Sex determination by morphology in New Holland Honeyeaters, *Phylidonyris novaehollandiae*: contrasting two popular techniques across regions

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Abstract

Sex determination of individuals is often required for ecological and behavioural studies but is difficult to carry out in the field for species that are only slightly dimorphic. To address this issue, researchers may use a variety of methods that rely solely on morphological measurements for sex determination. There are two main groups of morphological methods; (1) based on discriminant analysis, and (2) based on resolving mixed-modal distributions. Here, we use one method from each of the two groups to sex the slightly dimorphic New Holland Honeyeater, Phylidonyris novaehollandiae, *in South Australia, and we compare results of the two methods in relation to a genetic standard. We found that performance of both methods was comparable, but varied between populations. We also found regional differences in the best discriminating variables for morphological sex determination. This regional variation in performance of methods indicates that a single method for morphological sex determination cannot be applied across regions, even within species; furthermore, average morphological trait values should be reviewed across years given the possible role of selection or drift to influence phenotype. We suggest that when accurate sex discrimination is important, an alternate, reliable method, such as anatomical or genetic identification, should be favoured above morphological methods.*

INTRODUCTION

Sex determination of individuals is often required for ecological and behavioural studies where sex ratio, paternity, and parental care are commonly investigated. For sexually dimorphic species, visual inspection of individuals is sufficient for determining sex. However, many bird species lack consistently observable sexual dichromatism or dimorphism. In such cases, sex may be determined non-invasively using behavioural cues (Baeyens 1981), or by the presence of a brood patch on incubating females or a protruding cloaca in males. However, these approaches are usually only possible with sexually active individuals during the breeding season.

The difficulties of sex determination for species with only slight sexual dimorphism may be overcome by examining morphological measurements. Two techniques that can be used to determine sex based solely on morphological measurements are cited above all others: (1) more traditional methods based on resolving mixed-modal distributions (Disney 1966; Rogers et al. 1986; Rogers and Rogers 1995; Higgins and Peter 2002; Twedt 2004; Morgan 2005), and (2) a more recently emerging method that utilises discriminant analysis (Phillips and Furness 1997; Bavoux et al. 2006; Kesler, Lopes and Haig 2006; Alarcos et al. 2007; Hermosell et al. 2007; Jakubas and Wojczulanis 2007; Svagelj and Quintana 2007; Kochert and McKinley 2008; Pitzer et al. 2008). Discriminant analysis explores the predictive ability of a number of user-defined independent variables on a single categorical dependent variable. The best linear combination of traits is used to calculate the discriminant function. The probability that an individual with given measurements will belong to either sex can be calculated from the discriminant function. On the other hand, for the mixed modal method, researchers identify the single best predictor trait, and the mean and standard deviation for each sex for this trait are used to construct normal density curves. The probability that an individual with a given measurement will

belong to either sex can be calculated from these curves. This information is used to develop upper and lower cut-off values for which any individual having a measurement above or below can be assigned a sex with a given margin of uncertainty chosen by the user.

The New Holland Honeyeater, *Phylidonyris novaehollandiae,* is a common Australian passerine that plays a key role in ecosystem function (Ford & Paton 1977; Ford, Paton, and Neville 1979; Paton 1981, 1982; Driskell and Christidis 2004). Sex determination has been an ongoing challenge in *P. novaehollandiae* (Disney 1966; Rogers et al. 1986; Pyke and Armstrong 1993; Rogers and Rogers 1995). Disney (1966) reported a morphological sex difference in *P. novaehollandiae* and proposed that measurement of the extreme wingspan could discriminate between the sexes. However, taking this measurement in live, wild birds is difficult and can trigger undesired stress response in small birds; hence, this measurement is seldom taken. A more common measurement that has been used for sex determination in birds, including honeyeaters, is the bill-head length measurement (Rooke 1976). Many highly regarded bird manuals report use of bill-head length with the mixed modal method for sex discrimination (Rogers et al. 1986; Schodde and Mason 1999; Higgins and Peter 2002); however, reliable criteria for *P. novaehollandiae* have not been published.

