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Maternal effects of egg size on emu *Dromaius novaehollandiae* egg composition and hatchling phenotype

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Summary

Parental investment in eggs and, consequently, in offspring can profoundly influence the phenotype, survival and ultimately evolutionary fitness of an organism. Avian eggs are excellent model systems to examine maternal allocation of energy translated through egg size variation. We used the natural range in emu *Dromaius novaehollandiae* egg size, from 400 g to >700 g, to examine the influence of maternal investment in eggs on the morphology and physiology of hatchlings. Female emus provisioned larger eggs with a greater absolute amount of energy, nutrients and water in the yolk and albumen. Variation in maternal investment was reflected in differences in hatchling size, which increased isometrically with egg size. Egg size also influenced the physiology of developing emu embryos, such that late-term embryonic metabolic rate was positively correlated with egg size and embryos developing in larger eggs consumed more yolk during development. Large eggs produced hatchlings that were both heavier (yolk-free wet and dry mass) and structurally larger (tibiotarsus and culmen lengths) than hatchlings emerging from smaller eggs. As with many other precocial birds, larger hatchlings also contained more water, which was reflected in a greater blood volume. However, blood osmolality, hemoglobin content and hematocrit did not vary with hatchling mass. Emu maternal investment in offspring, measured by egg size and composition, is significantly correlated with the morphology and physiology of hatchlings and, in turn, may influence the success of these organisms during the first days of the juvenile stage.

Key words: emu, *Dromaius novaehollandiae*, egg, development, maternal effect, life history, allometry, scaling.

Introduction

Parental investment, particularly nutrients and energy allocated to eggs, can profoundly influence the development of embryos, the phenotypes and survival of hatchlings, and, therefore, evolutionary fitness of both offspring and parents. Parental investment in embryogenesis provides for the successful development of a zygote into a complete hatchling, and parental investment in care of the hatchling constitutes the energy and nutrients allocated to an egg beyond those needed to produce a hatchling and used by the hatchling to support growth and maintenance after emerging from the egg (Congdon, 1989). As phenotypes of oviparous mothers that affect phenotypes of their offspring, parental investment in offspring *via* eggs frequently has significant, and evolutionarily meaningful, maternal effects (Bernardo, 1996a,b). Reaching a full understanding of the magnitude of these maternal effects, and how they evolve, requires an examination of intraspecific variation in parental investment in eggs along with an examination of how embryos respond physiologically to the egg environments within which they develop.

The trajectory followed by an embryo from zygote to hatchling stages is influenced by an interaction between genetic instructions in the nuclei of the embryo's cells and conditions in the environment surrounding those cells. Conceptually similar to evolutionary paths blazed by populations of organisms through phenotypic space over several generations (Raup, 1966), developmental trajectories (Burggren, 1999) of oviparous amniotes can change as a result of biotic and abiotic factors encountered outside the eggshell and factors, initially maternal in origin, found within the eggs. Phenotypes of these embryos, developing toward hatching and toward metamorphosis into a more independent (e.g. self-feeding, thermoregulating and ambulatory) phase in their lives, are shaped both by genetic and environmental effects (Burggren, 1999). Acquiring in-depth knowledge of the sensitivity of developmental trajectories to environmental perturbations, including maternal investment of nutrients and energy in eggs, will improve our understanding of the genesis and importance of maternal effects manifested in phenotypes of hatchlings.

Requiring only heat and oxygen from the environment and containing all nutrients and water necessary to sustain developing embryos, avian eggs are attractive models for investigating effects of maternal investment on phenotypes of

embryos and hatchlings. Variation in the composition of avian eggs among species is correlated with functional maturity of hatchlings (Carey et al., 1980; Sotherland and Rahn, 1987). The quantity and composition of parental investment varies significantly within species and is frequently correlated with hatchling mass (Williams, 1994). Investigating how intraspecific variation in egg size and composition affects hatchling attributes can provide useful insights into the importance of maternal effects in oviparous amniotes.

In this study we examined consequences of natural variation in maternal investment – egg size and composition – on emu hatchling phenotypes. Emu eggs and hatchlings make good experimental subjects for a study of parental investment because they are large (egg mass approx. 600 g; hatchling mass approx. 400 g), facilitating measurements of hatchling characteristics (e.g. blood volume) that are otherwise difficult to quantify. In addition, intraspecific variation in egg mass from 400 g to >700 g provides a reasonably, but not unusual, wide range of egg size. Female emus lay between 5 and 20 eggs, typically incubated by the males during the breeding season. After emerging from their eggs the precocial hatchlings forage for food under guidance from the males (Davies, 1975). Thus, like other precocial birds (Williams, 1994; Hill, 1995), emus should produce eggs having component masses that vary isometrically with egg mass, as well as hatchlings, emerging from those eggs, that vary isometrically with egg mass. Therefore we tested the following hypotheses: (1) maternal investment, in the form of nutrients and water in eggs, is positively correlated with egg size and varies in such a way that the proportional composition of eggs remains constant; (2) morphological and physiological phenotypes of hatchlings correlate positively with egg size such that proportional composition of hatchlings remains constant regardless of hatchling size; (3) maternal investment in eggs provides for greater energy use in larger eggs during development while provisioning hatchlings with similar amounts of residual yolk regardless of hatchling size.

Materials and methods

Animals

Emu *Dromaius novaehollandiae* Latham eggs were randomly collected within 5 days after oviposition at the Cross Timbers Emu Ranch, Flower Mound, TX, USA from November 2000 to March 2001. At the time of egg collection, the female breeding population at Cross Timbers Emu Ranch was 45 female birds ranging in age from 3–7 years. Forty-nine emu eggs were used to determine egg composition, and 53 eggs were incubated to obtain measurements of hatchling phenotypes. Though we do not know the source of each egg, it is likely that more than one egg from some females was used in this study. All protocols used in this study were approved by the University of North Texas Animal Care and Use Committee.

