UNIVERSITY of OTAGO

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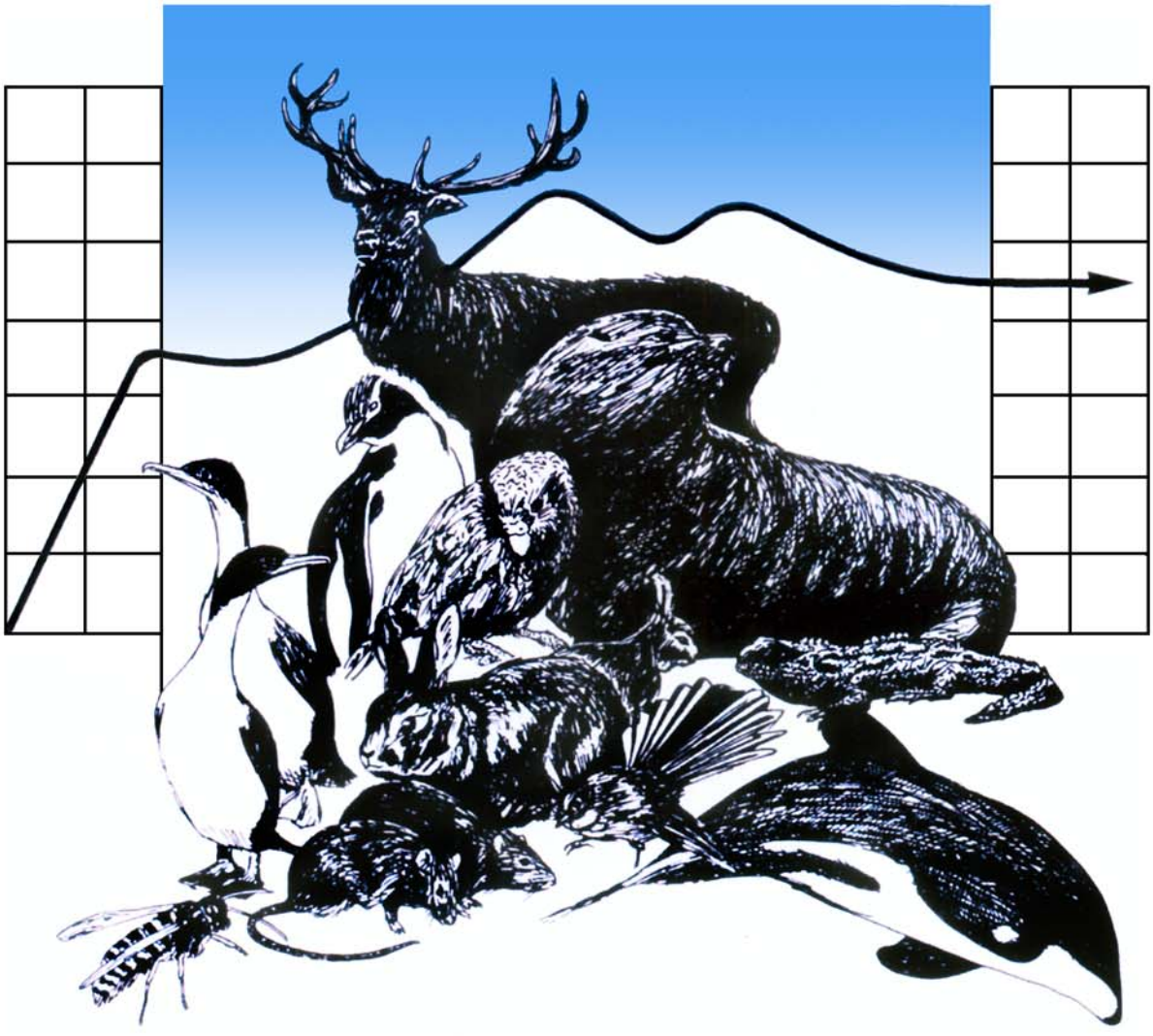
NEW ZEALAND

DEPARTMENT OF ZOOLOGY



WILDLIFE MANAGEMENT

Staging Kaki (Himantopus novaezelandiae) Embryos Using Embryonic Morphological Features

