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Size, shape and sex differences in three subspecies of Black-tailed Godwits *Limosa limosa*

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ABSTRACT

Capsule: Black-tailed Godwits *Limosa limosa* show sexual size dimorphism and size differences between the subspecies. The shape varies slightly between the subspecies, but not between the sexes.

Aims: To investigate whether and how the three subspecies of Black-tailed Godwits, and the sexes of these subspecies, differ in size and shape.

Methods: We collected body dimensions (lengths of the bill, total head, tarsus, tarsus-toe and wing) of adult Black-tailed Godwits from three locations (Iceland, the Netherlands and northwest Australia) corresponding to the breeding or wintering grounds of three known subspecies (*islandica*, *limosa* and *melanuioides*, respectively). Determining sex by molecular assays, we computed degrees of sexual size dimorphism. Using principal component analysis (PCA), we compared differences in size and shape among the different subspecies.

Results: The *limosa* subspecies was the largest and also showed the most significant sexual size dimorphism. Sexual size dimorphism was smallest for wing length and largest for bill length. The first two axes of the PCA that included all subspecies of both sexes explained 94% of the total variation. Most body dimensions were highly correlated with each other, but wing length varied independently of the other dimensions. Males and females differed only in size (the first axis). However, one of the two small subspecies, *islandica*, also differed in shape (the second axis) from *limosa* and *melanuioides*.

Conclusions: In all three subspecies of Black-tailed Godwits, females are larger than males. The fact that subspecies differed in the degree of size dimorphism and slightly in shape hints at sex-related differences in the ecological selection pressures between the different flyways.

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Intraspecific variation in body size has formed the basis for the assignment of subspecies and clines (Hamilton 1961, Rising & Somers 1989). However, body size is a composite of several different dimensions (Wishart 1979, Piersma 1984, Davies *et al.* 1988, Freeman & Jackson 1990). Although some studies suggest that overall body size would be well described by including body mass in a principal component analysis (PCA), body mass includes variable components, i.e. the mass of muscles and organs, which are dependent on ecological context and time of year (Piersma & Davidson 1991, Piersma & van Gils 2011). Moreover, organisms not only show variation in body size but also in body shape (Humphries *et al.* 1981). Indeed, shape has been considered a better indicator of intraspecific variation than size (Jolicoeur & Mosimann 1960). Technically, shape differences arise from differences in the ratios of various dimensions and are

usually quantified as the second axis in PCAs (Jolicoeur & Mosimann 1960, Sundberg 1989).

The best-studied biological category of size variation is the difference between the sexes (Payne 1984). Sexual dimorphism is also manifested in plumage colour and ornamental traits (Armenta *et al.* 2008), physiology (Giacomello *et al.* 2006), behaviour (Velando 2002) or any combination of these (Schroeder *et al.* 2008). Sexual dimorphism reflects the evolutionary responses to specific selection pressures that differ between males and females (Andersson 1994, Badyaev & Martin 2000). Obviously, selection pressures will also vary spatially and between environments (Gunnarsson *et al.* 2012, Alves *et al.* 2013). To infer whether there is any evidence that ecological selection pressures differ between flyways, we here examined differences in size and shape between the three known subspecies of a widespread

Paleartic shorebird species, the Black-tailed Godwit *Limosa limosa*. To examine whether such ecological difference could differ between the sexes, we also compared sexual size dimorphism.

Black-tailed Godwits *Limosa limosa* have a temperate breeding distribution that extends across the entire Palearctic (Groen *et al.* 2006, Gill *et al.* 2007). The small size of males is perhaps reflecting advantages to carrying out particular forms of aerial display (Jönsson & Alerstam 1990, Blomqvist *et al.* 1996), and possibly fecundity advantages for bigger females (Summers & Underhill 1991, Sandercock 1998, but see Verhoeven *et al.* 2019). In the two European breeding subspecies *limosa* and *islandica*, females are larger than males, but during the breeding season, males are more colourful (Schroeder *et al.* 2008, Gunnarsson *et al.* 2006). From west to east, the nominate subspecies *limosa* has been reported to show a clinal decline in body size (Prater *et al.* 1977, Groen & Yurlov 1999). There is little knowledge on the size and sexual dimorphism of the eastern subspecies *melanuroides*, which has a scattered breeding range across Mongolia and the sub-Arctic and Arctic regions of the Russian Far East (Engelmoer & Roselaar 1998, Trimbois *et al.* 2014). A preliminary study of the breeding population of *melanuroides* at Lake Baikal, Russia (Groen & Yurlov 1999), suggested that their body dimensions and plumage characteristics resemble *islandica* more than *limosa*. With significantly smaller body dimensions even than *islandica*, in particular the wing length of both sexes and tarsus length in females, this subspecies would be the smallest (Groen *et al.* 2006).

