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Secrets in the eyes of Black Oystercatchers: a new sexing technique

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ABSTRACT. Sexing oystercatchers in the field is difficult because males and females have identical plumage and are similar in size. Although Black Oystercatchers (*Haematopus bachmani*) are sexually dimorphic, using morphology to determine sex requires either capturing both pair members for comparison or using discriminant analyses to assign sex probabilistically based on morphometric traits. All adult Black Oystercatchers have bright yellow eyes, but some of them have dark specks, or eye flecks, in their irides. We hypothesized that this easily observable trait was sex-linked and could be used as a novel diagnostic tool for identifying sex. To test this, we compared data for oystercatchers from genetic molecular markers (CHD-W/CHD-Z and HINT-W/HINT-Z), morphometric analyses, and eye-fleck category (full eye flecks, slight eye flecks, and no eye flecks). Compared to molecular markers, we found that discriminant analyses based on morphological characteristics yielded variable results that were confounded by geographical differences in morphology. However, we found that eye flecks were sex-linked. Using an eye-fleck model where all females have full eye flecks and males have either slight eye flecks or no eye flecks, we correctly assigned the sex of 117 of 125 (94%) oystercatchers. Using discriminant analysis based on morphological characteristics, we correctly assigned the sex of 105 of 119 (88%) birds. Using the eye-fleck technique for sexing Black Oystercatchers may be preferable for some investigators because it is as accurate as discriminant analysis based on morphology and does not require capturing the birds.

SINOPSIS. **Secretos en los ojos de *Haematopus bachmani*: una nueva técnica de sexado**

El sexado de ostreros en el campo es sumamente difícil dado el caso de que tanto hembras como machos tienen plumaje idéntico y son similares en tamaño. Aunque los ostreros negros (*Haematopus bachmani*) son sexualmente dimórficos, el utilizar morfometría para determinar su sexo requiere capturar a ambos miembros de la pareja para compararlos, utilizando una análisis discriminativo a modo de asignar un sexo por probabilidad, basado en características morfométricas. Todos los adultos del ostrero negro tienen ojos amarillos y brillantes, pero algunos tienen manchas oscuras en el iris. Tomamos como hipótesis que estas peculiaridades observables estaban ligada al sexo, y que podían ser utilizadas como una herramienta novel de diagnóstico para identificar el sexo en dichas aves. Para poner a pruebas lo mencionado, comparamos datos de ostreros donde se utilizaron marcadores genéticos moleculares (CHD-W/CHD-Z y HINT-W/HINT-Z), análisis morfométrico, y categorías en las manchas en los ojos (manchas marcadas en los ojos, algunas manchitas en los ojos, sin manchas en los ojos). Comparado a marcadores moleculares, encontramos que el análisis discriminativo basado en características morfológicas ofrecía resultados variables asociados a diferencias morfológicas geográficas. Sin embargo, encontramos que las manchas en los ojos estaban ligadas al sexo. Utilizando un modelo de manchas en los ojos, en donde clasificamos como hembras aquellos individuos con manchas pronunciadas en los ojos y a machos con muy pocas manchitas o sin manchitas, le pudimos asignar correctamente el sexo a 117 de 125 (94%) individuos. Utilizando una análisis discriminativo basado en características morfológicas, le asignamos el sexo correctamente a 105 de 119 (88%) individuos. El utilizar la técnica de manchas en los ojos para el sexado de ostreros negros pudiera ser preferible para algunos investigadores porque es más exacto que el análisis discriminativo basado en morfología y porque no requiere que se tenga que capturar a las aves.

Key words: discriminant function, eye flecks, *Haematopus bachmani*, HINT-W/HINT-Z, molecular sexing, oystercatchers, shorebird

Determining the sex of shorebirds (Scolopacidae, Charadriidae, Haematopodidae, and Recurvirostridae) in the field can be difficult because male and female plumages are identical, or nearly so, in many species, including oystercatchers. For some species, sex can only be reliably assessed by inspecting reproductive tracts or using molecular sexing techniques. Eurasian Oystercatchers (*Haematopus ostralegus*) have been sexed using a discriminant function

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based on several morphological measurements. Although this method is 90–95% accurate, different equations may be needed in different locations and habitats because the bills of inland and coastal oystercatchers have different shapes (Zwarts et al. 1996).