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Abstract

The Black Stilt (kaki, *Himantopus novaezelandiae*) is a critical endangered wading bird whose range is limited to the Upper Mackenzie Basin in the South Island of New Zealand. Captive rearing and artificial incubation comprise a major part of the management plan for this species. The aim of this study is to use embryonic morphological features to order kaki embryos into an identifiable age sequence for use in artificial incubation. Morphometrics of kaki embryos from the last seven breeding seasons are used to form a series of normal stages by which to age kaki embryos throughout their incubation period. PCA analyses show head length, head width and total curved body length are the most reliable indicators of embryo age when used as a multivariate size measure. The importance of this developmental sequence and its application to artificial incubation are discussed. Also, recommendations are made for examining kaki embryos in the future.

Keywords: Kaki, *Himantopus novaezelandiae*, embryonic development, embryo aging, principal component analyses.

Introduction

Kaki (black stilt, *Himantopus novaezelandiae*) are a critically endangered wading bird found on braided rivers and wetlands in the central South Island of New Zealand. These birds are vulnerable to a range of threats including predation, habitat disturbance and hybridisation with the more common pied stilt (*H. himantopus*). Breeding is restricted to braided rivers in the Upper Waitaki Basin, where captive rearing is employed by the Department of Conservation (DoC) as part of an intensive management plan to increase the wild breeding population (Maloney and Murray, 2001). Eggs are removed from both the wild and captive birds and incubated artificially, and subsequently returning to the wild as juvenile or subadult chicks (Leseberg *et al,* 2004). To date, necropsied embryos are classified subjectively into incubation trimesters by their morphological features (E. Sancha, pers. comm.).

The aim of this study is to use embryonic morphological features to organise kaki embryos into an identifiable age sequence for use in artificial incubation. Developmental stages have been useful for improving artificial incubation techniques for other threatened New Zealand birds such as kiwi (S. Bassett, pers. comm). The ability to age embryos which die during incubation has allowed an increased understanding of the developmental process, incubation, and embryo mortality in these species. In this way, a series of normal stages could be applied to further enhance the survivability of kaki under captive management. It is predicted that the ability to age kaki embryos which die during incubation based on their morphological features will allow an increased understanding of development during artificial incubation in both kaki and other highly managed endangered avian species.

Because embryonic development is a continuous process with variation between individuals, normal stages do not fit exact developmental time increments. Instead they are based on easily recognisable morphological characteristics of the embryos (Padgett and Ivey, 1960). In their normal tables of development, Hamburger and Hamilton (1951) used structural differences in chicken embryos to differentiate between developmental stages. Where possible, several structural features are used to describe each developmental stage. Because these features change during development, different stages are characterised by different features (Starck & Ricklefs, 1998). Many of the criteria used by Hamburger and Hamilton (1951) in their embryonic descriptions are applicable to other precocial species such as kaki. Precocial chicks hatch covered with down, eyes open, organs of locomotion fairly well developed and are able to feed on their own (Blom & Lilja, 2005). Precocial species typically develop "demand" organs such as the brain, muscles, skeleton and feathers early on, while "supply" organs such as digestive organs develop at a slower rate (Blom & Lilja, 2005). Because of these structural similarities in embryonic development among precocial species, most of the recent work on staging embryonic development in these species is based on the work of Hamburger and Hamilton (1951) (Starck & Ricklefs, 1998). In each species, developmental stages are relative to incubation period, but are essentially the same set of morphological stages (Deeming, 2002). As the embryonic development of the domestic chicken is well documented, these developmental stages will be the primary reference work used to place kaki embryos into their correct stage of development.

Variation in embryonic growth rates among individuals can reduce the usefulness of these stages as developmental markers. Padgett and Ivey (1960) show that although the incubation period of quail is relatively constant, there is variation in the grade of development in embryos of the same age. This is particularly evident in

early stage embryos undergoing developmental differentiation, and late stages which are characterised primarily by growth (Starck & Ricklefs, 1998). This variation is a result of the many physiological and environmental factors affecting embryonic development and survivability both before and after incubation (Ricklefs, 1987). For example, the length of time an egg is retained in the oviduct following fertilisation is thought to influence mortality in early embryos, as does temperature and method of egg handling following collection in commercially reared species (Scott and Mackenzie, 1993). In their study on broiler breeder chickens, Scott and Mackenzie (1993) noted two distinct peaks in embryo mortality between stages 14 and 18 and stages 24 and 28. This is attributed to developmentally sensitive periods in incubation. Despite this variation between and within species, normal stages provide a useful framework for comparing embryonic development between individual embryos and between species (Padgett and Ivey, 1960).

