured to nearest 1 mm with a 1500 mm tailors tape measure; all others were measured to nearest 0.1 mm with a Wiha dialMax 4112102 Sliding Clock Vernier Calliper (Wiha Tools Ltd., Bromsgrove, UK).

Molecular sexing by polymerase chain reaction (PCR)

A drop of fresh blood was obtained by brachial vein venipuncture and spotted onto either a Whatman FTA® card or Whatman FTA® Elute card (GE Healthcare, Buckinghamshire, UK) for molecular sexing (Supplementary material 5). Each card was stored in a sealed bag containing silica gel beads with moisture indicator (Fisher Scientific, Leicestershire, UK) to preserve samples whilst in the field.

Data analysis

Molecular data were analysed using Genemapper v4 to determine individual sex. All statistical analyses were performed using R version 3.5.1 (RStudio Team 2016). The 'bill depth' biometric was rejected due to the percentage (15%) of birds that kept the mouth open during measuring.

Univariate biometric analysis

Summary statistics and two-sample *t*-tests were performed on 48 individuals and ten measurements to compare between males and females.

Principal component analyses

Due to the differences of age sampled, two principal component analyses (PCAs) were conducted: (i) using raw data to assess for size and shape of the individuals ('size and shape PCA') and (ii) size component was removed from all measurements to focus on shape ('shape PCA'). By removing the size component from all measurements, the risk of focusing on a difference in size due to any age difference could be avoided. Both analyses looked at the combined explanatory power of all variables in determining sex. Individuals with missing measurements, due to field constraints, were removed from the PCA to retain as large a set of biometrics as possible, leaving 43 individuals in the final analyses. Body weight was removed from the PCA due to the influence lifetime food availability may have on this variable, irrespective of sex.

Shape PCA

Prior to analysis of shape, measurements were normalised to size, using isometric calculations. These calculations allow us to estimate the size component from each measurement to later 'remove it' or normalise all measurements for size. Isometry considers whether proportions of components change during growth, acknowledging that as an individual matures its body parts increase in size but not all parts increase at the same rate. The size component of each nestling's set of biometrics were calculated using the Mosimann's (1970) formula:

$$I = 10 \text{ ^ } \text{mean}\,(\log 10\,(x))$$

where *I* represents 'isometry' and *x* is the biometric values for that nestling. Using the isometric values, the measurements were normalised to the size component.

Maturation analysis

Due to the possibility that growth rates of different body parts may be neither continuous nor comparable between individuals, it was important to find a proxy for individual maturity. To investigate the relationship between age and all other variables, regression plots of each biometric against age were produced. Any biometric that showed a positive relationship with age was then assessed by linear regression against all other biometrics to look for sex differentiation.

Results

Molecular analysis

Both PCR amplification methods produced consistent sex determination results.

Discrete biometric analysis

Measurements and sex of 43 golden eagle nestlings aged between 2 and 7.5 weeks of age were used (aged 3–5 weeks 1 male and 5 females; aged 4–5 weeks 12 males and 10 females; aged 6 weeks 11 males and 9 females). Whilst statistically significant differences were found for 5 of the linear measurements ($p < 0.002$, Table 1), there was considerable overlap in body weight and the nine linear measurements between males and females (Supplementary Fig. 2). The clearest differences were observed for tarsus depth; however, complete differentiation between sexes was only possible between the smallest males and largest females.

There were two statistically significant differences in Mosimann isometry/size values between the sexes for tarsometatarsus and tarsus depth ($p < 0.05$). However, the broad overlap observed for both measurements between sexes permits complete differentiation only for the smallest of males and largest of females (Fig. 1). Furthermore, statistically significant differences in biometric values following removal of size were found for culmen A, culmen B, hallux length, foot pad, tarsus width, and tarsus depth ($p < 0.05$); however, both sexes on all these measurements have a broad overlap. The rest of the measurements showed no significant difference between sexes ($p > 0.68$) (Fig. 1).

