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MOLECULAR VS. PHENOTYPIC SEXING IN RED KNOTS[1]

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Abstract. Using a reference collection of blood samples of Red Knots (*Calidris canutus*) from Florida and Alaska whose sex was determined by dissection, we demonstrate that the molecular method of sexing based on the size of polymerase chain reaction (PCR) products from the CHD genes after digestion with *Hae* III accurately identifies gender in this species. In males, the 110 basepair PCR product is cleaved into two smaller fragments, whereas in females this fragment remains uncut. Molecular sexing of other samples of Red Knots from the Dutch Wadden Sea, southern Brazil, and Delaware Bay revealed that methods using a discriminant function on size or plumage dimorphism are only partly effective in correctly assigning sex of birds. Given the low cost and accuracy of molecular sexing on very small blood samples, we anticipate that it will find increasing use in studies of the evolutionary ecology of shorebirds and other avian species.

Key words: Calidris canutus, *CHD genes, molecular sexing, morphometrics, plumage, Red Knots, shorebirds.*

Determination of sex of birds in the field provides the raw material for testing various theories in ecology and evolution including sex ratio evolution, mate choice, and sexual differences in resource utilization, reproductive investment, immunocompetence and behavior, and life history evolution (Short and Balaban 1994, Ellegren and Sheldon 1997). Many species of shorebirds are amenable to sexing because the sexes are dimorphic in plumage and/or size (Wymenga et al. 1990, Rogers 1995), but few studies have been done to independently verify the accuracy of phenotypic assignment of sex (Piersma and Jukema 1990, Zwarts et al. 1996). With the exception of highly sexually dimorphic species such as Ruff (*Philomachus pugnax*) and Bar-tailed Godwit (*Limosa lapponica*), it is difficult to determine sex in nonbreeding shorebirds when they have molted their dimorphic plumage of the breeding season into a monomorphic "basic" plumage. Other species are essentially monomorphic with regard to body dimensions and cannot be sexed except by dissection, laparotomy, or measuring the width of the cloaca during the egg laying period (Harrington and Morrison 1979, Maron and Myers 1984, Soloviev and Tomkovich 1995).

A recent solution to this problem has been provided by the refinement of molecular sexing techniques, in which a portion of the gene coding for a chromo-helicase-DNA-binding protein (CHD) is amplified by polymerase chain reaction (PCR) using universal primers that work on a wide range of bird species (Ellegren 1996, Griffiths et al. 1996, Ellegren and Sheldon 1997). The potential utility of this technique is that the sex of a banded bird can be unequivocally determined from a few drops of blood or from feather pulp taken in the field, and thus a study population of individually color-marked birds of known sex can be established.

In this study, we test the applicability of molecular sexing in a shorebird species, the Red Knot (*Calidris canutus*). Red Knots are long distance migrants that typically pass through staging sites en route to their arctic breeding and southerly wintering grounds in spring and fall, respectively (Piersma and Davidson 1992). The species has attracted much research attention (Piersma 1994, Harrington 1996), and is increasingly used as a model system in studies of bird migration (Gudmundsson and Alerstam 1998, Weber et al. 1998, Piersma and Baker 1999). Molecular sexing of Red Knots is highly desirable because the sexes overlap substantially in size (Tomkovich 1992, Tomkovich and Soloviev 1996), and thus an unknown fraction of a population sample cannot be sexed reliably by morphometric criteria alone. The sexes in this species are reputed to differ in breeding plumage, with females having barred feathers among the more intensely red breast feathers and a duller plumage on the back (Prokosch 1988). We show that Red Knots from four subspecies in the Americas and Europe can be sexed with the molecular method, and that morphometric measurements and plumage characters are of limited value

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as they correctly assign sex of less than 80% of the individuals in different samples.