Male and female Black Oystercatchers (*Haematopus bachmani*) also exhibit morphological differences (Jehl 1985, as cited by Andres and Falxa 1995), with the longer bills of females being most apparent. However, bill length alone is of limited use because of overlap between the sexes and differences in bill length among different breeding populations.

We observed a characteristic of adult Black Oystercatchers that appeared to be linked to sex, and could potentially provide a practical means of distinguishing sex in the field. Black Oystercatcher chicks initially have dark eye rings and dark irises. By their third year, they develop the orange eye ring and bright yellow iris characteristic of adults (Andres and Falxa 1995). However, some adults have darkened regions within the yellow iris. These dark regions that we call "eye flecks" have been observed in Black Oystercatchers, Eurasian Oystercatchers, American Oystercatchers (*Haematopus palliatus*), African Black Oystercatchers (*Haematopus moquini),* Blackish Oystercatchers (*Haematopus ater*), Magellanic Oystercatchers (*Haematopus leucopodus)*, Chatham Island Oystercatchers (*Haematopus chathamensis*), Variable Oystercatchers (*Haematopus unicolor*), South Island Pied Oystercatchers (*Haematopus finschi)* and Pied Oystercatcher (*Haematopus longirostris*) (M. van de Pol, and S. Murphy, L. Underhill, R. Woods, P. Moore, and A. Harrison, pers. comm.). We are currently unable to confirm if Sooty Oystercatchers (*Haematopus fuliginosus*) have eye flecks. For Black Oystercatchers, the presence of eye flecks appears to differ between the sexes, and we hypothesized that this trait could be used to unambiguously identify the sex of any adult bird.

To test our hypothesis, we determined the sex of Black Oystercatchers using two molecular methods. Assuming that our molecular results reflected the true sex of the birds, we then compared the accuracy of three other potential methods of determining sex, including (1) discriminant analysis using traditional morphometric traits, (2) discriminant analysis using morphometric traits and eye flecks, and (3) eye flecks alone. Here we present the results of our comparisons, discuss the relative efficacy and utility of each method of the origins of oystercatcher eye flecks.

METHODS

In 2004, a coordinated effort to capture and band Black Oystercatchers was initiated at four breeding areas in Alaska: Middleton Island (59.44°N 146.33°W), Harriman Fjord in Prince William Sound (61.05°N 148.32°W), the Beardslee Islands in Glacier Bay National Park (58.50°N 135.95°W), and Northwestern and Ailik Fjords in Kenai Fjords National Park (59.80°N 149.75°W, where banding commenced in 2003). All data presented here are from birds at these four locations.

Breeding birds were captured using noose mats, decoys, dip nets, and nest nooses (Morse et al. 2006, Tessler and Garding 2006). Each captured oystercatcher was banded with a USGS stainless steel leg band and a unique combination of colored-plastic leg bands. In addition, exposed bill length, head-bill length (tip of the bill to occipital process), diagonal tarsus length, and natural (unflattened) wing length were measured, and mass determined. All linear measurements were made using standard calipers and wing rules. Mass was measured with 1000-g hanging spring scales. In some instances, we did not record all measurements due to logistical problems. Multiple personnel took measurements at all field sites except Middleton Island, where a single person measured all captured birds.

Molecular markers. We obtained 50–100 μL of blood from the medial metatarsal vein of each captured oystercatcher using a 26-gauge (0.457 mm) needle and capillary tube. Blood was transferred into 1.5-mL vials containing 1 mL of field preservation buffer (Longmire Buffer; Longmire et al. 1988) and stored at ambient temperature (approximately 4–23°C) prior to laboratory analyses. We extracted DNA following protocols described by Medrano et al. (1990) and modified by Sonsthagen et al. (2004).

CHD sexing. CHD-W/CHD-Z was amplified via the Polymerase Chain Reaction (PCR) using the P2 and P8 primers identified by Griffiths et al. (1998), using protocols described by Handel et al. (2006). We visualized samples via

electrophoresis on a 25-cm, 6%-polyacrylamide gel on a LI-COR IR^2 automated sequencer (LI-COR, Inc., Lincoln, Nebraska). IR Dye-labeled tailed primers separated the product into either one (ZZ: 375 base pairs, bp) or two bands (ZW; 375 and 393 bp), indicating male or female, respectively. We scored the images using GeneImageIR^{TM} 4.05 software (Scanalytics, Fairfax, VA).