One factor impeding the development of morphological methods for sex determination has been the need for a large sample size to accurately represent the overall study population (Rogers and Rogers 1995). The development of primers for genetic sexing in birds (Griffiths et al. 1998; Kahn, St John and Quinn 1998; Jensen, Pernasetti and Durrant 2003) enables a reliable method for sex determination that can be easily applied to large sample sizes. This approach allows examination of morphological sex variation at a large scale. In *P. novaehollandiae*, further impediment in

development of methods arises through regional variation in body size (Latham 1790; Mathews 1918-27; Salomonsen 1966; Pyke and Armstrong 1993; Higgins and Peter 2002).

Our study area spans two geographically distinct regions, Fleurieu Peninsula and Kangaroo Island, for which previous body size variation has been observed (Mathews 1918-27). In this study on *P. novaehollandiae* in South Australia, we (1) genetically sex individual birds for which we have morphological data, (2) investigate the extent of sexual size dimorphism for all measured morphological traits in Kangaroo Island and Fleurieu Peninsula, (3) develop criteria for sex determination by the two morphologic methods (mixed-modal and discriminant analysis), and (4) compare the predictive power of both morphologic methods.

METHODS

We mist-netted birds across sites over a five-year period, between 2004 and 2008 (see also Oorebeek and Kleindorfer 2008a, b; Oorebeek, Sharrad and Kleindorfer 2009; Oorebeek and Kleindorfer 2009; Chapman et al. 2009). To minimise sampling bias across the sampling period, banding trips to each site were carried out annually during the same months (May, June, September). We caught and measured a total of 417 adult birds. Each bird was banded with an aluminium identification band, measured for morphological characteristics, and sampled for blood that was stored on FTA paper for DNA analysis (see Kleindorfer, Lambert and Paton 2006; Myers et al. 2009).

Morphologic data were collected at seven sites within South Australia: (1) Newland Head Conservation Park, (2) Scott Conservation Park, (3) Cox Scrub Conservation Park, (4) Sandy Creek Conservation Park, (5) Scott Creek Conservation Park, (6) Flinders Chase National Park, and (7) Pelican Lagoon Conservation Park. The sites span two geographically distinct regions; (1) Fleurieu Peninsula (FP), and (2) Kangaroo Island

(KI). Dominant flora at each site is described in Rix (1976), Ford and Paton (1977, 1982), Westphal et al. (2003), Kleindorfer, Lambert and Paton (2006), Schlotfeldt and Kleindorfer (2006), McGuire and Kleindorfer (2007), and Galligan and Kleindorfer (2008).

We determined sex of each bird by genetics using the polymerase chain reaction (PCR) method of Kahn, St John and Quinn (1998) and conditions outlined by Jensen, Pernasetti and Durrant (2003). Briefly, each PCR contained 1x Taq polymerase buffer, 4 mM MgCl2, 0.8 mM dNTPs, 0.16μM each primer, and 0.02 U/μL Taq polymerase (AmpliTaq Gold). The temperature profile included an initial denaturation at 94°C for 9 min; followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 6 min. All 417 birds had sex successfully assigned using this method. We assumed 100% accuracy of sex assignments; however, there is likely to be a small degree of error associated with this method (~ 0.5%) due to contaminating agents entering the PCR (Daniel et al. 2007). While there are methods available to minimise and essentially eradicate this error, they are costly and time consuming, and a 0.5% error is not likely to have a significant effect on the results. Therefore, we opted against using methods to alleviate this small degree of error. Template DNA for use in PCR was extracted from FTA using method #4 of the protocols outlined by Smith and Burgoyne (2004). Briefly, we took a small disc (1 mm^2) of blood-soaked FTA paper and submerged it in 500 mL lysis buffer solution (100mM Tris [free base], 0.1% sodium dodecyl sulphate [SDS]). After a thirty minute wash, we removed the supernatant and submerged the FTA disc in 500 mL DNAzol (guanidinium thiocyanate). After a ten minute wash, we removed the supernatant and washed the disc once in 95% ethanol, and twice in reverse osmosis water. Finally, we submerged the disc in 80 μL reverse osmosis water and incubated at 90°C for five minutes, to release the DNA from the disc.