METHODS

SAMPLES ANALYZED

Two samples of Red Knots in which the sex of each bird had been determined by dissection were chosen to serve as a reference to test the accuracy of the molecular sexing method. One sample of 12 birds was collected in San Marco Island in Florida on 30 January 1986, and the other sample of 13 birds was collected at the Yukon River Delta in Alaska on 5 May 1993. The subspecific status of the Florida birds is not fully resolved, but they are likely to be *C. c. roselaari,* the population of which purportedly breeds on Wrangel Island (Tomkovich 1990). The Alaska birds may belong either to *C. c. roselaari* or to *C. c. rogersi* which breeds in Alaska and Chukotka Peninsula and winters in Australasia (Barter 1992). For molecular sexing, DNA was extracted from tissue samples taken from birds immediately after death and frozen in liquid nitrogen.

To test the efficacy of phenotypic criteria commonly used in sexing Red Knots, we used blood samples of birds captured at different times of the year in the Dutch Wadden Sea. Upon capture, the birds were measured for the lengths of the bill (exposed culmen), total head–bill (from the back of the skull to the bill tip), wing (maximum stretched length), tarsus, and tarsus + middle toe (excluding the nail), and, when in breeding plumage, were scored for barring on the breast feathers (present = female, absent = male) and back color (predominantly faded reds and yellows = male, predominantly black feathers = female). The wing was measured with a ruler to the nearest mm, and the other measurements were recorded to the nearest 0.1 mm with dial calipers. All measurements and plumage assessments were made by T. Piersma. Birds were independently sexed by the molecular method using DNA isolated from small blood samples (10–100 μL) taken from the brachial vein and stored in 70% ethanol. A total of 116 birds, captured with mistnets at different sites in the western Dutch Wadden Sea, was examined. Six were captured as juveniles on 16 April 1998, and the remainder of the Wadden Sea sample was captured as adults (2 on 27 October 1994, 5 on 6 November 1994, and 103 in July–August 1995, 1996, and 1997). The July–August samples contained individuals of both *C. c. canutus* which breed in Siberia and winter in West Africa (Piersma et al. 1992), and *C. c. islandica* which breed in Greenland and northeast Canada and winter in Western Europe (Davidson and Wilson 1992). The Red Knots captured in October, November, and April were assigned to *C. c. islandica* because this is the only subspecies that winters in the Wadden Sea.

We also compared sexing methods in two samples of adult Red Knot *C. c. rufa* (Morrison and Harrington 1992, Harrington 1996), captured with cannon-nets during the spring migration in the western Atlantic flyway. One sample comprised 90 adult birds captured on the Atlantic Ocean beach adjacent to Lagoa do Peixe in Rio Grande do Sul Province in Brazil on 2–5 April 1997 (Baker et al. 1999), and the other comprised 85 Red Knots captured at Slaughter Beach, Delaware Bay, Delaware, on 22–24 May 1997. These samples were chosen because they provide an opportunity to evaluate phenotypic sexing techniques and to check for sex-specific differences in the timing of migration. All birds were measured and blood samples were taken and preserved in 70% ethanol.

MOLECULAR SEXING

The method of molecular sexing we employed was that of Griffiths et al. (1996), in which a short 110 base pair (bp) fragment in the DNA-binding region (see Fig. 1 in Kahn et al. 1998) of the highly conserved CHD gene was amplified using the P2-P3 primer pair. When used in a PCR reaction, the primers amplify products from two genes, one (CHD-W) located on the W chromosome and the other (CHD-NW) which is probably on the Z chromosome (Ellegren 1996, Griffiths et al. 1996, Griffiths and Korn 1997). In our assay, the restriction enzyme *Hae* III only cuts the product from the CHD-NW gene, and thus in males, the larger 110 bp band disappears because it is fragmented into pieces of 65 and 45 bp which are sometimes not visible on an agarose gel stained with ethidium bromide. Females retain this larger band on the gel representing a product from the CHD-W gene which does not contain the *Hae* III restriction site.