Egg components

Fresh egg mass was determined by drilling two small holes through the shell over the air cell, filling the air cell with water, and then weighing the eggs on a Denver Instruments (Denver, CO, USA) digital balance. Short of weighing eggs immediately after oviposition, this is the most reliable method of obtaining fresh egg mass (Ar and Rahn, 1980). Fresh eggs were then separated into shell, yolk and albumen following the methods described in Finkler et al. (1998). The intact yolk was weighed with the balance to determine yolk mass. Yolk, albumen and shell were then dried to a constant mass in a drying oven at 60°C. Shell mass was measured by weighing the dry shell on the balance, and albumen wet mass was determined by subtracting yolk wet mass and shell dry mass from the mass of the egg. Water contents of yolk and albumen were determined by subtracting dry mass of each from the respective wet mass; the sum of water mass in the yolk and water mass in the albumen yielded total water content of each egg. Mass of egg solids was computed by adding yolk and albumen dry masses.

Incubation

Eggs were stored at 4°C for no more than 7 days before incubation. Eggs were incubated in forced draft incubators with automatic rotation at Cross Timbers Emu Ranch until approximately day 40 of incubation. They were then transferred to the University of North Texas, where incubation continued until hatching in forced draft emu incubators (GQF Manufacturers, Savannah, GA, USA). Eggs were incubated at 36.5±1°C and a relative humidity of approximately 30%, corresponding to the relative humidity experienced in the nest. Prior to internal pipping, all eggs were transferred to a hatching incubator maintained at 36.5°C and a relative humidity of 35–40%.

Gas exchange of near-term embryos

Metabolic rates ($\dot{V}_{O_2}$) of 15 eggs were measured on day 46 of incubation (i.e. 92% of incubation) using a flow-through system similar to the methods of Dzialowski et al. (2002). Eggs were placed in individual PVC respirometers (approx. vol. 1 l) and then into a constant temperature chamber regulated at 37.5°C. Air was pumped through the individual chambers and flow was measured at the inflow side of the chambers using a calibrated Brooks (Hatfield, PA, USA) flow meter. Outflow O_2 concentration from each respirometer was measured using a Beckman OM11 O_2 analyzer (Anaheim, CA, USA). Inflow O_2 concentration to the respirometers was determined from the outflow of an empty respirometer. Metabolic rate (i.e. rate of oxygen consumption) was calculated using the equation of Hill (1972), corrected to STPD and expressed in units of ml O_2 h^{-1}.

Air cell P_{O_2} was measured in eight emu eggs on day 46 of incubation. On day 40 of incubation a 5 mm diameter hole was drilled in the air-cell end of each egg using a drill press. A square patch of 0.4 mm thick Thera-band™ latex was glued over the hole using Duro Quick Gel™ and the egg was replaced into the incubator for 6 days. Using a 1 ml syringe and a 27-gauge needle inserted through the latex, a 1 ml sample of gas was withdrawn from the air cell and then promptly

analyzed for P_{O_2} using a Cameron Instruments (Port Aransas, TX, USA) BGM2000 blood gas meter.

Eggshell conductance

We measured water vapor conductance (G_{H_2O}) of fresh eggs of mass 487–778 g (N=16). Eggs were initially weighed and then placed in individual desiccators (approx. vol. 6 l). Each desiccator contained an ample amount of Drierite™ desiccant in the bottom of the desiccator to ensure that water vapor pressure around each egg was near 0 kPa. The mass of each egg, desiccator temperature and atmospheric pressure were measured daily for 5 days. Whole eggshell G_{H_2O} was determined following the protocol of Ar et al. (1974). Finally, initial egg mass was measured as above by filling the air cell with water and then weighing the egg.

Hatchling morphology and composition

All measurements of morphology and composition were made on hatchlings that were less than 1 day old. Hatchlings were euthanized by exposure to either halothane or iso-flurane, and then weighed to the nearest 0.1 g to obtain hatchling mass (yolk-free hatchling mass plus residual yolk and yolk sac). The yolk sac was carefully dissected from each hatchling and weighed to measure the quantity of residual yolk; yolk-free hatchling mass was determined by subtracting residual yolk mass from hatchling mass. Culmen length and right tibiotarsus length were measured to the nearest 0.1 mm on each hatchling using digital calipers (Mitutoyo, Aurora, IL, USA) as a means of quantifying hatchling structural size. Heart, gizzard and liver were dissected from the body, weighed separately, and then dried to a constant mass in an oven at 60°C. The yolk sac and what remained of the hatchling were dried to a constant mass in a similar way. Water contents of the various components were determined by subtracting dry mass of each from the respective wet mass. Mass of yolk-free hatchling solids was computed by adding heart, gizzard and liver dry masses to the dry mass of the dissected carcass. We estimated the quantity of yolk consumed by an embryo during incubation by subtracting the measured dry yolk sac mass from the calculated mass of dry yolk that the egg from which a neonate hatched would have contained at the outset of incubation, using initial egg mass and the equation for dry yolk mass in Fig. 1.

Hematology and blood volume

To obtain blood for hematological measurements, hatchlings were anesthetized using halothane and blood was taken from the heart by direct cardiopuncture. Hemoglobin was measured with a Radiometer (Brønshøj, Denmark) OSM2 Hemoximeter. Hematocrit was measured by centrifuging blood in heparinized capillary tubes. Osmolality of the blood was measured using a Wescor (Logan, UT, USA) 5500 vapor pressure osmometer. Two measurements of each variable were made and averaged for each animal.

Blood volumes were measured in 11 hatchlings using the Evan's Blue dilution technique (El-Sayed et al., 1995).

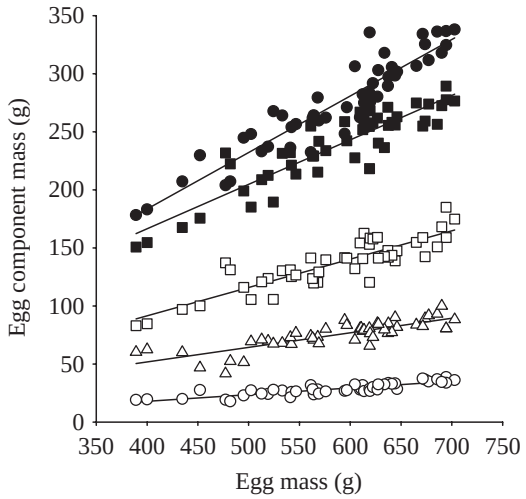

Fig. 1. Mass of emu egg components increase with fresh egg mass (M_e). Filled circles, albumen mass (M_a=0.49M_e–11.1; r^2=0.87); open circles, albumen dry mass (M_{ad}=0.06M_e–5.1; r^2=0.75); filled squares, yolk mass (M_y=0.48M_e+13.4; r^2=0.82); open squares, yolk dry mass (M_{yd}=0.24M_e–6.2; r^2=0.76); triangles, shell mass (M_s=0.13M_e+1.5; r^2=0.68).

