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A morphological model for sexing nestling peregrine falcons (*Falco peregrinus macropus*) verified through genetic analysis

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Abstract

In Australia, adult peregrine falcons (*Falco peregrinus macropus*) have monotypic plumage and display strong reversed sexual dimorphism (RSD), with females significantly larger than males. RSD is measurable amongst nestlings in the latter stages of their development and can therefore be used to differentiate between sexes. In the early stages of development, however, nestlings cannot be sexed with any degree of certainty as morphological differentiation between the sexes is not well developed. During this study we developed a model for sexing younger nestlings based on genetic analysis and morphometric data collected as part of a long-term banding study of this species. A discriminant function model based on morphological characteristics was developed for determining the sex of nestlings (*n*=150) in the field and was shown to be 96.0\% accurate. This predictive model was further tested against an independent morphometric data set taken from a second group of nestlings (*n*=131). The model correctly allocated sex to 96.2\% of this second group of nestlings. Sex can reliably be determined (98.6\% accurate) for nestlings which have a wing length of 9cm or greater using this model. Application of this model, therefore, allows the banding of younger nestlings, and as such significantly increases the period of time over which banding can occur. Another important implication of this model is that by banding nestlings earlier, they are less likely to branch / jump from the nest, therefore reducing the risk of injury to both the brood and the bander.

Keywords: peregrine falcon, molecular sexing, DNA, nestlings, morphometric measurements, Australia
Introduction

The peregrine falcon (Falco peregrinus) has a near global distribution, breeding on all continents except Antarctica (White et al. 1993). This has lead to morphological variation across the species’ near global range (White and Boyce 1988; Brown and Amadon 1989), generating 20 subspecies based on distinct colouration and sizes (White 1987). A wide variety of studies on the biology and ecology of peregrine falcons have been undertaken (e.g. Porter et al. 1987; Cade et al. 1988). No studies to date, however, have developed accurate field based methods for sexing nestlings prior to attaining asymptotic weights (Nisbet 1988; Olsen 1995).

Within Australia, adult peregrine falcons (Falco peregrinus macropus) are considered monotypic in plumage (Marchant and Higgins 1993; Olsen 1995), however, they do display a high level of reversed sexual dimorphism (RSD), with females significantly larger than males (Baker-Gabb 1984). Male and female adults of this species display virtually no overlap between commonly measured morphometric characteristics such as weight, wing length and culmen length (Baker-Gabb 1984). RSD is so significant in peregrine falcons that females warrant a larger sized leg band than males (Lowe 1989).

RSD is also clearly measurable amongst nestlings in the latter stages of their 35-40 day nestling period (Olsen 1995). Nestlings in the earliest stages of development, however, cannot be sexed with any level of certainty as RSD is not discernable. Banding nestlings of unknown gender poses a number of risks, with the most obvious relating to band size and subsequent injuries (Berggren and Low 2004) or band loss if the incorrect sized band is attached (Emison and Bren 1981).

To reduce the risk of birds being incorrectly sexed a field based model needs to be developed. This model needs to be accurate and easy to use to ensure that researchers adopt the model, especially when banding younger nestlings which display little or no size dimorphism. This study aims, therefore, to use genetically validated morphometric data to develop a model for determining the sex of nestling peregrine...
falcons and secondly, establish the minimum size/age at which this model can be accurately applied.

Methods

Study area

This study was conducted at 64 eyries from across Victoria, Australia (Figure 1). Sites surveyed were from each of the five geographical regions of Victoria as described in Emison et al. (1997). The altitude of sites ranged from 18 to 528 m above sea level and rainfall varied from 250 to 2,600 mm per year. The average maximum winter (initiation of breeding) temperatures range from $< 10^\circ$C at the higher elevations to $13^\circ$C along the coast and $17^\circ$C in the semi arid north-west.

This study involved taking blood samples, morphometric measurements and leg banding 150 peregrine falcon nestlings prior to fledging. This was undertaken over a two year period (2003-2004). Samples were taken from 51 nestlings in 2003 (25 male and 26 female) and 99 nestlings in 2004 (46 male and 53 female) over a wide geographic range (Figure 1). Nestlings sampled ranged in wing length from 6 to 26 cm. This range in wing length was selected to generate nestling growth curves commencing at pre-banding size and ceasing at fledging. Nestlings with a wing length shorter than 6 cm were excluded as they were considered too small to retain the recommended adult sized bands. Each nestling was measured only once during this study and all measurements were taken by the one researcher. All nestlings measured fledged successfully, with brood sizes ranging from 1-4 (mean 2.45, median 3 ± 0.83 STD). Nestling size was not influenced by brood size or hatch order (Hurley unpubl. data). As such the model was built only using nestlings from healthy clutches.