We examined eight morphological traits that have been shown to correlate with body size in birds (Rising and Somers 1989; Piersma and Davidson 1991; Senar and Pascual 1997): (1) bill-head length; (2) bill length from the tip of the bill to the base of the bill, where the feathers begin (bill-feathers length); (3) bill length from the tip of the bill to the anterior extreme of the nostril (bill-nostril length); (4) bill depth, measured at the base of the bill (bill depth); (5) bill width at the base of the bill (bill width); (6) tarsometatarsus length (tarsus); (7) length of the flattened wing (wing); and (8) mass. To determine the extent of sexual dimorphism in *P. novaehollandiae* and the influence of region, we carried out MANOVA using the eight morphological traits as independent variables and sex, as identified by genetics, as a fixed factor, as well as region (SPSS 14.0; SPSS Inc., Chicago, IL). The MANOVA showed a significant effect of sex (F = 52.510; P < 0.001; Wilk's Lambda = 0.491; Partial ETA2 = 0.509) and region (F = 17.589; P < 0.001; Wilk's Lambda = 0.743; Partial ETA2 = 0.257) on morphological variation, and no significant interaction effect between sex and region. This result suggests that, in this system, regions should be considered separately for the development of morphologic methods of sex determination; and this is how we proceeded.

To reduce measurement error, all morphological measurements were made by Sonia Kleindorfer (SK) and Margot Oorebeek (MO) (banding permit ABBBS 2601). Bill measurements of recaptured, remeasured birds (n=30) were used in t-tests with original measurements to analyse measurement error. Bill-head length showed the lowest measurement error (0.35%), while bill length nostril and bill length feathers showed higher error (1.9% and 3.4%, respectively). We therefore conservatively estimate that measurement error for any given trait was < 5%. To identify traits that showed significant variation between sexes in each region we carried out analysis of variance (ANOVA) for each morphological variable using sex

as a fixed factor, across regions. We applied Holm corrections (Holm 1979; Aickin and Gensler 1996) to ANOVA results to adjust for multiple comparisons that address a common null hypothesis (Rice 1989). We also calculated descriptive statistics for each morphological trait for each sex, across regions.

We calculated means, standard deviation, and Mahalanobis distance for each morphological variable across regions. Mahalanobis distance provides the difference between two means as the number of standard deviations different from zero. Therefore, the trait showing the largest Mahalanobis distance will have the greatest power for discrimination between sexes. The mean and standard deviation for each sex and trait that showed the largest Mahalanobis distance were used to construct a graph of normal densities. We assumed a normal distribution of measurements for each sex, and sex ratios equivalent to those of the sample population. The probability that a bird with any given measurement will be a given sex is obtained by dividing the ordinate of that sex at that measurement by the sum of the ordinates for both sexes.

To develop discriminant functions (d) for sex determination for each region we applied stepwise discriminant analysis to morphological variables. In the discriminant analysis, genetic sex was used as the dependent variable and the morphological measurements showing significant variation between regions by MANOVA were used as covariates.

From the discriminant function the probability (P) that a bird with a given morphology is male can be estimated as:

$$P_{male} = e^d \,/\, 1 - e^d$$

where d is the discriminant function and e is the base of the natural logarithms. The probability of being female is:

$$P_{female} = 1 - P_{male}$$

Based on these equations, we calculated the accuracy of assignments made using a minimum probability of correct sexing of 90%.

RESULTS

Table 1 shows results of the ANOVA carried out for each trait using sex as the fixed factor. All morphological measurements differed significantly between sexes. Examination of the descriptive statistics (Table 2) shows that all traits were larger in males than females. The trait that

Table 1. ANOVA of the effect of sex on morphology in New Holland Honeyeaters on the Fleurieu Peninsula and Kangaroo Island. For each trait at the two sites the Table presents the F-values (F), Partial ETA² (Part ETA²), P-values, and corresponding Holmed P-values (P_H).