Methods

Embryos

A total of 45 embryos have been stored at the kaki captive rearing unit since the 1998/99 breeding season. These embryos have been stored individually in 10% buffered formalin. Each embryo was removed from this solution and rinsed gently under running tap water for 3 to 5 minutes. Beginning with the largest embryo, measurements were taken with the embryo placed in a small shallow dish. For the smaller more delicate embryos, a small amount of water was added to prevent damage to the tissues. A paintbrush and dental tools were used when handling embryos. Embryos which had either a crossed bill, crossed or clubbed feet, or were malpositioned when necropsied were included in the staging sequence, but were not used as marker embryos on account of their abnormal morphology.

Morphological measurements

Body weight was measured to within 0.1g using electronic scales. Total curved body length was measured in all embryos from number 15 through to 45 using a piece of cotton placed from the cere to the end of the tail, the cotton was then measured using a ruler with a millimetre scale.

The following linear measurements were taken using vernier callipers with a millimetre scale: head width was taken directly behind the eye in embryos 9 to 45. Head length was measured from the cere to the back of the head in embryos 8 to 45. Wing and leg bones were easily distinguishable and able to be measured in embryos 15 to 45. Upper bill length was taken from the cere to the tip of the bill in embryos 17 to 45. Bill gape was measured in embryos 17 to 45 across the cere between the corners of the mouth opening. Eye diameter was measured in embryos 15 to 45 as the length between the two corners of the eye. Eyeball diameter was measured in embryos 2 to 45 where the eyeball was clearly visible. Leg depth and leg width were measured in embryos 28 to 45 as the depth and width of the tarsus bone respectively. These measurements were taken in the centre of the bone, equidistant from both joints. Body width was taken across the widest point of the abdomen in embryos 6 to 25. Tail length was measured along the underside of the tail in embryos 10 to 24. Right wing bud lengths were measured in embryos 6 to 11. Right leg bud lengths were measured in embryos 6 to 13.

Each measurement was taken on every embryo possible, with embryo size, preserved embryo position, presence/absence of morphological features and damage to embryos acting as limiting factors. Embryos were often viewed under a dissecting microscope to measure smaller morphological features. Measurements were taken on the right side of each embryo where possible, if this was impossible left side measurements were taken and this noted.
Embryos were photographed using an Olympus μ 800 digital camera, then placed in a 70% ethanol solution for future preservation. Protective clothing was worn and the lab space well ventilated when using formalin and ethanol. It should be noted that specimens stored in formalin are smaller than fresh embryos (Padgett and Ivey, 1960). Therefore the measurements and weights taken during this study are preserved measurements and weights, and will therefore be slightly smaller than fresh measurements.

Statistical analysis

Embryos were separated for analysis into those that were large enough to have a total curved body length measurement (embryos 15 to 45) and those too small for this measurement (VSN International, 2005). Principal Component Analysis (PCA) using a correlation matrix was performed on the morphological measurements for the large embryo dataset (embryos 15 to 45) using the GenStat multivariate statistics package in order to reduce the large number of correlated variables into a smaller number of uncorrelated components. The factor scores for PCA Axis 1 can be used as a multivariate size measure for the kaki embryos (Blackwell, 2002). Successive PCA analyses were performed to find the combination of measurements best described embryo "size", with the analysis that explained the greatest amount of variance on PCA axis 1 chosen as the final model.

Results

Given the small number of very young kaki embryos available for this study, the staging sequence begins at day 6 of incubation. Size and development are first estimated based on morphological appearance, and each embryo is then assigned a number from 1 to 45, youngest to oldest.