Size and shape PCA

The first principal component (PCA1), dominated by wing length, had a loading weight of 94%, whereas the second only 3%. Plotting the 2 components against each other did

Table 1 Summary statistics for ten biometric measurements of female (F) and male (M) golden eagle nestlings (*Aquila chrysaetos*) sampled in Scotland in 2017–2018

Variable	*N*	Sex	Mean	Max	Min	Range	SD	*p* Value
Body weight (g)	23	F	3320.48	4650	1400	1400–4650	857.29	0.09
	24	M	2890.48	4300	1500	1500–4300	708.05	
Culmen length A (mm)	26	F	34.03	38.20	25.90	25.90–38.20	3.21	0.04
	24	M	32.15	36.00	24.90	24.90–36	2.75	
Culmen length B (mm)	25	F	58.26	64.10	47.20	47.20–64.10	5.25	0.08
	24	M	55.60	64.80	47.00	47–64.80	4.63	
Hallux length (mm)	25	F	38.75	45.10	27.00	27–45.10	4.75	0.03
	24	M	36.04	41.60	27.00	27–41.60	3.57	
Foot pad (mm)	23	F	134.43	154.00	95.00	95–154	14.63	0.03
	25	M	124.88	140.00	91.00	91–140	12.33	
Wing length (mm)	26	F	290.43	430.00	112.00	112–430	83.54	0.78
	25	M	296.88	420.00	160.00	160–420	73.11	
Tibiotarsus (mm)	23	F	175.98	222.00	113.50	113.50–222	24.16	0.72
	24	M	173.57	200.00	123.30	123.30–200	20.62	
Tarsometatarsus (mm)	24	F	119.72	140.00	87.00	87–140	13.22	0.77
	24	M	121.04	140.00	83.20	83.20–140	16.11	
Tarsus width (mm)	25	F	15.57	18.80	12.10	12.10–18.80	1.61	0.01
	24	M	14.15	16.80	10.20	10.20–16.80	1.74	
Tarsus depth (mm)	25	F	17.08	20.30	12.20	12.20–20.30	1.74	< 0.002
	24	M	15.15	17.70	10.30	10.30–17.70	2.18	

The standard deviations and *p* values originated from *T*-test performed on the means of the two sexes