In this study, we compared the body measurements of individual Black-tailed Godwits from three locations corresponding to three subspecies (*islandica* from breeding areas in Iceland, *limosa* from breeding areas in the Netherlands and *melanuroides* from nonbreeding areas in northwest Australia). The birds were unambiguously sexed by molecular assays of their DNA so that we could make robust comparisons between the sexes for the different dimensions.

Methods

Birds belonging to the *islandica* subspecies breeding in Iceland (65.67°N 14.78°W) and the *limosa* subspecies breeding in the Netherlands (52.98°N 5.4°E), were caught in walk-in traps, mist-nets and clap-nets. Nonbreeding individuals of *melanuroides* were captured by cannon-net at Roebuck Bay in northwest Australia (17.94°S 122.25°E). Captured birds were fitted with a uniquely numbered metal ring and colour rings, they were measured and a small (10–50 µl) blood

sample was taken from the brachial vein for subsequent molecular sexing. In some Australian cases, and for all birds from Iceland, sex was based on DNA extracted from a few chest feathers. Both blood and feather samples from the Netherlands and northwest Australia were stored in 96% ethanol at –20°C or –80°C, feather samples from Iceland were stored dry.

Body dimensions

Five linear dimensions (the bill, total head, tarsus, tarsus plus toe and wing length) of adult Black-tailed Godwits were taken in the field. The lengths of bill, total head (i.e. the length from the back of the head to the tip of the bill), tarsus and tarsus-toe length (i.e. tarsus plus mid-toe without nail) were measured to nearest 0.1 mm; the wing (i.e. the flattened wing chord; Evans 1986) was measured to nearest 1 mm.

Possible differences in wing length of *melanuroides* and *limosa/islandica* due to the differences in stage and time of the year were examined by plotting wing length on the day of the year for *melanuroides*. In both sexes, wing length showed systematic changes across the nonbreeding season from July to April of the next year ($R^2 = 0.1129$, $P < 0.001$).

Molecular sexing

For the blood samples of *limosa* and *melanuroides*, DNA extraction using the ammonium acetate (AmAc) method was performed as described in Richardson *et al.* (2001). For the feathers from northwest Australia, a modified DNA extraction protocol was applied as follows: three feathers from ethanol were air-dried, the roots of the feathers were cut off and placed into a microcentrifuge tube. Then submerged in 205 µl of proteinase solution (25 µl proteinase K, 180 µl Buffer ATL). Feathers were finely cut with a pair of scissors whilst inside the tube, after digested at 55°C for 2 nights, the sample was centrifuged at 6000 g for 5 min, then 200 µl of the supernatant was transferred into a new microcentrifuge tube, after mixed with 180 µl Buffer AL and incubated at 70°C for 10 min, 200 µl ethanol (96–100%) was added and vortexed. To precipitate the DNA from the supernatant, each sample was applied to the column inside a collection tube, centrifuged at 6000 g for 1 min, flow-through was put back to the column and centrifuged again. Then 500 µl Buffer AW1 was added to the column and centrifuged at 6000 g for 1 min, followed by 500 µl Buffer AW2 and centrifuged at 20 000 g for 3 min. A new collection tube was used after the rinse; the column was air-dried, a volume of 50 µl pre-heated (70°C) buffer AE was added and incubated

at room temperature for 5 min before centrifuged at 6000 g for 1 min. This step was repeated once to maximize the collection of DNA. The DNA concentration was stored at -20°C . A pair of modified primers 2602F/2669R (van der Velde *et al.* 2017) was used for amplification of DNA through polymerase chain reaction (PCR). PCR products were visualized on a 2% agarose gel, males with ZZ genotype show only one bar on the agarose gel, whereas females of the genotype ZW have two bars. The molecular sexing techniques of *islandica* are fully described in Gunnarsson *et al.* (2006).

Totals of 276 Black-tailed Godwits (138 males and 138 females) from the Netherlands, 84 (38 males and 46 females) from Iceland and 178 (94 males and 84 females) from northwest Australia were successfully sexed. For the samples from the Netherlands and Australia, 55 and 35 samples (20% of each total sample size) were selected randomly to re-run the PCR and found to be 100% repeatable. Samples from Iceland were tested at least twice, with the results being entirely consistent.