Morphological measurements were analysed using Principal Component Analysis, and these scores are used as a multivariate size measure for the embryos. The PCA using head length, head width and total curved body length explained 98% of the variation in embryo size on PCA Axis 1, with PCA axis 2 only explaining a further 1% of this variation. Therefore PCA Axis 1 was chosen as the final model, describing the relative size of each embryo based on these three morphological measurements (fig. 7). All three variables are strongly negatively correlated with PCA Axis 1 (fig. 7). Photographs and morphometrics of each embryo were used to adjust this age sequence (fig. 7).
The main developmental events in the chicken sequence were identified and used to determine the three marker embryos based on key stages of embryo development. The corresponding kaki stage for these three marker embryos is calculated as a proportion of the total incubation period of the kaki (25 days under artificial incubation). The first marker is kaki embryo number 20 which exhibits the webbed feet and bill formation similar to that of a day 7 chicken embryo, and corresponds to a day 8.5 kaki embryo (fig. 2):

6 days/ 21 (total incubation period of chicken) = 0.28
0.28 x 25 (total incubation period of kaki) = day 8.3 kaki (rounded to 8.5 days).

The second marker is kaki embryo number 29 with the feather formation, head and eye development similar to that of a day 10.5 chicken corresponds to a day 12.5 kaki (fig.4). Embryo number 45 (fig. 6) was the only embryo which died hatching and had a fully absorbed yolk sac, so this was classified as day 25 (point of hatch).

Three distinct developmental gaps are identified in this current kaki sequence (fig. 7). Taking these gaps into consideration, embryos are assigned ages of equal time increments in the 25 day incubation period based on their photographs, using the marker embryos to "anchor" the sequence. These embryo ages are then plotted against their corresponding PCA scores to compare morphometric and photographic age sequences (fig. 8). For example, kaki embryo 15, with the degree of eye pigmentation and limb bud formation of a day 5 chicken, and the absence of cranial protuberances which characterise this stage is equivalent to a day 6 kaki based on extrapolation from the marker embryos (fig.1). Kaki embryo number 20, developmentally equivalent to a day 7 chicken is equal to a day 8.5 kaki (fig. 2). Kaki embryo number 26 is developmentally equivalent to a day 10 chicken, and subsequently is aged at day 11.5 in the kaki sequence (fig.3). Kaki embryo number 38 is equal to a day 17 chicken, which equates to a day 20 kaki (fig.5).

There are some anomalies in the embryo age ranking (fig.8). For example, embryo 32 has a lower PCA score than embryos 35 and 34 directly above it in the age sequence (fig. 8), despite being a less developed embryo. Embryo 36 has a lower PCA score but is less developed than embryo 37 immediately above it in age ranking. Embryo 37 was malpositioned when necropsied, so will be small considering its developmental stage (see fig. 10 appendix). Embryo 43 has a higher PCA score than both embryos 45 and 44 either side of it in the age sequence (fig. 8). This is attributed to natural variation in size between individuals.

