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Ostrich (*Struthio camelus*) embryonic development from 7 to 42 days of incubation

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ABSTRACT

1. Hatching success of ostrich eggs is poor (50–60% of fertile eggs). The current study was designed to identify the timing of key stages in the development of the ostrich embryo.
2. Growth of both embryo and wing length during 42 d of incubation was comparable and approximately linear, with a more or less weekly doubling in size up to 35 d of incubation.
3. The embryo eye size increased more rapidly than beak length and reached a maximum of ~16.2 mm by 28 d of incubation, whereas beak length increased continuously until hatching at 42 d.
4. Linear regression equations were derived from morphometric measurements of embryos between 7 and 42 d.
5. Information stemming from these results can be used to estimate the age of dead-in-shell embryos in an attempt to identify timing of incubation problems that potentially result in low hatchability of fertile eggs.

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Introduction

The success of artificial incubation of ostrich eggs has a major impact on chick production and consequently on the financial success of the industry. A major problem with artificial incubation of ostrich eggs is that hatching success is poor compared to that of domestic poultry, with hatchability figures of only around 50–60% of fertile eggs set (Brown *et al.*, 1996; Deeming and Ar, 1999; Van Schalkwyk *et al.*, 2000). The observed low hatching rate is consequently of great concern to the industry. Attaining good rates of hatchability requires correct diagnosis of problems and timing of these during embryonic development of artificially incubated eggs, but the developmental stages of the ostrich embryo need to be well described to allow this. The ostrich embryo undergoes a complex pattern of growth and differentiation in a series of developmental steps over the incubation period of 42 d. Ostriches are precocial birds (Brown and Prior, 1999), which undergo a longer phase of tissue maturation than altricial species (Ricklefts and Starck, 1998). The first 42 normal developmental stages in domestic fowl (chickens), as described by Hamburger and Hamilton (1951), can be applied equally well to altricial and precocial development. As a result, the well-described embryonic stages of chickens have previously served as a reference for other less-well-studied avian species. Richardson *et al.* (1998), however, showed differences in embryonic development between species. Limited observations on ostrich embryos suggest that the basic pattern of embryonic development differs little from that of the chicken (Deeming *et al.*, 1996; Ar and Gefen, 1998). The incubation period of ostriches is exactly double that of chickens, and a "rule of thumb" is that any particular stage of ostrich embryonic development can be obtained by reference to the corresponding stage of development in the chicken.

The embryo undergoes two distinct phases of development. The first is the differentiation stage, which takes place during the first half of development (Deeming, 1997). This period is characterised by the formation of new structures and conforms closely to the equivalent incubation stage of the chicken (Gefen and Ar, 2001). There are, however, differences in the second half, with this phase of development being characterised mainly by growth, specifically changes in the beak, wing, and leg length, as well as the wet weight of the embryo (Gefen and Ar, 2001). On the basis of this, Gefen and Ar (2001) suggested that embryonic age estimation of one species cannot necessarily be inferred from relative changes in linear dimensions of another species, although they caution that this observation is based on a limited number of observations for each embryonic age.

An important tool for identifying incubation problems that potentially cause low hatchability is knowledge of the age and stage of development of the embryo at the time of death (Ar and Gefen, 1998). Gefen and Ar (2001) presented equations for estimating the embryonic age of the ostrich during the second half of incubation using morphometric measurements. Again, however, these were based on a limited sample. In this study, we extend the work of Gefen and Ar (2001) by describing the stages of the development of ostrich embryos during the incubation period and derive equations from measurements of developing embryos from a substantially larger sample of eggs. This may allow for more accurate determination of age at mortality.