Morphometric data collection

Eleven morphometric features were measured for each nestling, however, only five of these were useful for determining gender as they are commonly used by field researchers. The five features measured were; body mass, wing chord length, tip-cere length, tarsus length and head-bill length.
Body mass was measured using spring Pesola balances accurate to ±1 g, ±5 g and ± 10 g for nestlings weighing up to 600, 1,000 and over 1,000 g respectively (nestling body masses recorded ranged from 40 - 1,153 g).

Wing chord was taken as a straight line between the carpal joint and the tip of the 8th (longest) primary laid, not flattened or straightened along a stainless steel butted rule (Lowe 1989). Wing length increases linearly with age, at a steady rate, consistent for both sexes regardless of nutritional status (Olsen and Olsen 1987). Wing chord length has therefore been utilized as a surrogate for absolute age in this study.

The tip-cere, tarsus and head measurements were each taken to the nearest 0.1 mm with Mitutoyo Digimatic (model number CD-6”) digital callipers (±0.01mm, max. 150mm).

Tip-cere length was taken as a measure of the chord from the front of the cere to the tip of the upper mandible. Tarsus length was measured from the posterior notch between the tibia-fibula and the tarso-metatarsus to the anterior notch between the tarso-metatarsus and third toe joint. This measurement was taken by gently holding the tibia and tarsus in a right angle and holding the metatarsi flexed in a right angle. The combined head and bill measurement was taken from the tip of the upper mandible to the rear of the occipital condyles at the rear and base of the skull.

**Molecular sexing**

Blood samples were drawn using a 26 gauge needle and 3.0 ml syringe from the brachial vein of 150 nestlings. Whole blood (50 µL) was preserved in 99% ethanol (1 mL), and stored at -20ºC until analysis. DNA was extracted from blood samples using Proteinase K digestion followed by extraction with ammonium acetate (Nicholls et al. 2000). DNA was also extracted (ammonium acetate extraction) from muscle tissue of peregrine falcons of known sex via dissection (Museum of Victoria tissue collection). One male (MV3492, registration B24290) and two females (MV4434, registration B31577 and MV4148, registration B26424) were used to validate the genetic sexing protocol.
PCR amplicons were prepared using the 2550F and 2718R primers (Fridolsson and Ellegren 1999). These primers provide a universal method for molecular sexing of non-ratite birds which is based on the detection of a constant size difference between the chromo-helicase-DNA binding protein CHD1W and CHD1Z (Fridolsson and Ellegren 1999).

PCR reactions were performed in 12.5 µL volumes on a Palmer Cycler (Corbett Research) Thermal Cycler using 0.05 U/µl Hot Star Taq (Qiagen), 0.1 mM dNTP’s, 1.5 mM MgCl₂ (Qiagen), 0.6 µM of primers 2550F (5’-GTTACTGATTCGTCTACGAGA-3’) and 2718R (5’-ATTGAAATGATCCAGTGCTTG-3’) and 1µL DNA template. The thermal profile comprised an initial denaturing step of 95°C (15 min), followed by 40 cycles of 30 s denaturation at 95°C, 30 s annealing at 40°C, 30 s extension at 72°C, followed by a final extension 72°C (5 min).

Preferential amplification of the shorter CHD1 (W) intron led to no detectable CHD1 (Z) production in females in the case of \textit{F.p. macropus}. The single female product was due to CHD1 (W) amplification out competing that of the CHD1 (Z) when both templates were present as targets for PCR (Fridolsson and Ellegren 1999). Amplification of the CHD1 (W) and CHD1 (Z) genes revealed a size difference of 150bp, which was clearly detectable when run on a 1.2% agarose gel run in standard TBE buffer and visualised by ethidium bromide staining.