Genomic DNA was extracted from blood samples using standard procedures (Sambrook et al. 1989). Blood samples were homogenized in a solution of STE buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0), 0.1% SDS, and 10 μg ml^{-1} proteinase K, and incubated overnight at 55°C. The homogenate was extracted twice with Tris-saturated phenol and once with chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated in NaCl and ethanol, and resuspended in sterile distilled water. The concentration of DNA was determined using a Hoefer TKO100 Dedicated MiniFluorometer, as per the manufacturer's instructions. DNA samples were amplified using the PCR in a total reaction volume of 25 μl, consisting of 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 50 μM each dNTP; 0.4 μM each of primers P2 and P3; and 1 Unit *Taq* DNA polymerase (Boehringer Mannheim). A total of 200 ng of genomic DNA was used as the initial template for amplification for each sample. The following thermal cycle profile was carried out using a Perkin Elmer DNA 480 Thermal Cycler: 93°C for 30 sec, 48°C for 30 sec; and 72°C for 60 sec, for a total of 40 cycles.

Following amplification, 8.5 μl of PCR product was digested overnight at 37°C with 5 Units of *Hae* III (Promega) in 1x Promega restriction buffer 3 in a total reaction volume of 10 μl. The digested PCR product and an aliquot of the remaining undigested PCR product were subjected to electrophoresis through a 4% agarose gel in 1x TA buffer, and visualized using ethidium bromide and UV illumination. The gender of each individual was scored on the basis of the presence or absence of the 110 bp band in the gel following digestion of the PCR product.

PHENOTYPIC SEXING

To quantify the magnitude of sexual dimorphism in size, we computed means and standard deviations of

the morphometric variables for each of the samples. All variables appeared normally distributed (checked by means of cumulative normality plots), and thus the raw data were used. To maximally separate the sexes, we constructed a two-group discriminant function using all variables or combinations thereof. The success of the discriminant function in sexing birds was evaluated with a classification matrix, although this estimate is specific to the sample used and would likely be lower when sex is assigned with this discriminant function to birds in an independent sample. Thus it represents a maximal estimate of the success of this method in assigning sex. The efficacy of plumage characters in sexing birds was investigated by comparing their assigned sex to that determined by molecular sexing. We note that not all birds in a sample can be sexed using plumage characters, and thus we recorded the success of the method with and without the birds of unknown sex. In the sample from the Dutch Wadden Sea, both the barring on the breast feathers and the color of the back were recorded, but in Lagoa do Peixe and Delaware Bay, sex was assigned only with the presence or absence of barring.

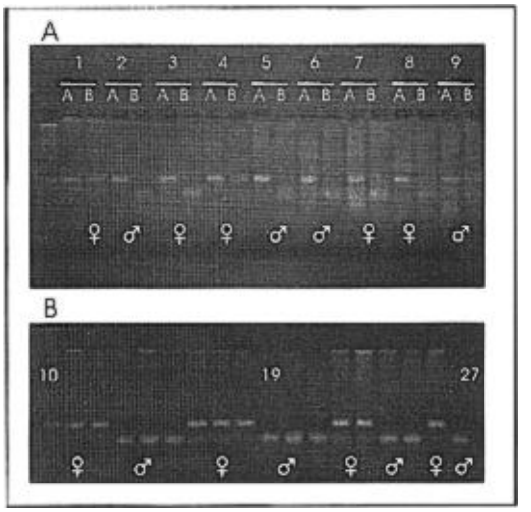

FIGURE 1. Agarose gel separation of PCR products amplified from the CHD genes in Red Knots with the P2-P3 primer-pair of Griffiths et al. (1996). In (A), undigested and *Hae* III-digested products from birds of the Florida sample that were sexed by dissection are run in lanes A and B, respectively, and sex is indicated on the gel. (B) only shows digested DNA profiles of 18 birds of unknown sex from the Dutch Wadden Sea in which it is clearly visible that males have only the lower band (the 65 bp digestion product from the CHD-NW locus). Females have this product too, but the intensity of the fluorescence is only half that in males.