Material and methods

Eggs that originated from the commercial ostrich flock at the Oudtshoorn Research Farm, South Africa, during 2009 were used for the study. Cloete *et al.* (1998, 2008) and

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Bunter and Cloete (2004) described the origin of flock and the management of the breeding birds. Incubation practices for the eggs have been described previously (Van Schalkwyk, 1998; Van Schalkwyk *et al.*, 1999; Brand *et al.*, 2007). For this study, only eggs of the South African Black genotype were used. The methods of egg collection, sanitation and storage on the research farm followed procedures described previously (Van Schalkwyk, 1998; Van Schalkwyk *et al.*, 1999; Brand *et al.*, 2007). All eggs collected were stored for 3 d at 17°C and relative humidity (RH) of 75% prior to setting into the incubator. The 3-d storage time was chosen in accordance with the findings of Brand *et al.* (2007, 2012), which suggested that the best hatching results are from eggs stored for 3–4 d. Because some ostrich farmers set eggs horizontally in the incubator and some others set them vertically, the eggs were randomly divided into two groups: one group was set horizontally (n = 114) and the other group was set vertically (n = 114), with the air cell up to assess whether setting position affects development. When placing the groups of eggs horizontally into the incubator, a sticker was placed on the eggshell to indicate the topside of the egg and to ensure that, upon opening the eggs, all eggs were consistently opened at the same location to be able to describe the position of the embryo. For eggs incubated vertically, a sticker was put on the part of the eggshell facing forward. Eggs were placed randomly throughout the same Buckeye incubator and incubated at 36.2°C and 24% RH. The eggs set horizontally were turned through a 90° angle on their long axis. The top of the eggs set vertically (containing the air cell) were turned through 90°. The incubator was set to turn eggs automatically through 90° hourly.

Between 21 and 34 eggs of each age were processed to investigate developmental changes that had taken place at each of d 7, 14, 21, 28, 35 and 42 of incubation. Eggs were weighed and then opened by breaking the eggshell at the region of the air cell and removing the membranes covering the embryo. Only eggs containing live embryos were considered for data collection. The qualitative stage of embryonic development, as described by Gefen and Ar (2001), was noted. After separation from the yolk and embryonic fluid, the embryo was weighed to the nearest gram. Embryos from eggs incubated for 7 d were too small to measure manually and so were placed under an Olympus SZ-61 microscope with an LG-PS2 light guide illumination system for a clear image as described by Brand *et al.* (2014). Digital images of the developing embryos were taken with a ColorView camera mounted on the microscope. After the developing embryos were photographed, the AnalySIS program (Soft Imaging System, 1999) was used to measure embryo length and eye size. Embryo length of 7-d incubated eggs was obtained by running a thread from the tip of the tail along the spinal cord and the blood line in the brain area to the defined point on the head edge. The measurement unit was 1 mm. The embryos of eggs incubated for ≥14 d were removed from the eggshell to measure the eyeball diameter, beak length (from the feather line where the beak begins to the tip of the upper beak), leg length (from the body–leg joint to the tip of the claw), upper wing length (humerus – from the body joint to the elbow), lower wing length (from radius to phalanges – the elbow to the tip of the second digit) and embryo length (from the top of the head to the tip of the tail). Only the extremities on the left side of the embryo were measured. These measurements were carried out with a digital calliper to an accuracy of 10 µm. Photos were taken to examine the morphological changes that took place. Because ostrich eggs normally start to hatch between d 41 and d 42 of incubation, all the final measurements were taken on live chicks after hatch. On opening the eggs, the head position of the embryo was noted by dividing the opened area into 4 sectors relative to the air cell and the placement of the sticker attached upon setting.

These data were then analysed to identify trends associated with incubation period (7, 14, 21, 28, 35 and 42 d), using least squares analyses in ASREML (Gilmour *et al.*, 2009). The position of the egg during incubation (vertical or horizontal) was also included in the analysis and interacted with day of incubation. Differences in egg size were accounted for by the inclusion of the initial length, width and weight of the egg as linear covariates in the analyses. The distribution of eggs between treatments was not always balanced, thus requiring the application of least squares procedures to account for uneven subclasses. Differences between comparable means were discerned with the least significant difference method on the provision that it was protected by a significant F-value in the ANOVA (Snedecor and Cochran, 1967). Chi-square procedures (Van Ark, 1990) were used to assess the effects of setting position on hatchability.