Hatchlings were anesthetized with iso-flurane and attached to a ventilator that maintained an iso-flurane concentration of 1% in the inspired air. Both the right and left jugular veins were exposed and non-occlusively canulated with tips of 26-gauge needles attached to PE50 tubing. The right jugular vein was used as the injection site for the Evan's Blue solution, and the left jugular vein was used to withdraw subsequent blood samples. Initially, 500 μl of blood was withdrawn into a heparinized syringe from the right jugular vein. This was followed by an injection of 400 μl of an Evan's Blue solution (5 mg ml^{-1} dissolved in 0.9% heparinized saline) into the right jugular vein. The Evan's Blue injection was followed by a 200 μl injection of heparinized saline to wash the tubing. Samples of blood were then taken from the left jugular vein at 10, 15 and 20 min after the initial injection of Evan's Blue.

After each blood sample was collected, a portion of blood from the sample was added to an equal amount of heprainized saline and centrifuged for 15 min. All volumes were gravimetrically determined using a Denver Instruments digital balance to increase measurement accuracy. A 200 μl sample of the supernatant was added to 800 μl of heprainized saline and the absorbance was measured at 610 nm using a Bausch and Lomb (Rochester, NY, USA) Spectronic 88 spectrophotometer. A subsample of plasma from the initial blood sample, taken before injection of Evan's Blue, was used to create a blank for zeroing the spectrophotometer for each hatchling's measurement.

A standard curve (r^2=0.93) relating absorbance to Evan's Blue concentration was generated using plasma from four additional hatchlings. Blood volumes were calculated from the measured Evan's Blue concentrations according to the methods in El-Sayed et al. (1995).

Statistical analyses

Linear regressions of parameters on egg mass and yolk-free hatchling mass were carried out using SPSS 11.0. Additionally, log–log regressions were performed on data to determine if component masses varied in simple linear proportion to body mass (slope of log–log regression, b=1.0) or if component masses showed a positive (b>1.0) or negative (b<1.0) allometry with egg mass or hatchling mass. The regressions were considered to vary in simple proportion to body mass if the 95% confidence interval of the slope of the log–log regression included 1. In order for the log–log relationship to hold true the intercept of the untransformed data must pass through the origin. Prior to log–log transformation all relationships were examined using non-linear power fits ($y=y_0+ax^b$; Sigmaplot 8.02) to the untransformed data. The 50% confidence interval for the intercept (y_0) was used to determine if it differed significantly from zero. Log–log regressions were carried out on data when the intercept was not significantly different from zero. A significance level of P<0.05 was adopted for all regressions. Linear regression equations are provided in the figure legends and when determined allometric slopes are provided in the text. All values are presented as means ± S.D. except for the slopes of the log–log regressions, which are presented as the slope (b) ±95% confidence interval.

Results

Egg composition

The mass of each egg component increased significantly as egg mass (586.17±78.06 g; N=49) increased from approximately 400 g to 700 g (Fig. 1). Albumen wet mass (274.00±40.75 g; N=49) increased significantly ($F_{1,47}$=309; P<0.001) with egg mass as did albumen dry mass (28.43±5.21 g; N=47; $F_{1,45}$=135; P<0.001). Yolk wet mass (237.66±33.07 g; N=49) increased significantly ($F_{1,47}$=208; P<0.001) with egg mass as did yolk dry mass (136.36±21.93 g; N=47; $F_{1,45}$=146; P<0.001). Like the other two major components of fresh eggs, shell mass (75.12±11.90 g; N=49) increased significantly ($F_{1,47}$=99; P<0.001) with egg mass.

The relative contribution of albumen and yolk to the eggs did not vary with initial egg mass. Slopes (b) of the log–log regressions of log albumen wet mass (b=1.04±0.13; r^2=0.87), log dry albumen mass (b=1.22±0.22; r^2=0.75), log yolk wet mass (b=0.93±0.14; r^2=0.82), and log dry yolk mass (b=1.05±0.18; r^2=0.76) against log initial egg mass were not significantly different from 1. As a result, fraction of yolk in the contents (0.47±0.03), typically correlated with developmental maturity of hatchlings (Sotherland and Rahn 1987), did not change ($F_{1,47}$=0.49; P=0.49) with egg mass.

Water and solid content of eggs increased with egg mass (Fig. 2), but the fraction of water and solids did not vary significantly over the range of egg masses examined. Water in eggs (343.02±46.21 g; N=45) increased significantly ($F_{1,43}$=648; P<0.001) with egg mass, as did the solid content of eggs (163.97±25.84 g; N=45; $F_{1,43}$=245; P<0.001).

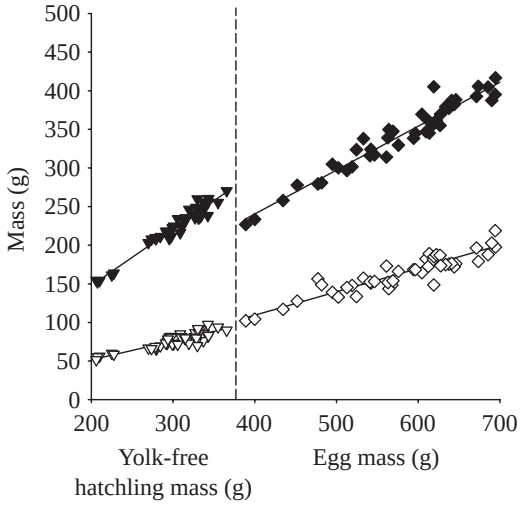

Fig. 2. Mass of water and solids in yolk-free hatchlings and eggs increase with yolk-free hatchling mass (M_{yfh}) and with fresh egg mass (M_e). Filled inverted triangles, yolk-free hatchling water content (M_{yfhw}=0.7M_{yfh}+2.2; r^2=0.95); filled diamonds, egg water content (M_{ew}=0.57M_e+13.4; r^2=0.94). Open inverted triangles, yolk-free hatchling solids (M_{yfhs}=0.25M_{yfh}+1.0; r^2=0.76); open diamonds, egg solids (M_{es}=0.3M_e–11.6; r^2=0.85).