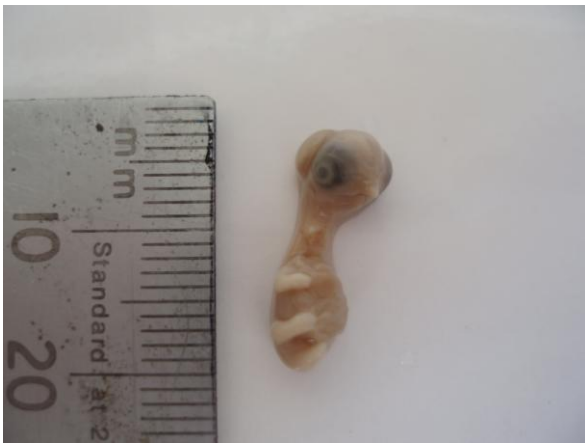

Figure 1: day 6

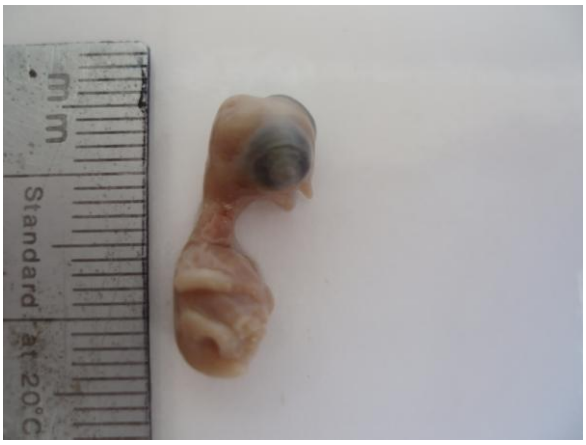

Figure 2: day 8.5

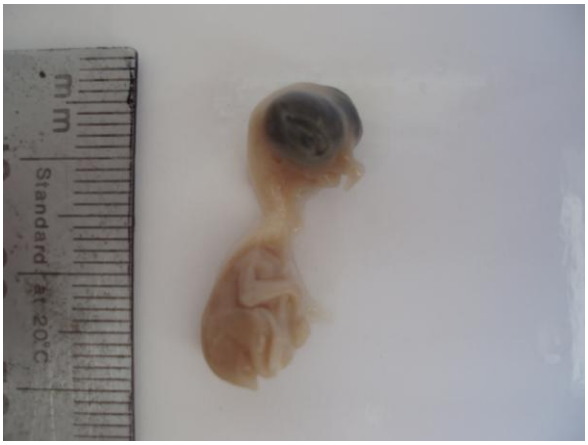

Figure 3: day 11.5

Figure 4: day 12.5

Figure 5: day 20

Figure 6: day 25

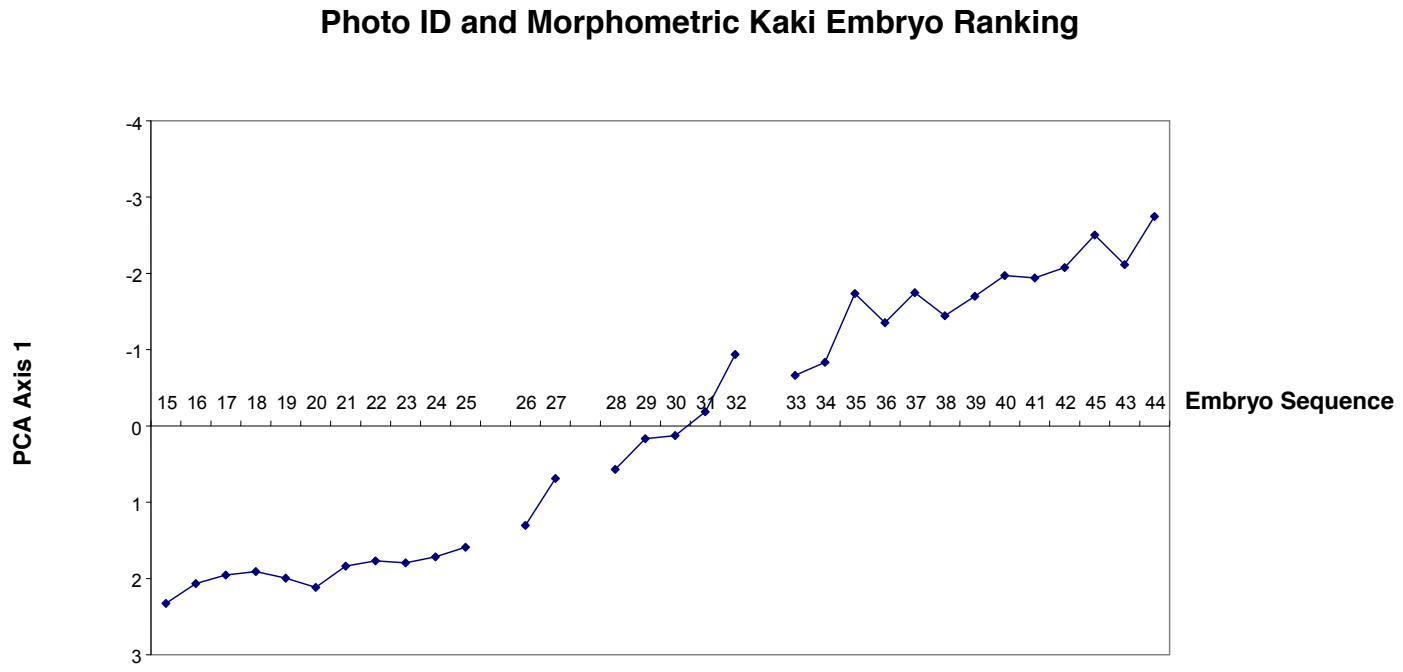

Figure 7: Ranked kaki embryo order using PCA values, adjusted using the morphometric photo sequence.