Results

Description of embryonic development

After 7 d of incubation, the tail bud of the embryo started to curve, with the tip pointing forward towards the anterior end. The embryo had reoriented from lying ventrally on the yolk surface to turning onto its side and floating within the fluid-filled amniotic sac above the yolk surface. The allantois had grown out on the right side of the embryo's gut and was variable in size, while the eyes were faintly grey in colour. By d 14, the vascular network and the amniotic sack enclosing the embryo covered the upper surface of the yolk. The beak was distinct at this stage, with the maxillary about twice the length of the mandible. The distinct grooves between the two toes and the three digits of the legs and the wings, respectively, were clearly visible at this stage. Rudimentary feathers were evident after 21 d of incubation. The eyeballs had been covered with eyelids to an oval opening. The embryo had sunken into a depression in the yolk surface.

By d 28, claws appeared on the toes of the embryo. The embryo was turned with its spine parallel to the long axis of the egg, with the head bent towards the breast and tucked between the legs. By 35 d of incubation, the beak was orientated towards the right and the legs were pulled closer to the body, with the feet positioned adjacent to the neck. The embryo was covered with a thick coat of feathers. The first chicks started to pip on d 40, with most pipping occurring on d 41 and 42. At this stage, the ostrich embryo was fully grown, with the beak pointing right over the wing, the right foot next to the beak and the left foot behind the head. The yolk sac had been fully retracted into the body cavity, and all the albumen had been used. Internal pipping occurred when the chick penetrated the membranes next to the air cell with its beak, followed by external pipping. A

combination of pecking, kicking and body extension movements then allowed chicks to hatch. It subsequently took up to 12 h for the chick to break completely free from the shell.

Setting and incubation position, embryo size and weight

Incubation position of eggs (vertical vs. horizontal) generally did not affect the measurements of the developing embryo throughout the 42-d incubation period. Results are consequently presented as the means of all eggs set. Embryos in eggs incubated both horizontally and vertically started to turn with their spines parallel to the long axis of the egg on d 28 of incubation. Hatching results showed no difference in hatchability between fertile eggs set in the horizontal position (13/18 = 0.72) and eggs set in the vertical position (11/18 = 0.61) (χ^2 = 0.13; df = 1; P = 0.72). On opening (n = 84), most embryos in the horizontally incubated eggs were positioned with their heads in the direction of the air cell (69%). All the embryos in the vertically incubated eggs (n = 84) were orientated with their heads towards the top (air cell) part of the egg and the distribution for the 4 quadrants was about equal (23–33% per quadrant). The orientation of embryos relative to the 4 quadrants was about equally distributed (21–31% per quadrant).

The measurements for all components of the developing embryo are presented in Table 1. Initial weights of eggs used in the trail ranged between 1405 and 1466 g. Embryo length increased by 81% from 91 to 166 mm between 21 and 28 d of incubation, while leg length doubled from 47 to 96 mm. Embryo weight increased by more than 7-fold from 21 to 156 g over the same period. By 35 d of incubation, the yolk sac had been retracted about halfway into the abdominal cavity, and about 74% of the albumen had been used. The length of the embryo and the leg during the 42 d of incubation was parallel and approximately linear (Table 1). Both embryo length (14.9–235 mm) and embryo leg length (12.4–139 mm) nearly doubled for each week of incubation up to 35 d of incubation. Smaller length increases occurred during the last week of incubation amounting to 267 and 181 mm, respectively, for embryo length and leg length.

Embryonic weight increased from 29% to 64% of initial egg weight during the last week of incubation (Table 1). Embryo eye size increased more rapidly than the beak length and reached its maximum at about 16.2 mm by 28 d of incubation, whereas the beak length continued to increase until chicks hatched at 42 d.

The weight of the embryo increased exponentially in the 42 d incubation period, with a slow rate of increase between d 7 and d 21 of incubation followed by a rapid rate of increase thereafter (Table 1).

Prediction of embryo age from linear body measurements

The age at which eggs were opened was regressed upon embryo length, leg length, upper wing length and lower wing length to derive a predictive tool to estimate the approximate age of dead-in-shell embryos at death. It was evident that simple linear regressions fitted the data well, as reflected by correlation coefficients ranging from 0.93 for lower wing length to 0.98 for leg length (Table 2).