Approximately 71% of water in eggs was found in the albumen (244.56±36.70 g; N=47), which was composed of an invariant fraction of water (0.90±0.01; $F_{1,45}$=1.8; P=0.19). Similarly, neither the fraction of water in the yolk (0.42±0.03; N=47) nor the overall fraction of water in the eggs (0.68±0.02; N=45) changed significantly with egg mass. However, the total amount of water in the yolk (99.93±13.84 g; N=47) increased significantly ($F_{1,45}$=66; P<0.001) with egg mass as did the total amount of water in the albumen ($F_{1,45}$=265; P<0.001).

Gas exchange of near-term embryos

Metabolic rate (107.8±11.6 ml O_2 h^{-1}; N=15) of pre-pip embryos, measured on day 46, was positively correlated with initial egg mass M_e ($F_{1,13}$=7.7; P=0.016; $\dot{V}_{O_2}$=0.11M_e+33.9; r^2=0.37) and with the yolk-free mass of the hatchlings M_h when they emerged from the same eggs ($F_{1,13}$=14; P=0.003; $\dot{V}_{O_2}$=0.20M_h+48.8; r^2=0.51). Metabolic rate scaled with a negative allometry with egg mass (b=0.56±0.41) and yolk-free hatchling mass (b=0.50±0.25). In a separate set of eggs, eggshell water vapor conductance (447.4±76.7 mg kPa^{-1} day^{-1}; N=14; G_{H_2O}=0.09M_e+4.5; r^2=0.51) increased significantly ($F_{1,13}$=9.4; P=0.01) with initial egg mass. In contrast, pre-pip air cell P_{O_2} (15.0±0.8 kPa; N=8) did not vary significantly with egg mass ($F_{1,6}$=2.4; P=0.17) (not shown).

Hatchling morphology and composition

Hatchling mass (yolk-free hatchling plus residual yolk) increased with egg mass (Fig. 3). Hatchling mass (403.59±45.67 g; N=48) increased significantly ($F_{1,46}$=214; P<0.001) with egg mass, as did yolk-free hatchling mass

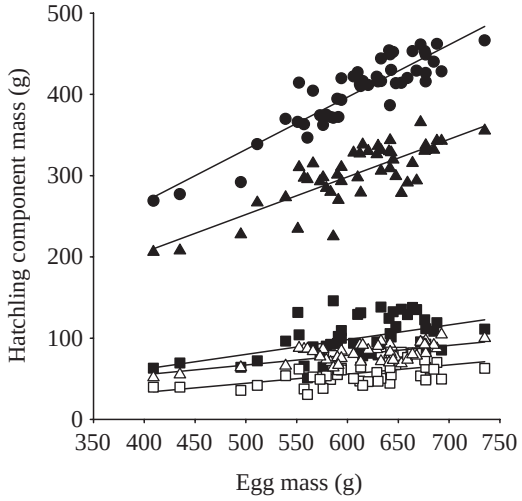

Fig. 3. Mass of emu hatchling components increase with fresh egg mass (M_e). Filled circles, hatchling (yolk-free hatchling + residual yolk) mass (M_h=0.64M_e+9.6; r^2=0.82); filled triangles, yolk-free hatchling mass (M_{yfh}=0.46M_e+20.5; r^2=0.64); open triangles, yolk-free hatchling dry mass (M_{yfhd}=0.12M_e+6.7; r^2=0.43); filled squares, residual yolk mass (M_{ry}=0.18M_e–10.9; r^2=0.24); open squares, residual yolk dry mass (M_{ryd}=0.11M_e–12.0; r^2=0.28).

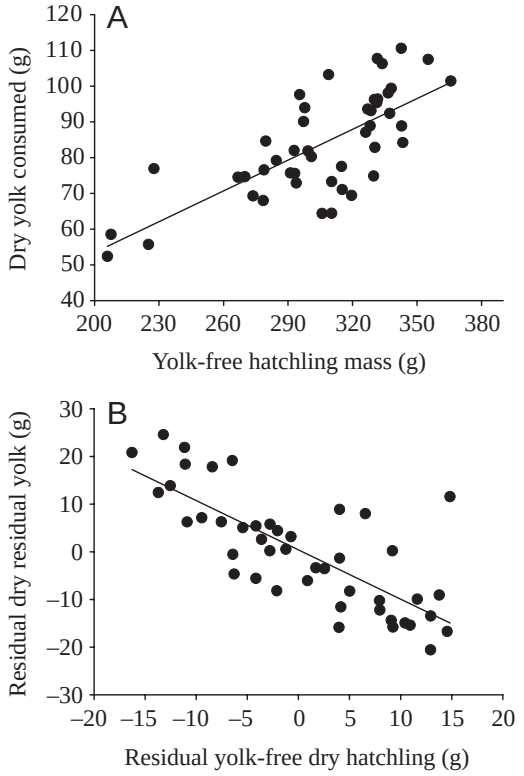

Fig. 4. (A) Mass of dry yolk solids consumed (predicted initial egg yolk solids – measured residual yolk solids) by embryos during development increases with yolk-free hatchling mass (M_{yc}=0.29M_{yfh}–3.94; r^2=0.52). (B) Mass of residual yolk solids *rM* decreased as mass of yolk-free hatchling increased irrespective of initial egg mass (rM_{ryd}=–1.03rM_{yfhd}+0.41; r^2=0.58). Statistical residuals from the regression of the mass of residual yolk solids and the mass of yolk-free hatchling on initial egg mass were obtained from regression equations in Figs 1 and 3 and plotted against each other, revealing a trade-off between retaining residual yolk and producing a hatchling.