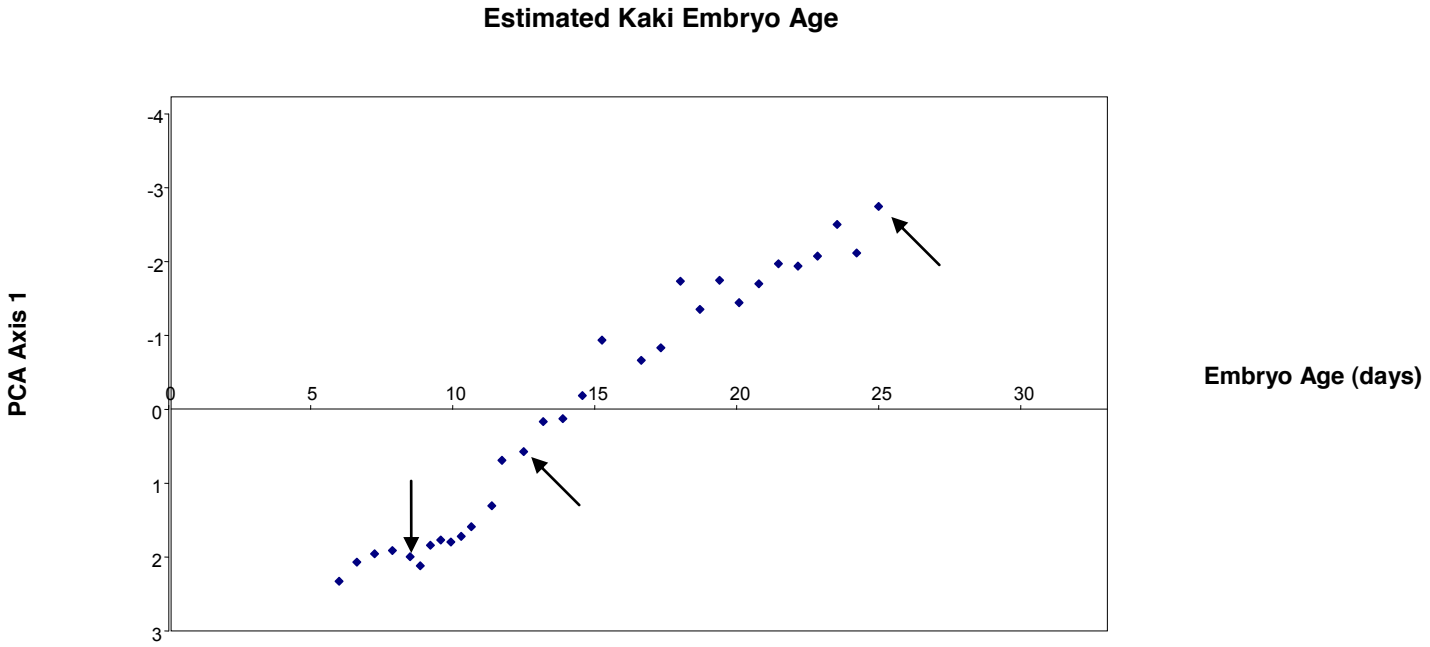

Figure 8: Estimated kaki embryo age (days) based on 3 marker embryos (arrowed).

Discussion

This project provides a straightforward sequence which can be referred to when undertaking egg necropsies during artificial incubation. This sequence will also be useful for field managers, for example in determining the age of embryos from abandoned eggs. Overall, this sequence provides a reference for aging kaki embryos, and is designed to improve the information gained from necropsies. For example, if all embryos are failing at the same developmental stage, the age of these embryos can be ascertained, and the problem potentially identified from this information. Defined developmental stages will provide clearer insight into factors affecting hatchability during artificial incubation. These factors are important to identify so improvements can be made and overall survivability increased. Defined stages are useful for clarifying the developmental process of a species so any distinguishing developmental features are identified and necropsy analysis accurate (Deeming, 1995a). For example, the embryonic development of the barn owl initially lags that of the chicken (Kőppl *et al*, 2005). Also, the mid stages of emu (*Dromaius novaehollandiae*) development are more advanced than that of the chicken (S. Bassett, pers. comm.).