Discussion

General embryonic development

The general appearance of the embryos at 7 d of incubation in the present study corresponds with the report by Gefen and Ar (2001). The appearance of the ostrich embryo on d 7 was also similar to stage HH20 (70–72 h) in chickens (Hamburger and Hamilton, 1951) and stage 33 for ducks (Dupuy *et al.*, 2002), while the positioning of the ostrich embryo corresponded with reports on the position of chicken embryos (Buhr and Rowland, 1997). In the present study, as well as in previous studies (Ar and Gefen, 1998; Gefen and Ar, 2001), the appearance of eye pigment in ostrich embryos began after 7–8 d of incubation. Similarly, the groove between the two toes was visible on d 14 of incubation, and the eyelids also started to cover the eyeballs. Hamilton (1952) observed that chicken embryos started to turn lengthwise between d 12 and d 16 of incubation. Both Buhr and Rowland (1997) and Gefen and Ar (2001) described the turning of ostrich embryos at d 28 of incubation, as well as the appearance of toe claws and fine feathers. These observations were consistent with the results from the present study.

Setting and incubation position, embryo size and weight

No literature regarding the effect of incubation position on embryonic development or orientation in eggs could be found for ostriches. However, Van Schalkwyk *et al.* (2000) found that the hatchability of fertile eggs was relatively low but unaffected by setting in either the vertical or the horizontal position for 6 weeks. The present result is consistent with this. The hatchability of eggs incubated horizontally accordingly did not differ from that of eggs incubated

Table 1. Least squares means (±SE) for weights and measurements of developing ostrich embryos for eggs incubated for 7–42 d.

		Mean ± SE					
Measured traits	*n*	7 d	14 d	21 d	28 d	35 d	42 d
Embryo length (mm)	24–31	14.9 ± 2.56	36.9 ± 2.56	91.4 ± 2.76	166 ± 2.55	235 ± 2.56	267 ± 2.94
Eye size (mm)	15–31	0.77 ± 0.32	8.60 ± 0.24	15.5 ± 0.26	16.2 ± 024	16.3 ± 0.24	15.9 ± 0.29
Beak length (mm)	19–30	-	3.37 ± 0.44	10.2 ± 0.36	16.5 ± 0.33	20.8 ± 0.33	21.7 ± 0.38
Leg length (mm)	19–30	-	12.4 ± 1.63	47.3 ± 1.75	95.8 ± 1.62	139 ± 1.63	181.3 ± 1.92
Upper wing (mm)	19–29	-	5.05 ± 0.89	16.3 ± 0.73	29.0 ± 0.66	37.7 ± 0.67	43.3 ± 0.77
Lower wing (mm)	19–29	-	6.32 ± 0.61	16.2 ± 0.50	26.7 ± 0.45	35.6 ± 0.47	43.4 ± 0.53
Embryo weight (g)	24–31	0.17 ± 0.15	2.78 ± 0.23	21.0 ± 0.83	156 ± 4.73	399 ± 11.9	910 ± 31.1

Egg weight and measurements at setting were included as linear covariates in the analysis to account for differences in egg size.
-: No data.
n: Minimum and maximum numbers of records means were derived from in each cell in rows.

Table 2. Regression and correlation coefficients describing the linear regressions of embryo age on linear body measurements (embryo length, leg length and wing length).

Trait	Number of records	Intercept ± SE	Slope ± SE	Correlation coefficient
Embryo length (mm)	176	8.48 ± 0.41	0.120 ± 0.002	0.97
Leg length (mm)	146	13.11 ± 0.32	0.159 ± 0.003	0.98
Upper wing length (mm)	134	11.21 ± 0.61	0.649 ± 0.019	0.95
Lower wing length (mm)	134	10.88 ± 0.71	0.692 ± 0.023	0.93

Embryo age can be estimated by substituting actual on-farm body measurements in these equations. All regression coefficients were highly significant ($P < 0.001$).

vertically in poultry (Van de Ven *et al.*, 2011). Takeshita and Mcdaniel (1982), however, reported that early embryonic development of poultry embryos was improved in those eggs that were incubated horizontally. These results are not consistent with the present findings for ostriches but indicate scope for a more detailed study in future. It needs to be stated that the number of chicks hatched at the end of incubation was too low to detect moderate to small effects on the hatchability of fertile eggs set.