(303.47±37.06 g; N=48; $F_{1,46}$=83.6; P<0.001) and residual yolk mass (100.13±23.96 g; N=48; $F_{1,46}$=14.3; P=0.001). Dry mass of yolk-free hatchling (80.76±11.73 g; N=45) and dry mass of residual yolk (57.13±13.93 g; N=46) also increased significantly ($F_{1,43}$=32.8; P<0.001 and $F_{1,44}$=17; P<0.001 respectively) with egg mass. Hatchling mass increased in simple linear proportion to egg mass. The slopes (b) of the log–log regressions of log yolk-free hatchling wet mass (b=0.96±0.20; r^2=0.66) and log yolk-free hatchling dry mass (b=0.94±0.29; r^2=0.47) against log initial egg mass were not significantly different from 1.

Large hatchlings were composed of more water and solids than were small hatchlings (Fig. 2), but the fraction of water in hatchlings remained unchanged regardless of hatchling size. Mass of water in yolk-free hatchlings (225.20±27.2 g; N=44) increased significantly ($F_{1,43}$=747.2; P<0.001) with mass of yolk-free hatchlings, but the fraction of water in those hatchlings (0.74±0.02; N=44) did not vary significantly ($F_{1,43}$=0.05; P=0.82) with hatchling mass. Mass of solids in hatchlings (i.e. dry mass of yolk-free hatchling) also increased significantly ($F_{1,43}$=120; P<0.001) with hatchling mass.

Yolk consumed by developing embryos, i.e. difference between mass of the dry yolk (estimated using measured initial egg mass and the equation for dry yolk mass provided in Fig. 1) and measured mass of residual yolk remaining in the yolk-sac (83.64±14.44; N=46) increased significantly ($F_{1,44}$=48.4; P<0.001) with yolk-free hatchling mass (Fig. 4A). The combination of initial yolk mass increasing with egg mass and yolk consumed increasing with yolk-free hatchling mass yielded a constant residual yolk mass across all hatchling masses (mean dry residual yolk 57.8±14.6). However, the statistical residuals from regressions of yolk-free hatchling dry mass and residual yolk dry mass on egg mass revealed that, independent of initial egg mass, larger hatchlings had less residual yolk upon hatching than smaller hatchlings (Fig. 4B).

Linear dimensions of heavier hatchlings were greater than those of lighter hatchlings (Fig. 5). Length of both the right tibiotarsus (70.44±3.79 mm; N=48; $F_{1,46}$=107.12; P<0.001) and culmen (36.78±1.90 mm; N=48; $F_{1,46}$=9.6; P=0.003) increased significantly with yolk-free hatchling mass.

Wet and dry masses of heart, liver, and gizzard all increased significantly with yolk-free hatchling mass (Table 1).

Hematology of hatchlings

Blood volume (27.8±7.0 ml; N=11), which constituted an essentially constant proportion (approximately 9.2%) of the yolk-free hatchling mass, increased significantly ($F_{1,9}$=12; P=0.007) with yolk-free hatchling mass (Fig. 6). The increase

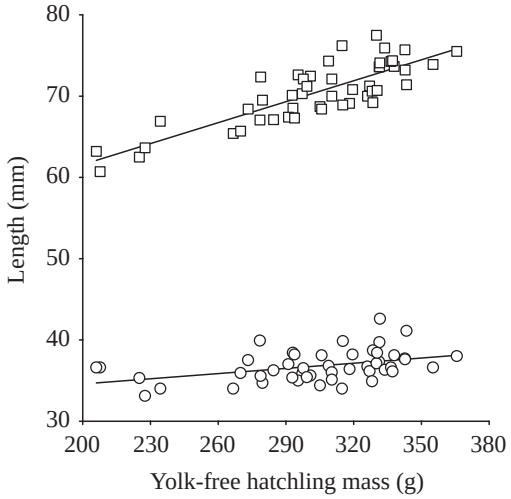

Fig. 5. Linear dimensions of hatchling emus increase with yolk-free hatchling mass (M_{yfh}). Squares, right tibiotarsus length (L_t=0.09M_{yfh}+44.4; r^2=0.70); circles, culmen length (L_c=0.02M_{yfh}+30.3; r^2=0.17).

in blood volume was proportional to the increase in yolk-free hatchling mass (b=1.05±0.46; r^2=0.69). However, none of the other blood parameters measured [osmolality (304.6±15.6 mOsm kg^{-1}; N=44), hematocrit (38.4±4.1%; N=45), or hemoglobin concentration (12.9±1.8 g%; N=44)] varied significantly with yolk-free hatchling mass (Fig. 6).