A total of 25 morphological measurements were taken on each embryo, with head length, head width and total curved body length the most useful in determining the developmental sequence for kaki. However, it is not clear during data collection which measurements will be the most useful, and it is preferable to be able to exclude inadequate measurements during data analysis than it is to not have enough descriptive measurements (S. Bassett, pers. comm.). Based on the PCA results, all three of the morphometric variables are strongly negatively correlated with PCA Axis 1 values in that they all increase as each embryo grows (fig. 7). Therefore these three measurements are suitable for aging kaki embryos. Photographs of each embryo provided further details of morphology which were used to adjust the age sequence derived from the PCA values (fig. 7). It is recommended that a combination of measurements and visible developmental markers be used to age embryos in the future (table 1 appendix).

The sequence was able to be refined with the use of marker embryos to 'anchor' it (fig.8). Ideally more than three marker embryos would be identified to anchor the sequence, but due to gaps in the series of available embryos it was difficult to pinpoint corresponding ages between the chicken and kaki. Future necropsies may provide suitable marker embryos and the sequence can be refined using these. Also, more individuals of the same developmental stage are required from future necropsies in order to quantify variation in embryos of the same age. A degree of variation is obvious in this developmental sequence, for example embryo 32 has a lower PCA score than embryos 35 and 34 directly above it in the age sequence (fig. 8), despite being a less developed embryo. The ability to quantify this variation will allow the age sequence to be further refined.

Many of the obvious developmental markers used by Hamburger and Hamilton (1951) cannot be applied to this study. For example, position of the embryo relative to yolk inside the egg is a reliable indicator of age in the domestic fowl (Freeman and Vince, 1974), but cannot be used here as the embryos have been removed from the eggs prior to this research being undertaken. Instead bill and feather formation, head and eyelid development are used. There are three marker embryos available in this data set whose easily identifiable characteristics allow them to be staged according to chicken morphology (Hamburger and Hamilton, 1951).

This method of transposing the chicken stage onto kaki embryos is the same as used by Deeming (1995b) in his staging of ostrich embryos, and Suzanne Bassett used a similar criteria for extrapolating kiwi embryo ages based on emu developmental stages (S. Bassett, pers. comm.).

It should be noted that this staging sequence needs refining; it is by no means a complete developmental stage sequence. However, given the small sample of embryos available for analysis it is as comprehensive as the data allows. Key information that can only be attained from fresh embryos such as position at death will be very useful for refining this sequence in the future. There are alternative methods employed for aging other avian species which, for various reasons, were unable to be used in this study. For example, candling is used by Deeming (1995b) to provide developmental information for staging ostrich embryos. This method cannot be used to the same extent for kaki on account of the speckled shell, which inhibits the view of the developing embryo. From mid-incubation onwards, the finer vascular details cannot be seen as they can in other species (E. Sancha, pers. comm.). Also, embryo weight as a proportion of average chick weight at point of hatch is an ideal method used to age embryos. However, scales accurate to 0.001g are required to do this accurately for extremely young embryos (Bassett, pers. comm.)

It is not expected that field managers or even artificial incubation managers will age kaki embryos to within 2 days or less based on the criteria provided in this study. It is preferable to have broader staging criteria which allow less room for error. Appendix Table 1 is designed to be used as a key when staging kaki embryos. This key can be referred to for head width, head length and total curved body length size ranges for embryos at intervals throughout incubation. These measurements should be referred to in combination with the typical morphological characteristics for each developmental period, as this will provide a more accurate and comprehensive guide to aging.

Suggestions and recommendations

The most useful recommendation which can be made from this study is for more detailed notes to be recorded during necropsies. Information on developmental events such as position of an embryo relative to the yolk, closing of the navel and absorption of the yolk would provide more accurate information by which to age the embryo. Ideally, embryos would be examined when fresh, as the preservation process tends to shrink and harden tissues, fixing the embryo into a rigid position. Preservation also causes further structural damage to embryos such as the loss of feathers.

The most effective way to produce a comprehensive developmental sequence for kaki would be to conduct an experiment using pied stilts or even hybrid birds. This would involve euthanasing embryos at regular intervals (for example every two days) and cataloguing them as a reference point for kaki embryos which fail at that particular age.