No birds were sacrificed for gonadal inspection to confirm the accuracy of the CHD results. Rather, we confirmed CHD-sexing by using a second molecular sexing method targeting the histidine triad nucleotide binding protein gene family HINTZ/W [formerly *Wpkci*: Hori et al. (2000) and ASW : O'Neille et al. (2000); see Ceplitis and Ellegren (2004) for discussion of nomenclature]. Unlike most other genes located on the avian W-linked chromosome, the amino acid sequence of the HINTW gene differs substantially from its gametolog, the highly conserved HINTZ (Ceplitis and Ellegren 2004, Hori et al. 2000), located on the Z-chromosome. These differences were used to develop species-specific markers to sex Black Oystercatchers.

Development of HINT sexing markers. We amplified and sequenced a ca. 850-bp, and 1270-bp fragment within the Black Oystercatcher HINTZ (for 2 males) and HINTW (for 8 females) gene family, respectively, using the primers ASW12-D3 (5′-GGGTTATCCGAAGCAGAAGATTC) and ASW12-R2 (5′-GCCCAGGTTAGCAGCACACTT-3′) designed from the chicken (*Gallus gallus*) genome (see Drovetski 2002). Primers were synthesized with added universal sequences (M13F: CACGACGTTGTAAAACGAC; and M13Rev: GGATAACAATTTCACACAGG, respectively), to allow for simultaneous bidirectional sequencing (SBS^{TM}; LI-COR, Inc. 1999) using universal primers (Oetting et al. 1995). The PCR products were electrophoresed in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) against a 100-bp DNA ladder on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. We purified PCR products using a PEG precipitation (30% PEG 3350/1.5 M NaCl) protocol modified by S. L. Talbot (unpubl. data) from Kusukawa et al. (1990). Purified products were cycle-sequenced via SBS using a commercial kit (Sequitherm LCII 2.0®; Epicentre Technologies, Madison, WI). We used fluorescently labeled universal primers (LI-COR; M13F and M13Rev) to prime the SBS reaction. We electrophoresed SBS products on a 64-lane 41-cm 5.5% polyacrylamide gel on a LI-COR 4200L automated sequencer (LI-COR, Inc. 1999). We analyzed sequence data using LI-COR eSeq^{TM} imaging software and aligned them using AlignIR 2.0^{TM}.

The sequences generated using the ASW12-D3 and ASW12-R2 primers differed in sequence and size between male (ca 850-bp) and female (ca 1270-bp) Black Oystercatchers, with sequences generated from males assumed to be homologous with the chicken HINTZ gene, and those generated from females assumed to be homologous with the chicken HINTW gene. Sequences are deposited in Genbank (Accession Nos. EU556702, EU556703).

Molecular sexing using HINTW/Z. We used the size differences between HINTW and HINTZ to design primers (HintZWF [5′-TTCTGRTGAATCTGTAAGT – 3′] and HintZWR [5′-TSAAAASCTYAACTCCATT]) for use in sexing Black Oystercatchers (Fig. 3b). Primers were synthesized with added universal sequences (M13F) to allow for universal tailed genotyping (Oetting et al. 1995). We carried out PCR amplifications in a final volume of 10 μL that contained 1 μL DNA extract, 0.2 mM dNTPs, 0.1 μg BSA, 1X PCR buffer (Perkin Elmer Cetus I: PE Biosystems, Forest City, California), 10 *p*moles unlabeled tailed primers, 1.0 *p*mole fluorescently labeled M13F primer, and 0.2 units *Taq* polymerase (USB Biochemical, Cleveland, Ohio). The PCR reactions began at 94°C for 90 s and continued with 40 cycles each of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. We concluded each reaction with a final extension of 72°C for 30 min. We electrophoresed PCR products on a 48-well 25-cm 6% polyacrylamide gel on a LI-COR 4200LR automated sequencer or IR2 automated genotypes (LI-COR, Inc.). We determined size of fragments by reference against an M13 DNA sequence ladder.

Results of gel electrophoresis of the HINTW/HINT-Z amplification product revealed a 197 bp band for males and a 208-bp band for females, consistent with the observed nucleotide data obtained in the sequencing step. Molecularly determined sexes are treated as the true sex in all subsequent analyses.