RESULTS

The sex of the of 25 Red Knots from Florida (n = 12) and Alaska (n = 13) determined with molecular sexing exactly matched their sex by dissection and gonadal inspection (Fig. 1A). Males are readily distinguished when the digested PCR products are run out on agarose gels because the CHD-NW product is cleaved into two smaller products, the larger (65 bp) of which fluoresces brightly just above the primer-dimers. In contrast, females have an intact 110 bp CHD-W product which is not digested as it lacks a *Hae* III site. However, as is most clearly seen in Figure 1B, females (chromosomes WZ) also have the 65 bp digestion product, but the intensity of the fluorescence is only half that in males (chromosomes ZZ). This clearly suggests that the CHD-NW locus is on the Z-chromosome rather than the autosome.

Based on the molecularly sexed Red Knots from the Wadden Sea, Lagoa do Peixe, and Delaware Bay, the degree of sexual dimorphism in the various linear dimensions can be examined (Table 1). In all three samples, sexual dimorphism is most pronounced with respect to bill length; bill length in females averages about 7% longer than in males in the Wadden Sea and Lagoa do Peixe, and about 3% longer in Delaware Bay. Size dimorphism also is apparent in total head-bill, wing, tarsus, and tarsus+toe dimensions, where females are on average 1–5% larger than males. Despite considerable overlap in dimensions between birds from the Wadden Sea belonging to *C. c. canutus* and *C. c. islandica,* and the individuals of *C. c. rufa* from Lagoa do Peixe and Delaware Bay, the latter subspecies appears larger than the Old World subspecies. This is in accordance with Tomkovich's (1992) observation based on museum specimens sexed by dissection.

Using only plumage characters, we were unable to assign sex to a quarter of the 95 adults captured in July and August in the Dutch Wadden Sea. When these unsexed birds are included in the sample, only 54.7% of the adult Red Knots from July–August were correctly assigned to sex (Table 2), with males (75.7% correct) apparently being more distinct than females (41.4% correct). Using a discriminant function based on the most sexual dimorphic linear dimension (bill length), 72.4% of the birds were correctly assigned to sex, with females (79.4% correct) being more often correctly sexed than males (62.5% correct). Adding another four body size variables to the discriminant function increased the overall percentage of correctly sexed birds from 72.4% to 75.9%.

For Red Knots from Lagoa do Peixe, the presence or absence of barred breast feathers correctly identified the sex in 73.3% of the birds (Table 2). The discriminant function using only bill length did slightly better for the Lagoa do Peixe than for the Wadden Sea birds (75.6% vs. 72.4% correct, respectively). The inclusion of total-head and wing length increased the overall percentage correctly sexed to 80.0%. Addition of the barred feather criterion into the discriminant function had no effect on classification success.

Given the greater overlap in the linear dimensions of the sexes in Delaware Bay than in Lagoa do Peixe, not surprisingly the discriminant functions performed more poorly (Table 2). A discriminant function based on three dimensions still accurately identified only about 70% of the source group, and adding the barring criteria did not improve the situation. Using the absence or presence of barred breast feathers only, 63.5%

TABLE 1. Body dimensions of male and female Red Knots from the Dutch Wadden Sea, Lagoa do Peixe in southern Brazil, and Delaware Bay, USA, sexed with molecular techniques as outlined in the text. All dimensions in mm. The sample sizes for birds from the Wadden Sea were 48 males and 68 females (except for wing length where n was 47 and 66, respectively). For Lagoa do Peixe, n was 55 males and 35 females (all adult); the sample from Delaware Bay consisted of 40 males and 45 females (all adult). All differences between the sexes were statistically significant at $P < 0.01$ level (Student's t-test, pooled variance).