Acknowledgements

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Appendix A

Table 1: Morphological characteristics and size classes for embryos at intervals throughout incubation.

Embryo Age	Morphological Characteristics	Total curved body length	Head width	Head length
6-8 days	Webbed toes, ridges form Upper bill longer than lower	30-40mm	4-6 mm	5-7mm
9-10 days	Webbed toes with ridges Upper and lower bill even length Wing and leg joints form	35-45mm	4-6mm	6-8mm
11-12 days	Distinct toes Dark feathers form on dorsal and lateral surfaces	40-60mm	6-8mm	9-11mm
13-15 days	Dark feathers form around eye White feathers form on ventral surface	55-65mm	8-10mm	10.5-12.5mm
16-18 days	Fully feathered Distinct egg tooth Distinct toenails	70-100mm	10-12.5mm	15-17mm
19-20 days	Growth	85-100mm	10-12.5mm	15-17mm
21-22 days	Growth	90-110mm	11-13mm	17-18mm
23-25 days	Internally and externally pips Yolk sac absorbed Growth	105-115mm	11.5-13.5mm	17.5-20.5mm

Appendix B

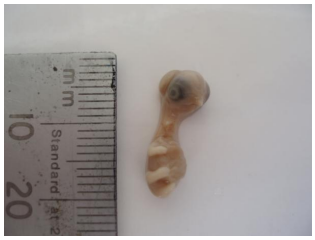

Figure 1: day 6(04-128)
Ridges on webbed toes barely visible
Upper bill longer than lower
No joints

Figure 2: day 8(03-48)
Webbed toes
2 concave ridges on each foot
Upper bill just longer than lower
Joints just formed

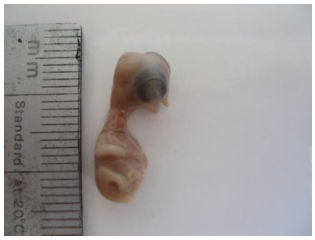

Figure 3: day 8.5(05-85)
Joints present
Upper and lower bill even
Webbed digits with ridges

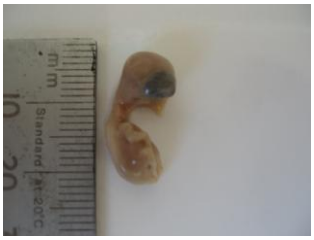

Figure 4: day 10.5(04-143)
joints present
Webbed digits with ridges
Bill longer

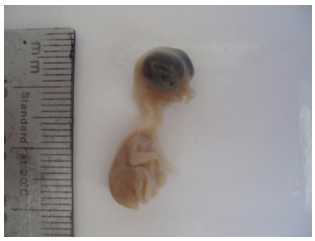

Figure 5: day 11.5(01-92)
Distinct toes
Single row of dorsal feather germs

Figure 6: day 12.5(01-26)
Dark feathers on lower dorsal and lateral surfaces only
Feather germs all over

Figure 7: day 14(embryo 30)
Feather germs all over
dark feathers on lower dorsal and
lateral surfaces and above upper eye lid
white feathers on ventral surface

Figure 8: day 15.5(00-18)
feathers on dorsal and lateral
surfaces and around eye
white feathers on ventral surface

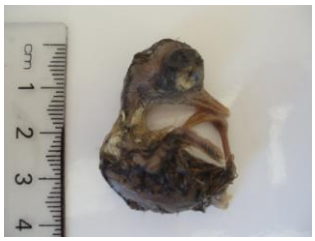

Figure 9: day 17.5 (05-M5)
Fully feathered
Distinct egg tooth
Distinct toenails

Figure 10: day 19(03-09)
fully feathered
Distinct egg tooth
Distinct toenails
Greater overall growth
Malpositioned

Figure 11: day 20(03-142)
Fully feathered
Papillae on foot pads??
Greater overall growth

Figure 12: day 21.5 (03-02)
fully feathered
Papillae on foot pads??
Greater overall growth

Figure 13: day 23.5(05-116)
Yolk not absorbed
Otherwise fully developed

Figure 14: day 25(04-83)
Fully absorbed yolk
Died hatching
Fully developed