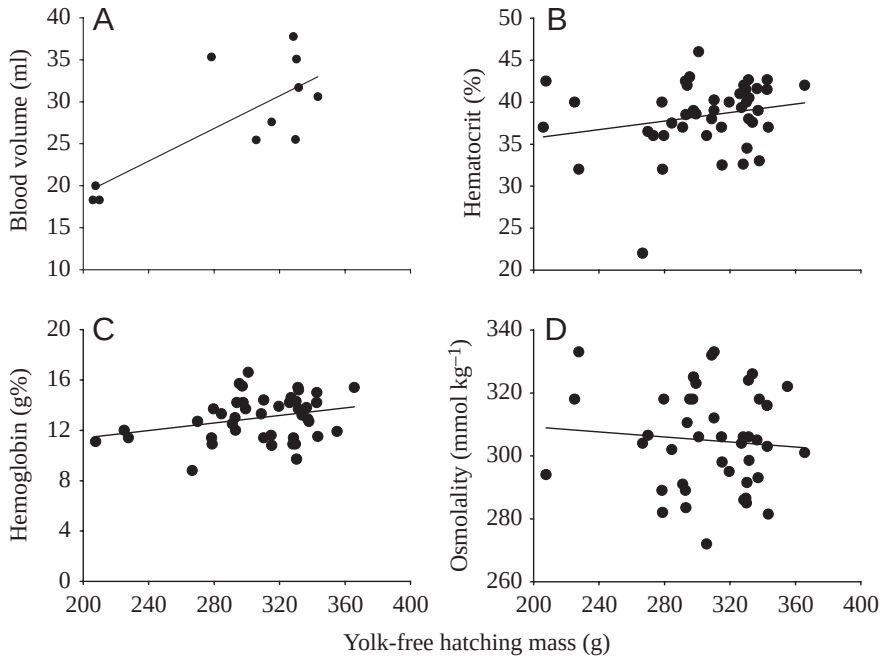

Fig. 6. Blood volume, blood osmolality, hematocrit and hemoglobin content plotted as a function of yolk-free hatchling mass (M_{yfh}). (A) Hatchling blood volume (V_b=0.09M_{yfh}+0.43; r^2=0.57), (B) hematocrit (Hct=0.02M_{yfh}+29.2; r^2=0.05), (C) hemoglobin (Hb=0.006M_{yfh}+9.4; r^2=0.03) and (D) blood osmolality (Osm=–0.03M_{yfh}+325.2; r^2=0.02).

Discussion

Consequences of parental investment (i.e. investment of energy and nutrients; Congdon, 1989) in reproduction, frequently manifested as maternal effects, can be observed as significant variation in the physiology, morphology and life history of organisms (Bernardo, 1996a,b). Female birds vary reproductive investment by allocating different amounts of albumen and yolk to the eggs they produce or by producing eggs of different size. Maternal investment in emu eggs, the third largest egg laid by extant birds, varied considerably (Fig. 1), with eggs differing in mass by as much as 300 g. This variation was reflected in concomitant variation in hatchling size and composition.

Yolk and albumen in eggs

One measure of parental investment in bird eggs typically is expressed as the fraction of yolk in the contents (FYC) of eggs. Emus in this study laid eggs containing nearly 50% yolk (FYC=0.47), which is within the range of yolk content for precocial birds but larger than that predicted for precocial eggs of the same mass. Sotherland and Rahn (1987) examined the relationship between egg mass and energy content for a wide variety of birds and found that FYC for precocial species ranges from 0.32 to 0.69. Based on the equation for yolk content in precocial species (Sotherland and Rahn, 1987), we predicted the FYC for an average sized emu egg (512 g wet contents) to be 0.39, which is less than the FYC of emu eggs measured in this study. Thus, female emus provision their eggs with relatively more yolk and less albumen than would be predicted for a typical large precocial egg. If we compare emu eggs with those of closely related species, emu eggs tend to have a larger FYC than either the ostrich *Struthio camelus* (1.2 kg egg, FYC=0.38; Romanoff and Romanoff, 1949) or cassowary *Casuarius casuarius* (546 g egg, FYC=0.42; Carey et al., 1980). This finding is not surprising, however, because the incubation period of the emu is longer than that of the ostrich, suggesting that emu embryos require more energy than ostrich embryos to complete incubation. In contrast, emu eggs have a lower FYC than the smaller kiwi eggs (*Apteryx australis*; 440 g egg, FYC=0.61; Reid, 1971; Calder et al., 1978), which has an incubation period about 25 days longer than the emu.

Another related comparison among species entails examining the contribution of albumen and, therefore, water (albumen in all bird eggs is about 90% water; Sotherland and Rahn, 1987) to avian egg contents. We suggest here that always focusing on yolk and FYC diverts attention from albumen and its important

Table 1. *Hatchling organ masses (g) and their regressions on yolk-free hatchling mass*

Component	Mean	S.D.	Slope	Intercept	*F*	*P*	r^2	*N*
Heart								
Wet	2.16	0.56	0.008	–0.386	20.33	<0.001	0.29	52
Dry	0.38	0.11	0.001	–0.04	12.6	<0.001	0.22	46
Liver								
Wet	8.30	1.65	0.024	0.97	18.9	<0.001	0.28	52
Dry	3.39	0.65	0.010	0.44	18.56	<0.001	0.30	46
Gizzard								
Wet	5.63	1.07	0.014	1.51	15.05	<0.001	0.23	52
Dry	1.05	0.29	0.003	0.30	4.7	0.036	0.10	46

contributions to embryo development and hatchling phenotype. The fraction of albumen in the contents (FAC=1–FYC) of eggs is very high (about 80%) in altricial species and drops to less than 50% in more precocial species. Emu egg contents are about half albumen (FAC=0.53), and at the middle of the range observed in ratites, where FAC varies between 0.4 (kiwi) to 0.6 (ostrich).

Scaling of egg composition

Maternal investment in avian eggs varies both interspecifically and intraspecifically in two ways. First, the absolute size of eggs and egg contents can vary among and within species. Second, the relative contribution of yolk, albumen and shell to the mass of an egg can vary with egg size and with maturity of neonate at hatching.

Intraspecifically, emu eggs exhibit isometric scaling between egg size and all egg components. Large eggs contained more yolk and albumen (Fig. 1) as well as water and solids (Fig. 2) than small eggs, but yolk and albumen mass increased isometrically with egg size; the slope of log–log regressions of these components on egg mass did not differ significantly from 1. Thus, emu eggs in this study followed the precocial pattern (Williams, 1994) where eggs of all sizes had the same relative amount of yolk and albumen.

A number of studies have examined intraspecific variation of egg composition and have revealed patterns of how yolk and albumen content vary with egg size along the altricial–precocial continuum (Sotherland et al., 1990; Williams, 1994; Hill, 1995; Carey, 1996). For most species of birds, the vast majority of which are altricial, variation in albumen mass accounts for most of the variation in egg mass, but yolk contributes more to variation in egg mass as FYC increases toward the precocial end of the altricial–precocial continuum (Sotherland et al., 1990). Williams (1994) reviewed 22 studies that had examined intraspecific variation in egg components and found that only half of these studies revealed an isometric relationship between egg size and either yolk or albumen content. Hill (1995) found that wet albumen mass and wet yolk mass tended to scale isometrically with egg mass in precocial species, whereas in altricial species albumen showed positive allometry (b>1.0) and yolk showed negative allometry (b<1.0). Thus, it seems that altricial species change egg size by increasing the amount of albumen while keeping yolk content relatively constant, whereas precocial species tend to alter egg size by increasing both yolk and albumen content with an increase in egg mass. Further support for this pattern has been observed in precocial wood ducks *Aix sponsa* (Kennamer et al., 1997) and ruddy ducks *Oxyura jamaicensis* (Pelayo and Clark, 2002), which lay eggs having yolk and albumen varying isometrically with egg mass, and in altricial great tit *Parus major* eggs, in which much of the variation in egg mass is attributable to variation in albumen mass (Lessells et al., 2002).