		Wadden Sea		Lagoa do Peixe		Delaware Bay	
Body dimensions	Sex	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Bill length	Males	32.3 ± 1.6	28.3–35.2	35.1 ± 1.5	31.2–38.0	34.3 ± 1.9	29.9–38.4
	Females	34.5 ± 1.7	30.2–38.1	37.6 ± 1.8	34.8–42.2	35.3 ± 1.6	31.3–38.7
	F/M ratio	1.07		1.07		1.03	
Total head length	Males	62.3 ± 1.8	57.6–66.0	63.6 ± 1.5	59.6–67.3	64.3 ± 1.9	59.2–68.0
	Females	64.7 ± 1.9	60.2–68.6	66.3 ± 1.7	63.2–70.6	65.5 ± 1.7	60.5–68.9
	F/M ratio	1.04		1.04		1.02	
Wing length	Males	167.7 ± 4.6	154–178	171.5 ± 3.9	154–180	169.5 ± 4.3	162–178
	Females	172.2 ± 5.3	155–181	173.9 ± 3.6	165–180	171.9 ± 3.1	163–178
	F/M ratio	1.03		1.01		1.01	
Tarsus length	Males	31.6 ± 1.2	29.2–33.7				
	Females	32.5 ± 1.4	28.5–35.6				
	F/M ratio	1.03					
Tarsus + middle toe length	Males	57.0 ± 1.4	53–60				
	Females	58.0 ± 1.9	53–62				
	F/M ratio	1.02					

TABLE 2. Performance of different phenotypic sexing methods on Red Knots from the Dutch Wadden Sea, Lagoa do Peixe in southern Brazil, and Delaware Bay, USA. See text for details of the different sex discrimination methods, and Table 1 for the sex-specific averages of the body dimensions in birds from the three locations.

Sampling site	Phenotypic sexing technique	Sample sizes		Percentage correctly sexed		
		Males	Females	Males	Females	All
Wadden Sea	Plumage characteristics (adults from July/August only)	37	58	75.7	41.4	54.7
	Discriminant function (using bill length only)	48	68	62.5	79.4	72.4
	Discriminant function (using bill, total head, wing, tarsus, and tarsus + middle toe length)	47	65	63.8	84.6	75.9
Lagoa do Peixe	Plumage character only (absence/presence of barring)	55	35	73.1	73.9	73.3
	Discriminant function (using bill length only)	55	35	85.5	60.8	75.6
	Discriminant function (using bill, total head, and wing length)	55	35	89.1	65.7	80.0
	Discriminant function (based on the previous three dimensions and presence/absence of barring)	55	35	89.1	65.7	80.0
Delaware Bay	Plumage character only (absence/presence of barring)	40	45	82.5	46.7	63.5
	Discriminant function (using bill length only)	40	45	60.0	66.7	63.5
	Discriminant function (using bill, total head, and wing length)	40	45	57.5	80.0	69.4
	Discriminant function (based on the previous three dimensions and presence/absence of barring)	40	45	65.0	68.9	67.1

of the birds were sexed correctly, an even lower proportion than in Lagoa do Peixe. Remarkably, success in determining the sex of birds with phenotypic characters was reversed in these two samples; in the sample from Brazil, males could best be sexed, whereas in the Wadden Sea and Delaware Bay females were sexed most successfully.

DISCUSSION

Our study has established that Red Knots can be conveniently and accurately sexed using agarose gel electrophoresis of digested products of the DNA-binding region in the CHD genes. The P2-P3 primer-pair designed by Griffiths et al. (1996) has wide applicability in birds, although, as noted by Kahn et al. (1998), it is by no means universal. For example, it does not work on Silver Gulls (*Larus novaehollandiae*) from New Zealand (A. Given, pers. comm.) because the *Hae* III cut site in the CHD-NW product is absent in virtually all individuals of this species examined to date, and other sex-specific cut-sites could not be located with other restriction enzymes. In such cases, the method of molecular sexing based on variation in the length of the intron between the helicase and DNA-binding region may provide a solution (Ellegren 1996, Kahn et al. 1998), and has the advantage that the additional step of digestion of the PCR product is eliminated. This also lowers the laboratory cost of molecular sexing from approximately U.S.$3.50 to U.S.$3.00 per bird. However, when we tried sexing knots on the basis of differences in the lengths of introns in the CHD-W and CHD-NW genes using the universal primers in Kahn et al. (1998), we were unable to distinguish the sexes absolutely in Red Knots because of the length variation in the CHD-NW intron.