Trait	Fleurieu Peninsula (N=336)				Kangaroo Island (N=79)			
	$F_{1,\,335}$	Part ETA²	P	P_H	$F_{1,\,78}$	Part ETA²	P	P_H
Bill-head length	326.66	0.494	<0.001	<0.008	113.32	0.592	<0.001	<0.008
Bill length feather	15.77	0.045	<0.001	<0.007	24.4	0.238	<0.001	<0.007
Bill length nostril	86.51	0.205	<0.001	<0.006	23.08	0.228	<0.001	<0.006
Bill depth	102.99	0.235	<0.001	<0.005	27.36	0.26	<0.001	<0.005
Bill width	58.45	0.149	<0.001	<0.004	18.76	0.194	<0.001	<0.004
Tarsus	83.82	0.2	<0.001	<0.003	19.19	0.197	<0.001	<0.003
Wing	215.96	0.392	<0.001	<0.002	151.87	0.661	<0.001	<0.002
Mass	125.84	0.273	<0.001	<0.001	85.64	0.523	<0.001	<0.001

explained the largest variation in the model for Fleurieu Peninsula was bill-head length (Part $ETA^2 = 0.494$), and for Kangaroo Island was wing length (Part $ETA^2 = 0.661$).

Table 2 shows sample size, mean, and standard deviation for morphological traits for each sex across regions. Two traits (bill-head length, wing length) were above the 90% level (Mahalanobis distance > 1.645) for Fleurieu Peninsula, while three traits (bill-head length, wing length, mass) were above the 90% level for Kangaroo Island. The trait that showed the least overlap between sexes for Fleurieu Peninsula was bill-head length, and for Kangaroo Island was wing length (see Figures 1 and 2).

Table 3 shows the percentage of the sample population correctly sexed, unsexed, and incorrectly sexed for each region applying criteria developed using the mixed-modal method at a minimum probability of correct sexing of 90%.

Table 4 shows the discriminant functions for each region, and the percentage of the sample population correctly sexed, unsexed, and incorrectly sexed for each region applying criteria developed using these discriminant functions at a minimum probability of correct sexing of 90%.

DISCUSSION

We found variation between sexes in *P. novaehollandiae* for all morphological variables in both the island and mainland regions in South Australia, with males as the larger sex. Male biased size dimorphism is common in birds (Bjorklund 1990; Ellrich, Salewski and Fiedler 2010) and has previously been observed in *P. novaehollandiae* (Disney 1966; Rooke 1976; Paton and Collins 1989). Size dimorphism is thought to be related to sexual selection and the reproductive role of each sex (Fairbairn 1997; Owens and Hartley 1998), although proximate causes are also possible (Potti and Merino 1997).

Table 2. Descriptive statistics describing the extent of sexual dimorphism in New Holland Honeyeaters on the Fleurieu Peninsula and Kangaroo Island. The mean values and standard deviations (SD) of each trait are given for males and females and the Mahalanobis distance (Mahal dist). The largest Mahalanobis distance is in bold.

Fleurieu Pensl.	Male (N=208)		Female (N=129)		
Trait	Mean	SD	Mean	SD	Mahal dist
Bill-head lng.	42	0.99	40	0.98	**2.028**
Bill lng. feather	23.7	1.86	22.9	1.71	0.444
Bill lng. nostril	10.6	0.65	9.9	0.54	1.151
Bill depth	5.2	0.27	4.9	0.26	1.128
Bill width	5.2	0.34	4.9	0.34	0.882
Tarsus	23.3	0.89	22.4	0.72	1.091
Wing	78.6	2.83	74	2.75	1.643
Mass	21.8	1.76	19.6	1.76	1.25

Kangaroo Isl.	Male (N=46)		Female (N=34)		
Trait	Mean	SD	Mean	SD	Mahal dist
Bill-head lng.	42.7	1.11	40.3	0.79	2.464
Bill lng. feather	24.7	1.52	23.1	1.27	1.132
Bill lng. nostril	10.9	0.7	10.2	0.58	1.079
Bill depth	5.2	0.31	4.9	0.28	1.01
Bill width	5.2	0.35	4.9	0.32	0.89
Tarsus	24	0.97	23	0.9	1.064
Wing	79.7	2.43	73.4	2.08	**2.762**
Mass	20.8	1.32	18.3	1.05	2.075

P. novaehollandiae exhibit sex specific breeding behaviour; male *P. novaehollandiae* maintain and defend territories during the breeding season, and larger males are more successful at territory defence (McFarland 1985; Clarke and Clarke 1999). Males that maintain a territory produce more offspring than males that do not maintain a territory. This mating strategy appears to be a likely candidate mechanism for driving selection toward larger males.