The mean embryo weight and mean embryo length in the present study at 14 d of incubation (1.16 g and 27.7 mm, respectively) corresponded with the results from Gefen and Ar (2001). The mean weight of 21 g after 21 d of incubation for ostrich embryos was also similar to that reported by Gefen and Ar (2001), as were lower wing lengths. Although observations on the development of the embryo up to 28 d of incubation matched results from Gefen and Ar (2001), the means for the beak (16.5 mm vs. 10.5 mm) and leg length (95.8 mm vs. 34.5 mm) in the present study are much higher. The difference in measurements can be contributed to both the larger sample size in the present study (19–30 samples vs. 2 samples) and the egg size. Egg weight plays an important role in the size and weight of the developing embryo, but unfortunately there is no mention in Gefen and Ar (2001) on the weights of the eggs used in their study. It, thus, is possible that eggs used in current study are larger (between 1405 and 1466 g). Gefen and Ar (2001) also reported an average embryo weight of 145 g at 28 d of incubation, which is comparable with the 156 g recorded for embryos in the present study at a similar age of incubation.

Gefen and Ar (2001) reported a mean embryo weight of 359 g ($n = 3$) at 34 d of incubation. The corresponding mean at 36 d was 439 g ($n = 2$). A mean of 399 g after 35 d of incubation in the present study was intermediate and thus in broad agreement with previous results. In contrast to embryo weights at earlier stages, the mean chick weight of 910 g after 42 d of incubation was substantially higher than the weight of 680 g at 40 d of incubation reported by Gefen and Ar (2001). Again the difference could be contributed to the bigger sample size, as well as the slightly lower incubation temperature (35.2 vs. 36.5). Higher incubation temperatures can contribute to a higher water loss percentage especially towards the end of incubation. Since pipping occurred from as early as 41 d of incubation in the current study, the hatchlings were classified as chicks on the 42nd d of the incubation period. It is conceded that embryo weight in the current study is based on a wet embryo, since several sources give the average day-old weight of Oudtshoorn ostrich chicks at 855–862 g (Bunter and Cloete, 2004; Cloete *et al.*, 2004, 2005).

The prediction of embryo age from linear body measurements

Commercial hatcheries may need a tool to allow them to predict the age of embryos from information obtained from dead-in-shell chicks. For such prediction, linear body measurements may be of utility, since measuring devices (ruler, tape measure and callipers) would be readily available and easy to use. Literature is limited on the use of body measurements in linear regression models for predicting the age of avian embryos. Browne (2006) identified three distinct developmental gaps in the kaki (*Himantopus novaezelandiae*) sequence to compare morphometric and photographic age sequences but found that, due to natural variation in size between individuals, more reference points were needed for more accurate predictions. The high correlation coefficients for predicting embryo age in the current study correspond well with the findings by Ar and Gefen (1998) and Gefen and Ar (2001). However, since we regressed embryo age on body measurements to allow the prediction of embryo age by on-farm measurements, it is not possible to compare the regression equations in this paper directly with those of Ar and Gefen (1998) and Gefen and Ar (2001). Kashmiri and Vatsalya (2012) also reported that the length of the beak and leg correlated highly with embryo weight in Japanese quail (*Coturnix japonica*) and was a useful tool in assessing incubation age. However, data from this study fitted simple linear regressions well, and it will be feasible to estimate embryo age. A study with more data points is required to account for variation between embryos and potentially for differences between genotypes.

Conclusions

The stages of development of the ostrich embryo in the present study were similar to the results reported by Gefen and Ar (2001). These results thus appear to be quite robust for ostrich eggs in general. Linear relationships stemming from readily obtained morphometric measurements can be used to identify the age of dead-in-shell embryos and consequently the stage or stages of incubation during which problems resulting in a low hatchability occurred.

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Disclosure statement

No potential conflict of interest was reported by the authors. AQ2

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