Comparison of the molecular sex of birds with their phenotypic sex assignment in samples of Red Knots from different seasons, subspecies, and stopover or wintering sites, indicated clearly that birds cannot be sexed accurately with commonly used mensural or plumage characters. Although the usual shorebird pattern of size dimorphism, in which females are larger on average than males, is present in Red Knots, the zone of overlap is too large for effective use of discriminant functions. *A posteriori* classification probabilities on new samples will be even less successful, and there is no way of dealing with the inevitable mis-

classification of very small females and very large males.

In contrast, the ability to accurately determine sex of birds with molecular sexing methods offers the exciting possibility of investigating sex ratios in different age classes, primary sex ratios, differential timing of migration of the sexes, and numerous other sex-related life history parameters of significance in ecology and evolution. For example, the sex ratio in the northward migrating sample of Red Knots captured at Lagoa do Peixe in 1997 was strongly biased in favor of males, and likely indicates that many males migrate ahead of females in the southern part of the western Atlantic flyway. However, the sample captured on 22–24 May 1997 in Delaware Bay immediately prior to the departure of birds for their arctic breeding grounds in Canada had a 1:1 sex ratio, suggesting that the sexes increasingly synchronize their migration as the breeding period approaches. Another example is provided by a large mortality of Red Knots we observed in southern Brazil (Baker et al. 1999). Sexing by dissection of the casualties and molecular sexing of the surviving birds indicated that males succumbed disproportionately to the unknown mortality agent (T. Piersma and A. J. Baker, unpubl. data). We anticipate many new ecological applications with molecular sexing methods in shorebird populations.

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MOLT CHRONOLOGY OF AMERICAN COOTS IN WINTER[1]

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Abstract. We examined molt chronology of American Coots (*Fulica americana*) wintering in Alabama. Molt intensity was quantified by converting percentages of developing feathers sampled in 10 feather regions to molt scores (Total Molt Score) and by estimating percentage of total feather mass undergoing molt (%MOLT). Percent occurrence of molting birds was greatest in December (95%) and lowest in January (28%). Molt intensity was higher during October–December than in January and February, with lowest molt intensity occurring in January. This pattern was consistent between Total Molt Score and %MOLT, however, the magnitude of values for Total Molt Score was twice that for %MOLT. We assert that %MOLT provides a more biologically meaningful assessment of molt. Low molt intensity coupled with adequate protein and energy content of the diet resulted in minimal additional nutrient demands due to molt for wintering coots at Guntersville Reservoir.

Key words: American Coot, Fulica americana, *molt, nutrition, winter.*

Periodic replacement of feathers is essential to the protection, thermoregulation, locomotion, and communication functions of avian plumage. Because molt is nutritionally costly (Murphy 1996), its timing in the annual cycle has important ecological implications. For many species, the timing of molt represents a tradeoff between the need to replace worn or inappropriate plumage and allocation of nutrients to other important events in the annual cycle (Moore et al. 1982). For example, Darwin's finches typically molt on a regular cycle, but suspend molt to nest when food availability increases (Grant 1986). For Northern Pintails (*Anas acuta*) and Mallards (*A. platyrhynchos*), molt is delayed in winters of poor habitat conditions and/or low food availability (Miller 1986, Heitmeyer 1987). Clearly, knowledge of the timing and intensity of molt is critical for a complete understanding of nutritional requirements throughout the annual cycle.

American Coots (*Fulica americana*) breed throughout much of the northern U.S. and southern Canada, and winter in large numbers across the southern U.S., Mexico, and Central America (Alisauskas and Arnold 1994). Despite their abundance and ubiquitous distribution, chronology of molt for this species is poorly documented. In a study of captive coots, Gullion (1953) reported one complete wing molt per year occurring in late summer, but did not present data for molt of body plumage. Although wing molt is complete in free-living coots before fall migration, molt in some body regions still is occurring when these birds

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