Table 3. The potential accuracy of sex assignments with the mixed-modal method on the Fleurieu Peninsula and Kangaroo Island. Cut-off values and percentage of the population correctly sexed, unsexed, and incorrectly sexed at a minimum correct sexing of 90%. See Table 2 for a list of the means ± s.d. for males and females.

Region	Variable	Cut-off values	Sex		
			Correct	Unknown	Incorrect
Fleurieu Peninsula	Bill-head length	♀ ≤39.6 mm ♂ ≥41.0 mm	70.6%	21.4%	8.0%
Kangaroo Island	Wing length	♀ ≤74.5 mm ♂ ≥77.9 mm	76.2%	22.5%	1.3%

We found size variation in birds between Kangaroo Island and Fleurieu Peninsula, consistent with findings by Mathews (1918-27) and Rogers et al. (1986). Kangaroo Island birds were larger for all measured traits with the exception of bill depth and width, which did not differ between regions, and body mass, for which Kangaroo Island birds had lower mass. This regional morphological variation may represent local adaptation through natural selection as a result of regional environmental variation (see Myers, Brown and Kleindorfer 2010; Myers 2011; Myers, Donnellan and Kleindorfer in review), given that island resources are generally depauperate compared with the mainland (Robinson-Wolrath and Owens 2003; Lomolino 2005).

The morphological trait that showed the most variation between the sexes was different in each region. In Fleurieu Peninsula, bill-head length was most different between the sexes, whereas on Kangaroo Island, wing length was most different between the sexes. These two traits are commonly cited in the literature as being useful for discriminating sex, probably due to the low intrinsic error of these measurements relative to other common measurements. Discriminant analysis for each region estimated that a linear combination of traits was a better predictor of sex than any single trait on its own. The linear combination of traits for Fleurieu Peninsula included bill-head length, bill-feather length, bill depth, and wing length, and for Kangaroo Island included bill-head length, wing length, and mass. Comparing the two morphologic methods directly, at a minimum correct sexing of 90%, the mixed-modal method correctly sexed a larger proportion of the population than the discriminant analysis method for the Fleurieu Peninsula sample. However, almost twice as many samples were incorrectly sexed using the mixed-modal method. For the Kangaroo Island sample, the discriminant analysis method correctly sexed a larger proportion of the population than the mixed-modal method.

Table 4. The potential accuracy of sex assignments using the discriminant analysis method on the Fleurieu Peninsula (FP) and Kangaroo Island (KI), South Australia. The Table shows the multivariate discriminant functions, and percentage of the population assignable with 90%

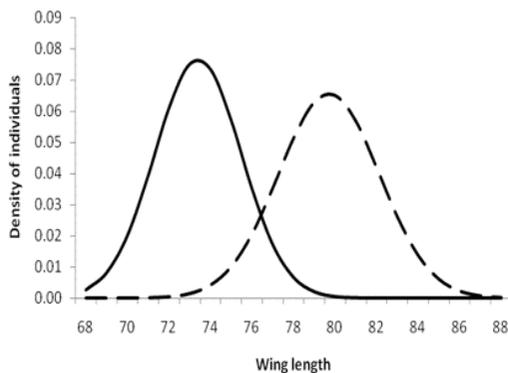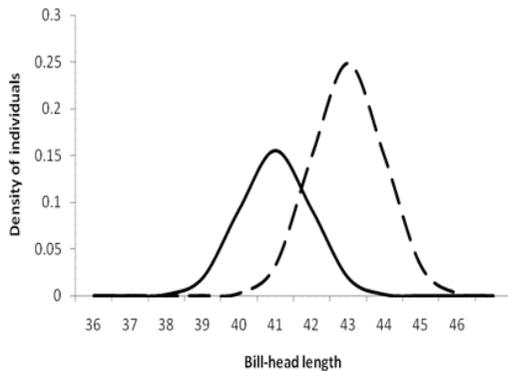| Region	Discriminant Function	Sex		
		Correct	Unknown	Incorrect
Fleurieu Peninsula	D = 0.787(BHL) - 0.127(BFL) + 0.675(BD) + 0.139(wing) − 43.585	65.9%	29.4%	4.7%
Kangaroo Island	D = 0.542(BHL) + 0.243(wing) + 0.236(mass) - 45.956	88.7%	8.8%	2.5%