Eye flecks. In 2004, we recorded the presence or absence of eye flecks for Black Oystercatchers captured on Middleton Island and in Prince William Sound. However, for some birds, a simple yes (full eye flecks present) or no (eye flecks absent) was insufficient and such birds were categorized as having "slight eye flecks." After examination of photographs of these slight eye flecks, they were a posteriori defined as relatively small eye flecks for which no part of the fleck was completely black (Fig. 1). We photographed birds captured at Middleton Island in 2005 and at Prince William Sound and Glacier Bay in 2006 to better document eye flecks. We also photographed eight previously banded birds at Prince William Sound in 2006 using a 12-megapixel digital camera (Nikon, Tokyo, Japan) and a 500-mm f/4.5 lens (Sigma, Tokyo, Japan). Photographs of these birds, at distances up to 20 m, allowed us to determine their eye fleck status (Fig. 2). For analysis, each bird was assigned to one of three eye-fleck categories: full eye flecks, slight eye flecks, or no eye flecks.

Statistical analysis. To test the null hypothesis of no difference between sites, we used Levene's test for homogeneity of variance and Shapiro–Wilk tests for normality to determine if parametric assumptions were met. If not met, we used Kruskal–Wallis one-way nonparametric analysis of variance to test for possible differences. When all sample sets met the assumptions, we used ANOVA with the PROC GLM procedure to test for differences in all morphological measurements between the sexes, between sites, and between eye fleck categories. We also tested for a sex × site interaction. For testing for morphological differences within sex and eye fleck category, we pooled slight and flecked males due to the low sample size of flecked males ($N = 4$) and the similarity between these two groups when compared to nonflecked birds. Alpha for most tests was set at 0.05. We employed Bonferroni corrections when testing for

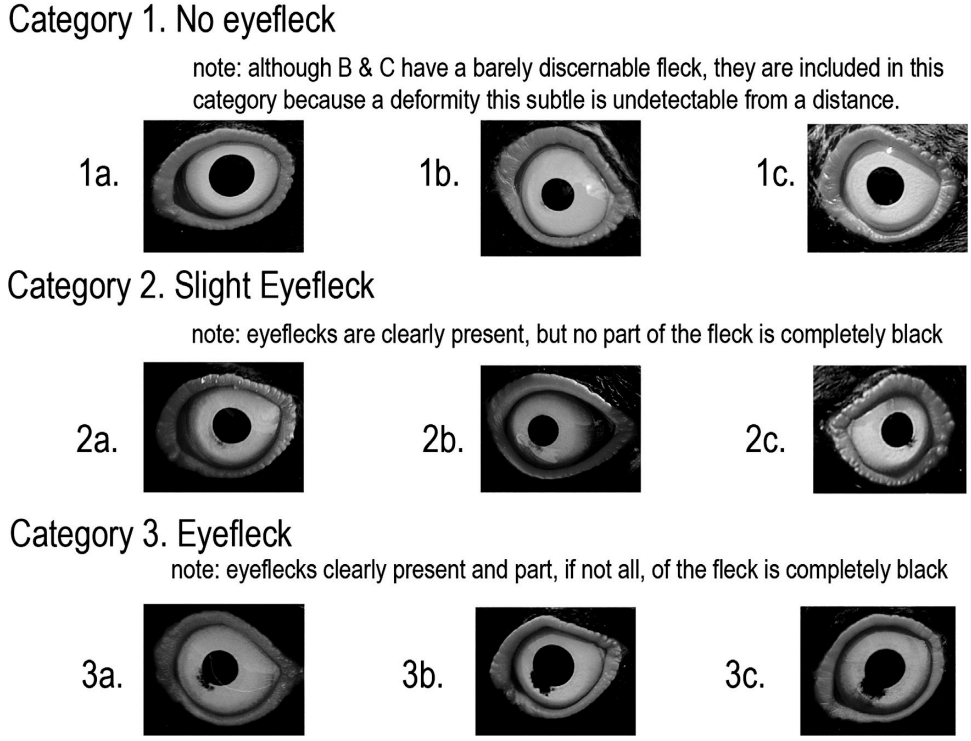

Fig. 1. Photographs illustrating variation and categorization of Black Oystercatcher eye flecks. Within each category, photographs illustrate the range of variation from least (A) to most (C).