\textit{Analysis and model development}

Discriminant function analysis was used to develop a model for predicting the sex of nestling peregrine falcons based on the morphometric data and genetically derived sexes. The final model was validated against the data used to derive the model and tested against a separate set of morphometric data from peregrine falcons of known sex which were not used to derive the model. SPSS version 12.1 (SPSS Inc, Chicago, Illinois) was used to conduct all statistical analyses.

\textit{Results}

\textit{Model development}

Morphometric data from the 79 female and 71 male peregrine falcon nestlings that were sexed using genetic techniques were used to develop a predictive model for
determining sex using discriminant function analysis. The morphometric measurements used in the model were wing chord (log_{10} cm), weight (log_{10} g), tarsus length (mm), head plus bill length (mm) and tip-cere (mm). These variables were selected because they are frequently measured by field researchers (e.g. Olendorf 1972; Arroyo et al. 2000; Balbontin et al. 2001) and are likely to differ between sexes (Baker-Gabb 1984; Olsen 1995). Overall, body mass (weight), tarsus length, head plus bill length and tip-cere length all differed significantly between the sexes, with nestling females tending to have larger measurements than males of the same age (Table 1). Wing length did not differ significantly between the sexes (Table 1), however, it was included in the model as it is an indicator of, and is directly proportional to, the age of the nestlings (Olsen and Olsen 1987; Olsen 1995).

The model discriminated between male and female groupings of peregrine falcons (Pillai’s trace = 0.750, df = 5,144, F-Ratio = 86.519, P<0.001). The mean discriminant score for males was -1.791 (SE = 0.107) and for females was 1.657 (S.E. = 0.121) (Figure 2). The function that best discriminated between male and female peregrine falcon nestlings was:

D_j = -39.930 – 16.830 (wing chord (log_{10} cm)) + 12.128 (weight (log_{10} g)) + 0.124 (head+bill length (mm)) + 0.130 (tarsus length (mm)) + 0.502 (tip-cere (mm))

Scores greater than zero were assigned as females and scores less than zero were assigned as males (Figure 2).

Validation of the model

The above formula was tested on 150 birds that were sexed by molecular techniques and used in the original model development. Overall, the model was able to correctly allocate the sex of 96.2% of this sample of birds (Figure 3). Applying the model to females with wing chord lengths less than 9 cm was extremely inefficient with only 20% (1 of 5 birds) correctly identified as female. Females with a wing length over 9
cm were correctly allocated 98.6% (73/74) of the time. Overall, the model worked extremely well for males with 98.6% (70/71) of all males correctly sexed (Figure 3).

This model was further tested by applying it to a separate set of 131 birds (66 females and 65 males) which had their sex confirmed by band recoveries later in life (via post-mortem of individuals recovered injured, deceased or observed as breeding adults that had been banded and measured as nestlings). Each individual was only measured once and all measurements were undertaken by the same researcher (VGH). The model correctly sexed 96.2% (126/131) of these birds. Overall the model was 98.5% (64/65) accurate for males and 93.9% (62/66) accurate for females. There was only one female with a wing length less than 9 cm, which was incorrectly classified by the model. If this bird is excluded from the results females with a wing length greater than 9 cm were correctly classified 95.4% (63/66) of the time.

Discussion

Correctly identifying the sex of raptor nestlings is an essential component of any banding project, especially with dimorphic species where band sizes differ between the sexes. This is often a difficult task as younger nestlings show very limited, if any, sexual size dimorphism.

Previous Australian studies on peregrine falcon nestlings have relied on determining sex with older nestlings (i.e. larger individuals) as RSD is more pronounced in larger nestlings where sex can be determined through morphometric features such as body weight or tarsus length compared to wing length (Emison and Bren 1981; Olsen et al. 1982; Olsen and Cockburn 1991; Mooney and Brothers 1993).

Wing length is directly proportional to age in nestling diurnal raptor species and formulae have been developed predicting age on wing length for nine raptor species in Australia (Olsen and Olsen 1987). As egg hatching is rarely monitored during most raptor banding studies, wing length can be used as a surrogate for the age (in days since hatching) of raptor nestlings. Our study aimed to determine the minimum age (wing length) at which nestlings can be sexed in order to increase the number of days
available for banding prior to fledging. The model developed here predicted sex most accurately when nestlings had a wing length of 9 cm or greater. When applied to the formula developed by Olsen and Olsen (1987) this translates to 15 days post hatching and allows for 20 days or 63% of the nestling period to accurately sex and band peregrine falcon nestlings at each nest.