Egg size and hatchling size

The developing emu embryo may partition the maternal investment of energy and nutrients into growth and maintenance of the developing body or into residual yolk. The energy and nutrients invested in an egg by a female that the embryo uses for growth and maintenance are parental investment in embryogenesis, whereas energy and nutrients left as residual yolk or hatchling fat deposits comprise parental investment in care of the hatchling (Congdon, 1989). Increased parental investment in larger emu eggs (Fig. 1) yielded larger hatchlings (Fig. 3) that tended to contain similar amounts of residual yolk as smaller hatchlings due to the fact that the larger hatchlings consumed more of their yolk during incubation (Fig. 4). Increased hatchling size is attributable to increased total water content (Fig. 2), increased dry mass (Fig. 2), and increased structural size as measured by the tibiotarsus and culmen lengths (Fig. 5). Heart, liver and gizzard masses were also larger in hatchlings from large eggs (Table 1). Thus, the increased maternal investment was used by the developing embryo for embryogenesis to yield a larger hatchling that had the same level of post-hatching care in the form of residual yolk, suggesting that egg size can be equated with egg quality in emus.

In contrast with our findings here, a review of the literature by Williams (1994) found that larger bird eggs produce heavier hatchlings, but not necessarily structurally larger hatchlings. However, many of the studies of the relationship between mass and structural size in hatchlings examined only hatchling mass including residual yolk and concluded that hatchlings from larger eggs were heavier because they contained more residual

yolk and not because they were structurally larger (Williams, 1994). Ankney (1980) found a significant positive relationship between egg size and length of the tarsus and culmen of lesser snow goose *Anser caerulescens* hatchlings, but the relationship between hatchling size and linear dimensions was not reported. Larger eggs laid by the thick-billed murre produced heavier hatchlings, but this was due mainly to increased water content or residual yolk and not due to increased linear dimensions (Birkhead and Nettleship, 1982). In a number of alcid species, most of the variance in hatchling mass in relation to pipped egg mass was attributed to differences in residual yolk rather than increased water content or dry hatchling mass (Birkhead and Nettleship, 1984). In the king eider *Somateria spectabilis*, large eggs produced larger hatchlings with larger wet and dry breast and leg muscle mass than the hatchlings produced from small eggs (Anderson and Alisauskas, 2002). However, the relative structural size of larger king eider hatchlings was less than that of small hatchlings. In the altricial blackbird *Turdus merula*, large eggs produced both heavier and larger hatchlings (Magrath, 1992).

Emu hatchlings had a large residual yolk, which was positively correlated with initial egg mass (Fig. 3) but not yolk-free hatching mass. Female emus provisioned eggs with enough yolk to support development and maintenance of embryos and to provide sufficient residual yolk to support activity and survival after hatching. By factoring out the effect of egg mass on both residual yolk dry mass and yolk-free hatchling dry mass (Fig. 4B) we found that larger-than-average hatchlings, at any egg mass, have less residual yolk than smaller-than-average hatchlings. Therefore, there appears to be a trade-off for the developing embryo: produce more tissue and hatch with less residual yolk or hatch smaller with more residual yolk.

In general, emu hatchlings have more residual yolk, as a fraction of the whole hatchling, than many of the other avian species studied (Vleck and Vleck, 1996). Whereas precocial hatchlings retain residual yolk of 0.15–0.18 of their wet mass and 0.28 of their dry mass (Carey, 1996; Vleck and Vleck, 1996), emu hatchlings in our study had residual yolk amounting to 0.25 of their wet mass and 0.42 of their dry mass. There is also sizeable residual yolk in the ostrich, accounting for 0.29 of wet mass and 0.56 of dry mass (Gefen and Ar, 2001). The ostrich and emu are both ratites, suggesting that members of this clade have noticeably high parental investment in hatchling care and residual yolk.

Though we did not examine survivorship consequences of the levels of residual yolk measured here, it is plausible that the large amount of residual yolk we measured would influence early growth and survival of these hatchlings because adult emus do not feed the young (Davies, 1975). Parental investment in hatchlings *via* yolk can provide hatchlings with energy used to grow and sufficient residual yolk (i.e. parental investment in care), which serves as a post-hatching source of nutrients and energy that can affect survivorship, especially during times of nutritional stress. A number of studies of precocial species (Kear, 1965; Ankney, 1980; Peach and Thomas, 1986; Thomas et al., 1988; Slattery and Alisauskas, 1995; Visser and Ricklefs, 1995; Dawson and Clark, 1996; Nager et al., 2000; Anderson and Alisauskas, 2001) have shown that an increase in residual yolk, correlated with increased egg size, results in increased hatchling survival under limited food conditions.

Water relations and hatchling mass

Water loss from avian eggs during incubation and metabolic water production by the embryos occur at rates that cause the hydration of egg contents at the end of incubation to be similar to that at the beginning of incubation (Ar and Rahn, 1980); these coincident rates also cause hatchlings and the eggs from which they emerge to have similar water contents (Sotherland and Rahn, 1987). Emu eggs contained on average 68% water, which comprised 74% of the hatchlings they produced (Fig. 2). Both of these values are in close agreement with the water content of precocial eggs and hatchlings (Sotherland and Rahn, 1987). There was an isometric increase in the water content of both the egg and the yolk-free hatchling with an increase in initial egg mass and yolk-free hatchling mass (Fig. 2); a similar relationship was observed in the dry mass of eggs and yolk-free hatchling solids (Fig. 2). Japanese quail *Coturnix coturnix* hatchling water content scales isometrically with egg size, but the proportion of water in laughing gull *Larus atricilla* chicks increases with a positive allometry such that larger hatchlings are composed of more water (Ricklefs et al., 1978).