Fig. 2. Photograph of a pair of Black Oystercatchers taken from about 20 m. The banded bird with an eye fleck in this photograph was a known female from molecular markers. The unflecked bird is presumably her male mate.

differences between sites to control for the number of tests (Zar 1999).

We used a stepwise model selection (PROC STEPDISC) to determine the morphological characteristics that contributed most to discriminating the sexes. Morphological traits entered into our model were bill length, total head length including bill (head-bill), diagonal tarsus, natural wing length, body mass, and eye fleck category. The STEPDISC procedure was run four times using different combinations of parameters: (1) all measured morphological characteristics with eye flecks excluded from the model to determine the most useful morphometric variables, (2) all morphological characteristics including eye flecks, (3) and (4) the same as (1) and (2), but excluding diagonal tarsus because it was not measured on several birds in our sample. After determining the variables that contributed to the model for each STEPDISC procedure, we performed a discriminant analysis with cross-validation to assign sex according to the model selected by the STEPDISC procedure. An additional model (5) including only eye flecks was used for the discriminant function. We report the partial correlation coefficient (r) for variables included in the model. We used chi-square statistics to test for model efficacy and bias. We used the Statistical Analysis System (SAS Institute 2004) for all analyses.

RESULTS

We captured 212 adult Black Oystercatchers and determined their sex using molecular markers. Based on both CHD-Z/W and HINT-Z/W molecular markers, 114 oystercatchers were females and 98 were males. HINT-Z/W sexing results were in agreement with CHDZ/W results (Fig. 3). We found no polymorphism in the CHD-Z or CHD-W bands, although variability has been documented in other species (Dawson et al. 2001, S. Talbot, unpubl. data). Results of the molecular techniques were used to assign sex to individuals for the subsequent morphometric and eye fleck analyses.

Overall, we found significant differences between males and females for all measurements (Table 1). For females, bill and wing lengths differed among sites (Table 1). For males, all measurements except mass differed among sites (Table 1). No site × sex interaction was detected.

We recorded the eye fleck category for 125 birds, including 70 females and 55 males. All females had eye flecks; 66 (94%) had full eye flecks and four (6%) had slight eye flecks. Among males, 35 (64%) had no eye flecks, 16 (29%) had slight eye flecks, and four (7%) had full eye flecks. Within sexes, there was no significant difference in morphology among the eye fleck types ($P > 0.05$). The bill lengths of nonflecked

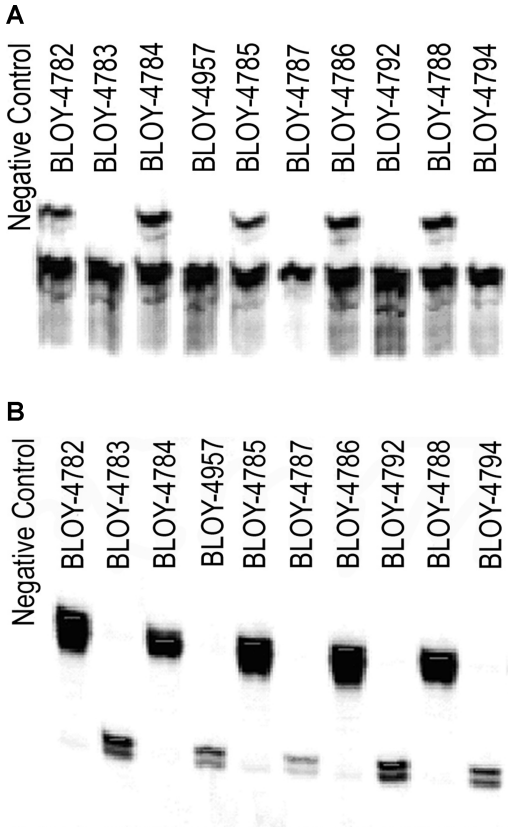

Fig. 3. A. CHD results for 10 Black Oystercatchers. Results for females yield two bands representing the Z (375 bp) and W (393 bp) chromosome. Males yield a single band, representing the Z (375 bp) chromosome. B. Results for the same 10 Black Oystercatchers for the HINT-W/HINT-Z gene. Both sexes yield a single band, 197 bp for males and 208 bp for females.

males were not significantly shorter than pooled slight and flecked males ($P = 0.07$).