N number of individuals sampled, *sex* based on molecular determination

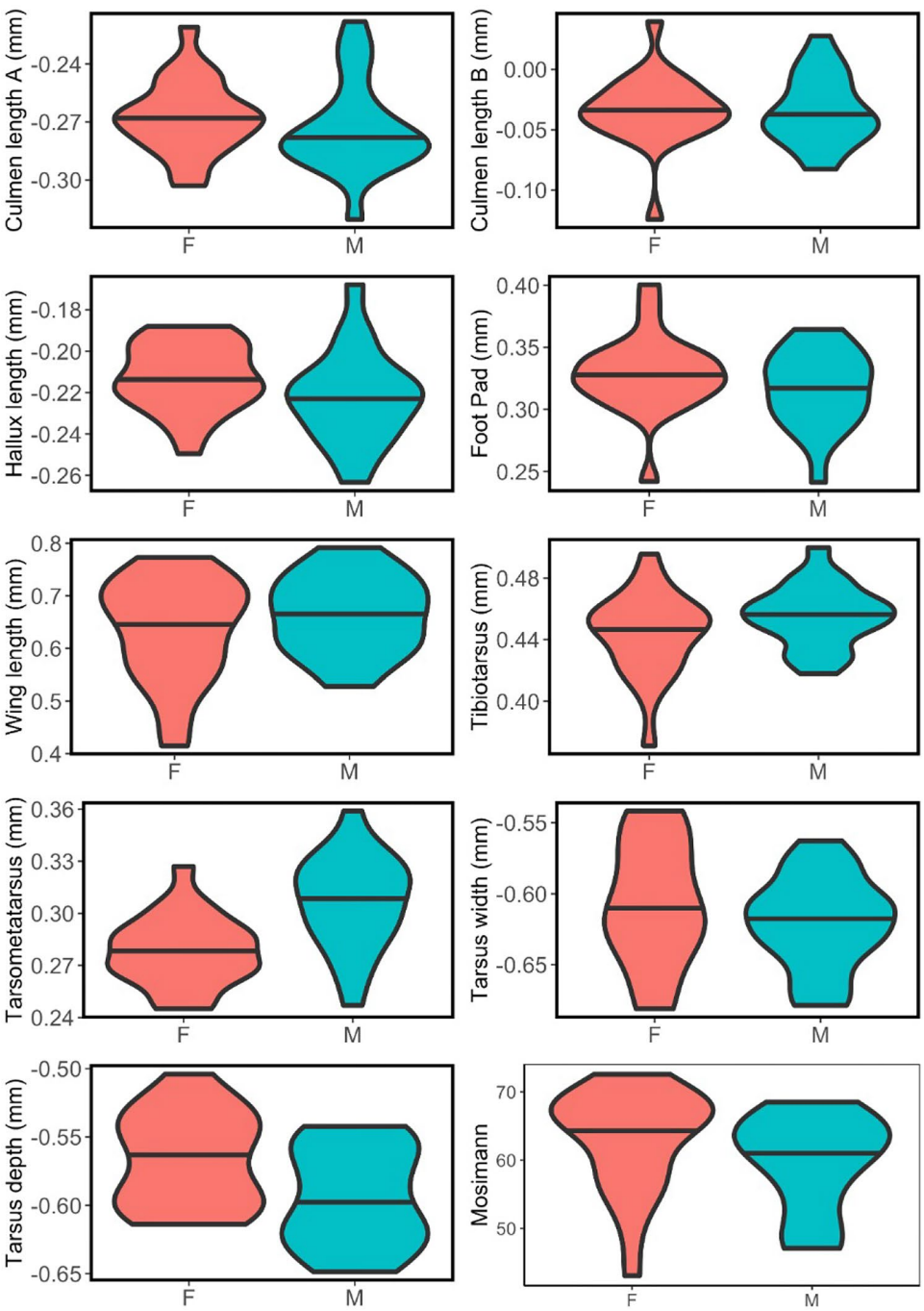

Fig. 1 Violin plots of nine biometrics created with the shape PCA data showing the distribution of each measurement and its frequency when considering shape only across the sampled population of Scottish golden eagle nestlings. The distribution of females is shown on the left (pink) and the distribution of males on the right (blue)

not lead to differentiation between sex based on the biometrics (Supplementary Fig. 3a). Whilst there was differentiation based on 3 age groups (< 4, 5–6 weeks, ≥ 7 weeks), considering sex within age group did not provide differentiation (Supplementary Fig. 3b).

Shape PCA

The shape PCA returned similar results to the size and shape PCA. PCA1 explained 66% of the variation with wing length, tarsus depth, and tarsus width having the highest loadings and PCA2 explaining 11%. Plotting the 2 shape PCA components against each other did not lead to differentiation between sex and sex within age group based on the biometrics (Supplementary Fig. 3c, d).

Maturation analysis

A positive relationship between age and wing length was found in the 10 individual biometrics ($R = 0.9$, $p < 0.002$, R^2

= 0.8129, $p < 0.002$) (Supplementary Fig. 4), indicating that wing length is a measurement that can be used as a proxy for maturity. Regression analyses of wing length against all other biometrics found no differentiation by sex ($p \leq 0.01$).

Discussion

Despite apparent statistically significant differences in the individual 10 biometrics between male and female golden eagle nestlings < 8 weeks old, the overlap of measurements indicates a lack of differentiation between sexes at an early age, and means individual biometrics cannot be used on their own to discriminate between the sexes in these young nestlings. Genetic diversity and natural pressures, which underpin natural selection, enable a wide variability in sizes in any natural population (Pfister and Stevens 2002). Consequently, it is unsurprising that larger than average male golden eagle nestlings can fall within the range of smaller than average females, and vice versa. Using 'wing length' as a proxy for maturity and age (Bortolotti 1984) across the growing period suggests that differentiation will only begin to resolve some time after seven and a half weeks of age, even when individuals are grouped into age categories. The examination of the data across the growing period suggests differentiation will only begin to resolve some time after seven and a half weeks of age, even when individuals are grouped into age categories, males and females overlap, preventing any clear sexual differentiation (Supplementary Fig. 3b, d).

The results also shed doubt on previously published growth curves, which use weight and foot size to infer the sex of nestlings to document differences in male and female nestling growth rates (Ellis 1979). Ellis' growth curves were later used by Watson (2010) as guidance for sexing golden eagle nestlings. These growth curves were created prior to molecular techniques becoming available and rely on the assumption that there are differences in weight between the sexes or use weight to make that characterisation. The overlap of weight data found between sexes in the present study shows that this linear variable does not discriminate between males and females aged under 7.5 weeks of age.

Studies of avian ecology, biology, behaviour and genetics frequently depend on reliable methods to sex individuals, as do practical conservation efforts such as the current golden eagle reinforcement project in Scotland. For example, the monitoring of sex ratios across a population can be used to indicate health problems in a population and/or environmental problems across an area (Hayes et al. 2002, 2010; Bouland et al. 2012; Székely et al. 2014). Similarly, the success of the current Scottish golden eagle translocation program to establish a new breeding population will, in part, be determined by ensuring appropriate sex ratios of the individuals released. Based on the findings of this work, the use of morphometric measurements is not appropriate for sexing golden eagle nestlings, and existing molecular tools should continue to be used until alternative methods of sex determination are developed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10344-022-01627-1.

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Code availability Not applicable.

Declarations

Ethics approval All procedures took place under the necessary Natural England/British Trust for Ornithology licence authority, Home Office license authority (Project license PB8A1D5C7) and ethical approval from the University of Edinburgh.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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