The quantity of water in avian eggs, found mainly in the albumen, has a significant influence on the mass of developing embryos and hatchlings. Variation in emu hatchling mass is attributable in part to variation in mass of water in yolk-free hatchlings (Fig. 2). Studies examining the effects of water loss from eggs during incubation have shown that differences in wet embryo mass tend to be correlated with water content of the embryo and that eggs losing the most water tend to produce embryos with the lowest mass (Davis et al., 1988; Tullett and Burton, 1982). Removing albumen from chicken *Gallus gallus* eggs caused a reduction in hatchling size (Hill, 1993; Finkler et al., 1998) and resulted in hatchlings with a reduced yolk-free wet body mass (Finkler et al., 1998). Though hatchlings emerging from eggs from which albumen had been removed were smaller (i.e. length of the tibiotarsus was shorter), much of the decrease in wet body mass was attributed to the presence of less water in the smaller hatchlings. The dry yolk-free body mass of hatchlings from control eggs was not different from that of hatchlings emerging from eggs from which albumen had been removed (Finkler et al., 1998). Thus, water availability in eggs may be one of the main determinates of yolk-free hatchling mass in precocial species. A similar relationship between water content and hatchling mass has been observed in turtle eggs, where increased levels of water in eggs result in increased hatchling and organ sizes (Packard, 1999; Packard et al., 1987, 2000; Packard and Packard, 2001).

Finkler et al. (1998) postulated that some of the observed variation in body mass, correlated with variation in water mass,

might be accounted for by variation in extracellular liquid volume, including blood volume. Blood volume of emu hatchlings increased isometrically with hatchling size (Fig. 6), but this increase in blood volume was not accompanied by variation in other hematological parameters (Fig. 6). Thus, a portion of the water found in larger emu hatchlings appears in a larger volume of blood

Metabolic rate, eggshell conductance, and air cell P_{O_2}

Maximum metabolic rates of bird embryos and their eggshell conductance are typically matched in such a way that levels of respiratory gases in the air cell vary over an amazingly narrow range at the end of incubation, regardless of egg size, length of incubation, or degree of hatchling maturity (Rahn and Paganelli, 1990). Metabolic rates of developing emu embryos reported here, which reach a plateau about 8 days prior to hatching (Vleck et al., 1980), agree with those reported previously by Beutel et al. (1983) and Vleck et al. (1980), and were significantly correlated with initial egg mass and yolk-free hatchling mass. Larger eggs produced larger hatchlings, and, not surprisingly, near-term embryos from larger eggs had greater overall metabolic rates than those from smaller eggs. Because metabolic rate of emu embryos and water vapor conductance of the eggs in which they developed covaried with egg mass, P_{O_2} in air cells of emu eggs did not vary with egg mass and were in close agreement with values calculated by Vleck et al. (1980). Using Fick's law of diffusion and our measurements of shell gas conductance and metabolic rate, we calculated that air cell P_{O_2} should have averaged about 14.3 kPa, which is less than 5% different from the values measured.

Consequences of egg size variation

Emu egg size influenced the morphological and physiological phenotypes of the resulting hatchlings. To summarize the consequences of emu egg size variation we used the regressions from the results and Figs 1–4 to predict a number of parameters for a small emu egg (450 g) and a 44% larger emu egg (650 g; Table 2). In support of our hypotheses, hatchling phenotypic characters measured here were 38–51% larger in hatchlings from the larger egg and scaled proportionally with egg size. Using the energy content of dry solids in eggs (29 kJ g^{-1}; Sotherland and Rahn, 1987), we predict that a female emu would invest 3596 kJ in a 450 g egg, whereas a 650 g egg would contain 48% more energy (5336 kJ). If mass-specific costs of producing eggs were the same for eggs of all sizes within a species, then a female ovipositing a 650 g egg would allocate nearly 50% more energy per egg than a female ovipositing a 450 g egg. We hypothesized that increased maternal investment in the form of increased yolk would result in larger hatchlings. Embryos in larger eggs received more parental investment in embryogenesis, which allowed them to consume more yolk solids and grow larger during incubation but have sufficient yolk reserves to support them as hatchlings (Table 2).

Table 2. *Predicted egg and hatchling components from 450 g and 650 g emu eggs*

	Egg mass (g)		% increase
Parameter	450	650	44
Wet yolk in egg (g)	184	260	41
Dry yolk in egg (g)	102	150	47
Wet albumen in egg (g)	209	307	47
Dry albumen in egg (g)	22	34	55
Water in egg (g)	270	384	42
Energy in egg (kJ)	3596	5336	48
Water-vapor conductance (mg day^{-1} $Torr^{-1}$)	45	63	40
Air cell P_{O_2} (kPa)	14.4	15.3	6
MR near-term embryo (ml O_2 h^{-1})	83	105	26
Wet hatchling (g)	298	426	43
Yolk-free hatchling wet (g)	227	317	40
Yolk-free hatchling solids (g)	64	88	38
Water in yolk-free hatchling (g)	163	229	40
Yolk solids consumed (g)	63	95	51

Parameters that did not scale isometrically with egg mass were near term-embryo metabolic rate and air cell P_{O_2} (Table 2). Larger eggs had higher metabolic rates, but metabolism did not increase to the same extent with increases in egg mass as with hatchling body mass, suggesting that embryos in larger eggs may have responded more to limitations imposed by a relatively low eggshell conductance.

Our investigation revealed that female emus vary parental investment in their offspring through changes in the absolute amount of yolk and albumen in eggs, while keeping the proportion of the two constant. Embryos in larger eggs developed into hatchlings that were heavier and structurally larger than embryos in smaller eggs (i.e. greater parental investment in embryogenesis yielded larger hatchlings), but hatchlings from eggs of all sizes contained the same amount of residual yolk (i.e. emus invest similar parental care, *via* eggs, in their hatchlings). We do not know, however, if embryos that 'find' themselves in larger eggs, containing more resources and a larger gas exchange surface, respond by growing more or if embryos that would normally grow more are put into larger eggs. Further research is needed to elucidate more clearly how maternal phenotypes affect developmental trajectories and ultimately fitness.

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