Model efficacy varied from 86% to 99% (Table 2). Models that included eye flecks in the discriminant function were more accurate than those that did not, but not always significantly more accurate. The two most accurate methods (2 and 4) included a combination of eye flecks and morphology. Only one model (4) was biased in incorrectly assigning sex in males (14%) more often than females (1%).

DISCUSSION

Black Oystercatchers can be sexed using molecular markers, morphological characteristics, eye flecks, or a combination of the latter two. Major drawbacks to molecular sexing are the cost, especially if DNA samples are not required for other study objectives, and the inability to immediately determine the sex of an individual. It is also possible for human error to occur at any of the multiple steps from sample collection to result, including, transfer to the laboratory, DNA extraction, amplification, and electrophoresis. The use of discriminant analysis requires the capture and handling of birds, and regional and temporal morphometric differences may obscure results.

Using a model where all females have full eye flecks and all males have slight or no eye flecks, eye-fleck categorization was more accurate than morphometric analysis, correctly assigning sex 93.6% of the time. Although molecular markers provide the most accurate method for sexing oystercatchers, eye flecks can be used to sex adults in the field with reasonable accuracy and without capturing birds. Eye-fleck categorization would be particularly useful for studies requiring immediate sex identification in the field and when genetic sexing is not possible. Adding morphological measurements to eye-fleck data may slightly increase the chance of correctly sexing a Black Oystercatcher. Another method to increase accuracy would be to eliminate birds with slight eye flecks from sampling. Thirty percent of males and 6% of females were in this category. This would prevent females from being incorrectly sexed, but not the estimated 7% of males that would be classed as females. In addition, as with morphometric methods, the eye-fleck method cannot be use to sex chicks because they have completely darkened irises.

As with other oystercatchers (Baker 1974, Zwarts et al. 1996), discriminant analysis using body measurements can be used to sex Black Oystercatchers with reasonably high levels of accuracy (86–88%). However, as in other studies where the sex of birds was determined using morphological measurements from multiple sites (Zwarts et al. 1996, Jodice et al. 2000), we found population differences in linear and mass measurements that increased the overlap of the frequency distributions of the two sexes. Therefore, caution is needed if investigators wish to use a discriminant function to sex individuals from multiple populations.

The discriminant function with the highest probability (99%) of correctly sexing

Table 1. Comparison of the morphological characteristics of Black Oystercatchers captured at four locations in Alaska. Data shown as mean ± 1 SD (*N*).

Sex	Site	Bill (mm)	Head-bill (mm)	Tarsus (mm)	Wing (mm)	Mass (g)
Female	Glacier Bay	76.34 ± 3.76 (31)	120.26 ± 4.12 (31)	no data	255.7 ± 8.7 (31)	611.0 ± 42.5 (31)
	Kenai Fjords	75.54 ± 3.17 (19)	no data	no data	no data	633.4 ± 37.2 (19)
	Middleton Is.	73.28 ± 2.67 (46)	118.51 ± 3.00 (46)	53.00 ± 1.82 (45)	247.7 ± 4.3 (46)	625.4 ± 31.4 (45)
	Harriman Fjord	74.43 ± 2.87 (18)	117.79 ± 5.79 (18)	53.7 ± 2.16 (5)	256.2 ± 8.2 (18)	602.1 ± 69.6 (18)
	All sites	74.67 ± 3.33 (114)	118.95 ± 4.09 (95)	53.06 ± 1.84 (50)	251.9 ± 7.9 (95)	619.1 ± 44.1 (113)
Male	Glacier Bay	70.01 ± 3.00 (23)	114.50 ± 3.33 (23)	no data	255.0 ± 8.6 (23)	576.6 ± 30.5 (21)
	Kenai Fjords	69.01 ± 2.58 (27)	no data	no data	no data	593.5 ± 28.2 (27)
	Middleton Is.	67.39 ± 2.77 (36)	112.15 ± 3.24 (36)	51.43 ± 1.39 (36)	244.8 ± 3.9 (32)	573.2 ± 32.0 (33)
	Harriman Fjord	68.73 ± 1.94 (11)	113.57 ± 1.74 (12)	52.78 ± 0.68 (5)	252.3 ± 8.8 (12)	591.4 ± 31.7 (11)
	All sites	68.63 ± 2.90 (97)	113.15 ± 3.22 (71)	51.60 ± 1.40 (41)	249.7 ± 8.2 (67)	582.1 ± 31.4 (92)
Pr > *F* sex[a]		<0.001	<0.001	0.02	0.04	<0.001
Pr > *F* site[a]		<0.001	0.01	0.06	<0.001	0.04
Pr > *F* sex × site[a]		0.92	0.4	0.56	0.53	0.27
Pr > *F* site female[b]		<0.001	0.07	0.44	<0.001	0.08
Pr > *F* site male[c]		0.004	0.02	0.04	<0.001	0.05