Most studies on dimorphic raptors rely on morphometric measurements, in particular weight and wing length of adults and free flying juveniles, to accurately determine sex (Baker-Gabb 1984; Hartley and Mundy 2003; Delgado and Penteriani 2004; Bavoux et al. 2006). The use of a single feature, however, has been found to provide limited accuracy for some species even amongst adults, as was reported with footpad length in Northern spotted owls (*Strix occidentalis caurina*) (Fleming et al. 1991). Further, the use of a single feature, such as wing chord alone, has been challenged on statistical grounds and the degree of overlap of this feature between the sexes of adult saw-whet owls (*Aegolius acadicus*) (Mueller 1990). Although these features may become apparent in older chicks, they are extremely difficult to distinguish in very young nestlings. As a result, most studies attempting to sex nestling raptors have relied on using morphometric measurements of older nestlings approaching fledging. These were successfully developed for nestling bald eagles (*Haliaetus leucocephalus*) (Bortolotti 1984a; 1984b), brown falcons (*Falco berigora*) (McDonald 2003), short-eared owls (*Asio flammeus*) (Arroyo et al. 2000) and brown goshawks (*Accipiter fasciatus*) (Olsen et al. 1982).

Bortolotti (1984b) was able to allocate the sex of nestling bald eagles using size measurements (foot-pad length and bill depth) at 51.2% of nestling period when growth was almost complete. Arroyo et al. (2000) in their study on the dimorphic short-eared owl were able to sex nestlings (*n* = 16) on plumage features when the nestlings reached 12 days of age. The nestling period for this species is 30 days and therefore sex could be correctly assigned after 40% of the nestling period. A study on the growth of nestling brown goshawks found weight plotted against age, derived from wing length, showed a clear size separation (not genetically tested) amongst nestlings from 20 days of age or by as late as 65.6% of the nestling period (Olsen et al. 1982).
During this study we successfully developed a field model to reliably sex nestling peregrine falcons. This model has been genetically validated and correctly sexed 96.2% of field samples used. The model was able to predict the sex of nestlings at 36% of the nestling period. Correctly sexing raptor nestlings at 36% of the nestling period is a vast improvement on past studies as this increases the number of days over which nestlings can be sexed and banded with confidence. Another important implication of this model is that by banding nestlings earlier, they are less likely to branch/jump from the nest, therefore reducing the risk of injury to both the brood and the bander.

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References


Figures

Figure 1. Study sites where DNA and morphometric data were collected (2003 and 2004). Shaded area represents study area. Black dots represent nest sample sites.

Figure 2. Discriminant scores for male and female peregrine falcon F.p. macropus nestlings. Grey bars represent males and white bars represent females as determined by genetic analysis (n=79 females and 71 males). The reference line is at zero to indicate the pivot point between male and female classification.

Figure 3. Relationship between wing chord length and body mass in nestling peregrine falcons F. p. macropus as sexed by genetic analysis. Triangles represent males and circles represent females. Open symbols represent correct classification by the model whereas solid symbols represent incorrect classification. The vertical dotted line represents a wing length of 9 cm.
Table 1. Morphometric characters and total body mass (means ± 1 s.d.) for nestling peregrine falcons, and statistical comparisons between sexes. *n* = 150 (79 females, 71 males).

<table>
<thead>
<tr>
<th>Character</th>
<th>female</th>
<th>male</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wing chord (log₁₀ cm)</td>
<td>1.16 ± 0.14</td>
<td>1.13 ± 0.15</td>
<td>1.376</td>
<td>0.171</td>
</tr>
<tr>
<td>Body weight (log₁₀ g)</td>
<td>2.86 ± 0.09</td>
<td>2.73 ± 0.09</td>
<td>8.901</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tarsus length (mm)</td>
<td>48.12 ± 3.60</td>
<td>43.22 ± 3.22</td>
<td>8.742</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Head+bill length (mm)</td>
<td>63.15 ± 4.42</td>
<td>58.59 ± 3.78</td>
<td>6.759</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Culmen chord (mm)</td>
<td>24.60 ± 1.71</td>
<td>22.15 ± 1.47</td>
<td>9.377</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>