Figure 1. Normal density curves for bill-head length (mm) on the Fleurieu Peninsula (upper graph) and for wing length (mm) on Kangaroo Island (lower graph). Solid lines indicate females and dashed lines indicate males.
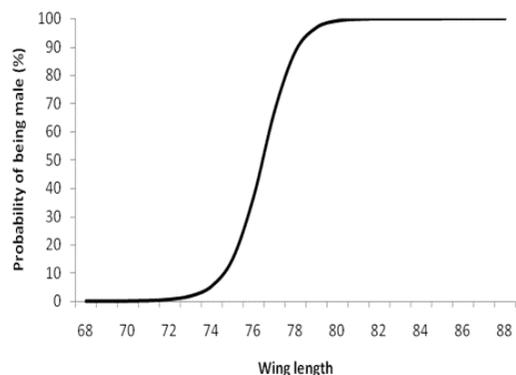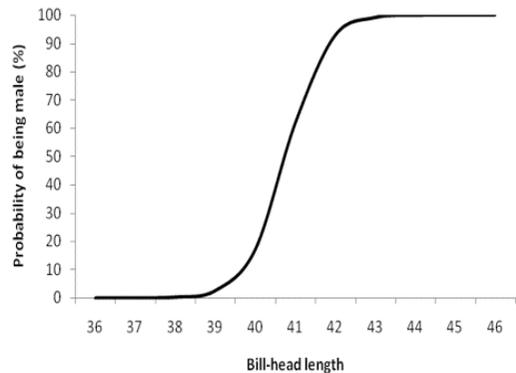
Figure 2. Probability curves that an individual will be male based on bill-head length (mm) on the Fleurieu Peninsula and based on wing length (mm) on Kangaroo Island.

These results showed that discriminant analysis and the mixed-modal method are comparable methods for sex determination in *P. novaehollandiae* in South Australia. Their performance will depend on the morphological characteristics of the population under examination.

We found regional variation in the traits that best discriminate sex, which indicates variation in sexual size dimorphism between Kangaroo Island and Fleurieu Peninsula. Although regional variation in morphologies has been previously observed (Latham 1790; Mathews 1918-27; Salomonsen 1966; Pyke and Armstrong 1993; Higgins and Peter 2002), it has not been interpreted in relation to sexual size dimorphism. Avian studies over the last

decade have provided evidence that sexual size dimorphism correlates with mating strategy and parental care (Fairbairn 1997; Benito and González-Solís 2007; Dale et al. 2007). The regional variation in size dimorphism that we observed in *P. novaehollandiae* may indicate region-specific selection pressures for parental care or foraging competition. However, this remains to be examined (but see Myers 2011). The extensive regional variation in morphologies observed in *P. novaehollandiae*, both in this study and previous studies, suggests that protocols for morphologic-based sex determination developed for a particular site may not be suitable for landscape-level sex determination. A suitable method and suitable measurements must be determined for any given area and time span given neutral and

adaptive changes in morphology (Price and Grant 1984; Schluter and Smith 1986; Reimchen 1995). Even within an area and time span, we were not able to accurately assign sex to the entire sample population based solely on morphological measurements. Morphologic methods for assigning sex are intrinsically error prone because there is a trade-off between the accuracy of sex determination and the percentage of the population that can be sexed (Twedt 2004). Therefore, when accurate sex discrimination is important, an alternate, reliable method, such as anatomical or genetic identification, should be favoured above morphologic methods; at the very least, if a morphologic method is used, an alternate reliable method should be applied for sexing individuals in the unavoidable overlap. This study has demonstrated, using South Australian *P. novaehollandiae*, the limitations of morphologic sexing methods. We argue that there is a good chance that these limitations apply to the majority of sexually monomorphic bird species (e.g., Van de Pol et al. 2009).

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