[a]Results for two-factor ANOVA with interaction testing for differences in sexes, sites, and sex × site interaction for each morphological characteristic.
[b,c]Results for single-factor ANOVA to test for site differences within each sex for each morphological characteristic.

Table 2. Stepwise model selection and discriminant function in determining the sex of Black Oystercatchers results of five, two-step analyses[a].

	Stepwise selection			Discriminant function		
	Variables included	Variables selected (partial r^2)	Individuals (N)	Cross-validation results (number correctly identified)	Individuals (N)	Percent correct
1	Tarsus, bill, head-bill, wing, weight	bill ($r = 0.57$)	61	108	125	86.4
2	Tarsus, bill, head-bill, wing, weight, eye fleck	eye fleck ($r = 0.90$) tarsus ($r = 0.13$) weight ($r = 0.04$)	61	64	65	98.5
3	Bill, head-bill, wing, weight	bill ($r = 0.51$) weight ($r = 0.06$)	115	105	119	88.2
4	Bill, head-bill, wing, weight, eye fleck	eye fleck ($r = 0.76$) bill ($r = 0.13$) weight ($r = 0.05$)	115	112	119	94.1
5	Eye fleck	n/a	n/a	117	125	93.6

[a]The stepwise model selection is first used to choose variables that contribute to the model. The variables chosen are then used for the discriminant function. Although all tests started with 125 total birds, both statistical procedures exclude individuals with any missing data. Because the stepwise selection was used to build a model from a larger suite of variables than the subsequent discriminant function, N differed between tests and is thus reported for each step. Only one variable was used in the fifth analysis, so stepwise selection was not used.

oystercatchers included eye-fleck category, tarsus length, and mass. However, two factors may have influenced this result. First, more than 85% of the oystercatchers included in this analysis were from Middleton Island, reducing the variance for each measurement by eliminating birds from other populations that differed significantly in size. In addition, all birds captured at Middleton Island site were measured by the same person, possibly reducing the variances for each sex and making it easier to discriminate between males and females. However, if males and females differed more in tarsus length and mass than those at the other locations, we would likely have detected a significant sex × site interaction. Thus, we cannot rule out the importance of tarsus length in discriminating the sexes in Black Oystercatchers. However, because diagonal tarsus was not measured at some sites, the importance of tarsus length in discriminating the sex of Black Oystercatchers remains unclear and additional data were needed.

The cause of eye flecks in oystercatchers is currently unknown and, therefore, investigators should exercise caution in using our method to sex Black Oystercatchers at other times (e.g., the nonbreeding season) and locations. A pilot study of eye flecks in Eurasian Oystercatchers revealed some similarities and differences relative to Black Oystercatchers (Guzzetti and van de Pol, unpubl. data). In that species, females have larger eye flecks than males, but, because nearly all Eurasian Oystercatchers photographed had eye flecks, they were less useful in identifying sex. Clearly, additional study of Black Oystercatchers and other species of oystercatchers is warranted.

To our knowledge, we are the first investigators to use the HINTZ/W genes to determine the sex of a wild bird species. Although used in our study to confirm the results of CHDZ/W sexing, the HINTZ/W markers may be developed in other species, such as loons and some raptor species, for which CHDZ/W genes cannot be used to accurately determine sex.

Although more work needs to be done to understand eye flecks in oystercatchers, our study suggests that eye flecks may be useful for identifying the sex in Black Oystercatchers in the field. Whereas molecular markers are still the best method for sexing live oystercatchers, eye fleck categorization, at least at our study sites during the breeding season, can be used to quickly sex Black Oystercatchers with high accuracy without capturing the birds.

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LITERATURE CITED

ANDRES, B. A., AND G. A. FALXA. 1995. Black Oystercatcher (*Haematopus bachmani*). In: The Birds of North America, no. 155 (A. Poole, and F. Gill, eds.). The Academy of Natural Sciences, Philadelphia, PA, and The American Ornithologists' Union, Washington, D.C.