Statistics

We used PCA to describe sex and subspecies related to variation in size and shape. This ordination method is widely used in morphological studies (Humphries *et al.* 1981, Somers 1986, Bookstein 1989, Lockwood *et al.* 1998). The variables were converted and displayed as several independent principal components (PCs); from the equation of the correlation matrix, eigenvalues of all variables were determined (Table 1). PCs values that accumulate more than 80% of the variation were used and then loaded into a biplot as a vector. The origin of the biplot represents the average, the angles between vectors can be interpreted as their correlations: if vectors have the same direction then the correlation is 100%, but at an angle of 90 degrees the correlation is zero; vectors in opposite directions have negative correlations (Zuur *et al.* 2007). Vectors can be projected perpendicularly onto each of the axes to evaluate the relative size for that dimension. In morphological studies, the first component of the PCA

(PC 1) is generally interpreted as size, with PC 2 (the y-axis) as shape (Somers 1986, Sundberg 1989, Lockwood *et al.* 1998). As PCA cannot cope with missing values, this analysis only includes individuals with complete sets of measurements.

To describe the degree of sexual dimorphism (DSD), we used the formula for species where females are the larger sex introduced by Lovich & Gibbons (1992):

$$\text{DSD} = \left(\frac{\text{♀ mean body dimension}}{\text{♂ mean body dimension}} - 1 \right)$$

The larger the value, the greater the sex difference for that body dimension, with zero meaning that the sexes are of the equal size. The statistical analyses were performed in R studio 3.5.0 and JMP 11.0.

Results

In all three subspecies, and for all dimensions, females were larger than males (Table 2). Nevertheless, overlap still occurred for every dimension (Figure 1). Overall, *limosa* was the largest subspecies for every dimension relative to *melanuiroides*, and *islandica* had a body size close to the mean value of all subspecies. The largest DSD was for bill length and the least for wing length in each subspecies. The subspecies *limosa* and *melanuiroides* had similar DSD for the total head, tarsus to tarsus-toe length. The second-largest DSD in *islandica* was for tarsus length, followed by total head and tarsus-toe length (Table 3).

The first two PCs explained 94.0% of the total variation (Table 4). The vectors represent three layers of size and shape structure: (1) overall body dimensions and differences between (2) sexes and (3) subspecies (Figure 2). All vectors of body dimensions pointed to the right, indicating that all variables were highly correlated with each other except for wing length; females and males showed a negative (size) correlation; *limosa* and *melanuiroides* were different from *islandica*.

The second component, describing shape discrepancies among sexes and subspecies, explained 4.7% out of 94.0% of the total variation. The projection of sex on the second component showed that females and males have similar absolute values (see the origin of the biplot, Figure 2). The projection of subspecies on the second component indicates that the shape of *islandica* is different from *limosa* or *melanuiroides* which have similar shapes. Thus, the second component shows shape discrepancies between subspecies, and not between the sexes.

Table 1. Correlation coefficients for five morphometrics in Black-tailed Godwits *Limosa limosa*.

	Wing	Bill	Total head	Tarsus-toe	tarsus
Wing	–	0.7950	0.8420	0.8172	0.8057
Bill		–	0.9683	0.8577	0.8575
Total head			–	0.8828	0.8820
Tarsus-toe				–	0.9538
Tarsus					–

Table 2. Five body measurements of three subspecies of Black-tailed Godwits *Limosa limosa* corresponding to three locations: *limosa* of the Netherlands (NL), *islandica* of Iceland (ISL) and *melanuiroides* of northwest Australia (AUS). For the difference between the sexes in each population, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. NL: $n(\text{male}) = 138$, $n(\text{female}) = 138$; ISL: $n(\text{male}) = 38$, $n(\text{female}) = 46$; AUS: $n(\text{male}) = 94$, $n(\text{female}) = 84$.

Dimension	Population	Males			Females		
		Mean	sd	Range	Mean	sd	Range
Bill length(mm)	NL***	89.70	5.28	71–118	105.44	5.63	83–130
	ISL***	80.22	3.66	72–88	93.79	5.28	79–102
	AUS***	73.86	4.11	63–85	84.34	4.71	72–96
Total head length (mm)	NL***	128.17	5.54	111–152	145.32	6.06	122–164
	ISL***	119.38	4.09	111–129	134.09	5.71	120–144
	AUS***	107.33	5.05	94–121	118.57	6.00	103–133
Tarsus length (mm)	NL***	72.72	3.65	60–89	80.62	4.12	67–100
	ISL***	68.57	3.27	58–79	75.40	3.97	66–82
	AUS***	62.78	2.72	55–70	67.95	3.25	57–75
Tarsus-toe length (mm)	NL***	115.30	4.75	102–134	125.58	5.09	109–144
	ISL***	111.04	3.81	101–121	120.38	4.32	111–128
	AUS***	101.45	3.34	92–111	108.46	3.93	97–116
Wing length (mm)	NL***	214.34	5.48	199–241	225.18	5.72	206–245
	ISL***	213	4.73	203–223	225.71	5.57	211–234
	AUS***	194.38	7.21	179–209	204.67	6.59	158–219

Moreover, there were also differences between subspecies in the correlation structure. In *limosa*, the lengths of the bill, total head, tarsus and tarsus-toe

were highly correlated with each other, but not with wing length, which made the first four dimensions the best measure to describe size and shape. In *islandica*,

Figure 1. The Bill, total head, tarsus, tarsus-toe and wing length of female (♀) and male (♂) Black-tailed Godwits *Limosa limosa* from Iceland (ISL, *islandica*), The Netherlands (NL, *limosa*), NW Australia (AUS, *melanuiroides*).

Table 3. DSD of three subspecies of Black-tailed Godwits.

Trait	<i>islandica</i>	<i>limosa</i>	<i>melanuroides</i>
Bill	0.17	0.18	0.14
Total Head	0.12	0.13	0.10
Tarsus	0.15	0.19	0.08
Tarsus-toe	0.08	0.09	0.07
Wing	0.06	0.06	0.05
N males	38	138	94
N females	47	138	84
N total	85	276	178

Table 4. Eigenvalues and (cumulative) percentages for the four axes in the PCA of five morphological traits (length of the bill, total head, tarsus, tarsus-toe and wing) in Black-tailed Godwits of three subspecies.

Axis	Eigenvalue	Percentage	Cumulative Percentage
1	4.4675	89.350	89.350
2	0.2367	4.733	94.083
3	0.2225	4.451	98.534
4	0.0459	0.919	99.453
5	0.0274	0.547	100.000

the wing length was positively correlated with the other dimensions. Similarly, in *melanuroides* wing length was also correlated with the other dimensions, but this time negatively with respect to both axes of variation. Thus, *melanuroides* had the relatively shortest wing lengths of the three subspecies.

Discussion

We confirmed earlier reports (Prater *et al.* 1977, Groen & Yurlov 1999) that *limosa* is the largest subspecies and that *melanuroides* is the smallest, and that in all three subspecies of Black-tailed Godwits females are the larger sex. However, we also show, for the first time, that there are subspecies differences in the extent of sexual size dimorphism, with *limosa* showing the greatest dimorphism. This actually follows Rensch's rule (Rensch 1950) that sexual size dimorphism increases with body size in taxa in which males are the

Figure 2. PCA correlation biplot of linear dimensions of Black-tailed Godwits (length of the bill, total head, tarsus, tarsus-toe and wing) with two explanatory variables: sex and subspecies.

bigger sized sex and decreases with body size in those in which females are larger (Abouheif & Fairbairn 1997). Furthermore, although the shape differences expressed by the second PC were rather small, shape variations between subspecies do exist. Most of the variation in body dimensions between sexes and subspecies was explained by the first principle component, i.e. representing size rather than shape variation. Still, the shape of *islandica* was different from the other two.

Sexual size dimorphism is often thought to relate to sexual selection (Figuerola 1999, Sandercock 2001, Blondel *et al.* 2002), with only one hypothesis, the one of display agility, having been empirically examined in shorebirds. In Dunlin *Calidris alpina*, females preferred smaller males with better display flights (Blomqvist *et al.* 1996). Interestingly, in Icelandic Black-tailed Godwits, males of smaller body size with proportionally longer wings occupied breeding sites of higher quality and greater availability of females (Gunnarsson *et al.* 2012). This would select for smaller sized and relatively longer winged males, which could represent a selection pressure that explains the sexual size dimorphism in this subspecies. The question is whether this also applies to other subspecies.

In addition to sexual selection pressures, the difference between three subspecies may reflect differences in the kind of natural selection pressures encountered in the course of the year (Loonstra *et al.* 2018); such pressures may well differ (also in temporal ways) between flyways. Selection will manifest itself as differences in reproduction (on the breeding grounds) and survival (possibly measured indirectly as foraging efficiency and predator avoidance throughout the annual cycle). The finding that bill length consistently was the most sexually dimorphic trait implies that male and female Black-tailed Godwits have feeding strategies or food resources which differ between flyways. The importance of bill length as a niche differentiator has previously been established for several shorebird species including Oystercatchers *Haematopus ostralegus* (Durrell *et al.* 1993), Western Sandpipers *Calidris mauri* (Mathot *et al.* 2007), as well as Bar-tailed Godwits *Limosa lapponica* (Duijns *et al.* 2014, Zharikov & Skilleter 2002) and Icelandic Black-tailed Godwits (Alves *et al.* 2013).

The present study on size and shape variations between subspecies and sexes of Black-tailed Godwits suggest that the flyway-specific ecological conditions, and downstream selection pressures, faced by Black-tailed Godwits in the course of the year actually lead to morphological differences between the flyways. We propose that Black-tailed Godwits represent a tractable study system to comparatively study how ecological

conditions affect the microevolution of body size and shape.

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