BAKER, A. J. 1974. Criteria for ageing and sexing New Zealand oystercatchers. New Zealand Journal of Marine and Freshwater Research 8: 211–221.

CEPLITIS, H., AND H. ELLEGREN. 2004. Adaptive molecular evolution of HINTW, a female-specific gene in birds. Molecular Biology and Evolution 21: 249–254.

DAWSON, D. A., S. DARBY, F. M. HUNTER, A. P. KRUPA, I. L. JONES, AND T. BURKE. 2001. A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. Molecular Ecology Notes 1: 201–204.

DROVETSKI, S. V. 2002. Molecular phylogeny of grouse: individual and combined performance of W-linked, autosomal, and mitochondrial loci. Systematic Biology 51: 930–935.

GRIFFITHS, R., M. C. DOUBLE, K. ORR, AND R. J. G. DAWSON. 1998. A DNA test to sex most birds. Molecular Ecology 7: 1071–1075.

HANDEL, C. M., L. M. PAJOT, S. L. TALBOT, AND G. K. SAGE. 2006. Use of buccal swabs for sampling DNA from nesting and adult birds. Wildlife Society Bulletin 34: 1094–1100.

HORI, T., S. ASAKAWA, Y. ITOH, N. SHIMIZU, AND S. MIZUNO. 2000. *Wpkci*, encoding an altered form of *PKC*I, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. Molecular Biology of the Cell 11: 3654–3660.

JODICE, P. G. R., R. B. LANCTOT, V. A. GILL, D. D. ROBY, AND S. A. HATCH. 2000. Sexing adult Black-legged Kittiwakes by DNA, behavior, and morphology. Waterbirds 23: 405–415.

KUSUKAWA, N., T. UEMORI, K. ASADA, AND I. KATO. 1990. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. BioTechniques 9: 66–72.

Li-Cor, Inc. 1999. DNA sequencing manual. Global edition, IR^2 system. Publication number 9910-150, LI-COR, Lincoln, NE.

LONGMIRE, J. L., A. K. LEWIS, N.C. BROWN, J. M. BUCKINGHAM, L. M. CLARK, M. D. JONES, L. J. MEINCKE, J. MEYNE, R. L. RATLIFF, F. A. RAY, R. P. WAGNER, AND R. K. MOYZIS. 1988. Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. Genomics 2: 14–24.

MEDRANO, J. F., E. AASEN, AND L. SHARROW. 1990. DNA extraction from nucleated red blood cells. Biotechniques 8: 43.

MORSE, J. A., A. N. POWELL, AND M. D. TETREAU. 2006. Productivity of Black Oystercatchers: effects of recreational disturbance in a national park. Condor 108: 623–633.

OETTING, W. S., H. K. LEE, D. J. FLANDERS, G. L. WEISNER, T. A. SELLERS, AND R. A. KING. 1995. Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics 30: 450–458.

O'NEILL, M., M. BINDER, C. SMITH, J. ANDREWS, K. REED, M. SMITH, C. MILLAR, D. LANBERT, AND A. SINCLAIR. 2000. ASW: a gene with conserved avian W-linkage and female specific expression in chick embryo gonad. Development Genes and Evolution 210: 243–249.

SAS INSTITUTE. 2004. SAS/STAT user's guide. Version 9.1. SAS Institute, Cary, NC.

SONSTHAGEN, S.A., S. L. TALBOT, AND C. M. WHITE. 2004. Gene flow and genetic characterization of Northern Goshawks breeding in Utah. Condor 106: 826–836.

TESSLER, D. F., AND L. S. GARDING. 2006. Black Oystercatcher distribution and productivity in the Beardslee Islands, Glacier Bay National Park and Preserve, Alaska. 2005 Annual Report for Glacier Bay National Park and Preserve, Gustavus, AK.

ZAR, J. H. 1999. Biostatistical analysis, 4th edition. Prentice Hall, Englewood Cliffs, NJ.

ZWARTS, L., J. B. HULSCHER, K. KOOPMAN, AND P. M. ZEGERS. 1996. Discriminating the sex of oystercatchers (*Haematopus ostralegus)*. Ardea 84: 1–12.