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Quantitative and Morphological Measures May Predict Growth and Mortality During Prenatal Growth in Japanese Quails

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Abstract: Growth pattern and mortality rate during the embryonic phase of avian species are difficult to recognize and predict. Determination of such measures and associated events may enhance our understanding of characteristics involved in the growth and hatching process. Furthermore, some quantitative measures could validate morphological determinants during the embryonic phase and predict the course of normal growth and alterations. Our aim was to characterize quantitative growth of embryos and to establish baseline embryonic standards for use in comparative and pathological research during the prenatal life of Japanese quail. Day 10 was a landmark timeline for initiation of extensive anatomical changes in growth and transformation. Wet and dry weights were positively correlated with each other and inversely correlated with water content ($p = 0.05$). Following d10, the water content decreased progressively, whereas, dry and wet weights increased with increasing age. Velocity of growth in wet and dry weights was evident starting d6, spiked at d11 and d15 and then declined before hatching on d16. Organic and inorganic contents of embryos were positively associated with age. Progressive increase in the organic to inorganic ratio with age was evident after d5, spiked on d9, d13 and d16. Accurate determinations of prenatal growth processes could serve as valuable tools in identifying morphological developments and characterization of prenatal growth and mortality, thus enhancing the reproductive efficiency of the breeding colony and the postnatal robustness of the offspring.

Key words: Japanese quail, embryonic growth, morphology, quantitative traits

INTRODUCTION

Numerous advantages of using Japanese quail in biomedical research have been reported (Reese and Reese, 1962; Dieterlen-Lievre, 1997; Huss, 2008; Scanes and Mcnabb, 2003). Advantages include cost effective management, rapid growth rate and sexual maturation, efficient feed conversion and a short prenatal life of 16-17 days at which time inert egg content is transformed into a fully developed chick (Spencer, 1968). The Japanese quail is being used extensively for both classroom teaching and conducting research serving as an experimental animal model for studies in comparative development, teratology, morphogenesis, organogenesis, tissue grafting, chimera production, toxicology and drug-testing (Poynter, 2009; Tsudzuki *et al*. 1998). They are also being used for studies evaluating environmental toxicants, reproductive toxicology, thermal stress, aging, altitude, virology, photoperiods, hyperoxic and hypoxic environments and hormonal challenges (Smith *et al*.1969; Lauber, 1975; Chaturvedi, 1993; Vatsalya and Arora,, 2012). In such studies, the measurement of treatment effects included growth rate, embryonic mass and timeline development of specific organs, structural malformations and embryonic deaths during the prenatal life as well as a variety of anatomical and physiological alterations during post-natal life. Therefore, accurate determinations of prenatal growth processes will be valuable tools in identifying structural formations. Although Romanoff (1967) has reviewed literature on quantitative embryonic development in various avian species, there is very little information available focused on quantitative growth and development of the Japanese quail. This study was therefore designed to:

1. Characterize quantitative growth of embryos during the prenatal life of Japanese quail;
2. Predict the embryonic mass (weight) in relation to incubation time period;
3. Establish baseline embryonic standards for use in comparative and pathological research with Japanese quail. With this information, we anticipate to advance information on prenatal events as function of age and variations in the processes leading to pathological events and mortality.

MATERIALS AND METHODS

Eggs of uniform size, shape and weight were collected from a breeding colony of Japanese quail having a fertility of over 98 percent for use in this study. The eggs were collected between 3:00 and 6:00 P.M. and held for 1-3 days in a holding room set at 50°F. Before incubation, the eggs were allowed to warm up to room temperature for about three hours before being

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transferred to the incubator set at 98.8°F and 65% relative humidity with a built-in egg turner. The eggs were collected in four consecutive batches in order to obtain enough embryos for the treatment. Each time the birds and eggs were handled with similar standard procedures. Starting with three days of incubation, 30-40 eggs were broken open daily up to d16 into shallow petri-dishes containing chick's Ringer solution. The eggs were transferred to hatching trays on d15. The embryos were devoid of membranes and transferred to a paper towel for removal of free floating water around the embryos and then placed in previously weighed aluminum cups and weighed to the nearest milligram with a Sartorius analytical balance. An ordinary teaspoon with perforations at the bottom was used to lift the embryos. Residual yolks unabsorbed into the bodies of the embryo were excluded from evaluation. Aluminum cups containing embryos were then transferred to an incubator set at 100°F for 2 days to obtain constant dry weights. A lab plant incinerator was used to determine ash content. The morphological changes in the embryos were recorded, photographed and preserved in Bouin's solution.

Various morphological landmarks of embryos were used for evaluating changes in size and appearance, comparing growth rate, estimating time of embryonic mortality, identifying deformities and for predicting size and weight of embryos from the incubation age. Subsequent to a historical milestone of staging chick embryo by Hamburger and Hamilton (1951), different investigators reported comparable morphological characteristics for staging other avian species, including the Japanese quail (See Ainsworth *et al*., 2010 for review). It was reported that embryonic staging of the Japanese quail embryo, based on the number of somites at given hours of incubation, was quite comparable with that of chickens up to 4-5 days of incubation. From then onward, the internal and external organs of quail grow faster than chick embryos completing ontogeny in 16-17 days, compared to 23 days in chickens.

In addition to somite count, a variety of embryonic and extra-embryonic morphometric parameters were used for determining embryonic growth rate and staging of embryos. These included one or more parameters used by various investigators for studying qualitative and quantitative events at various stages of development such as measuring the width of blastoderm, the width of one side of area vasculosa, the length of area pellucida, the length of embryo (from tip of head to notochord), weight of embryo, size of beak, leg and third digit, size of brain, crown-rump length, size of wing and limb buds (Table 2 and Fig. 1) and size of metatarsus. (Romanoff, 1967; Graham and Meier, 1975; Arora and Matsumoto, 1968; Arora, 1963, 2011; Mun and Kosin, 1960; Padgett and Ivey, 1960; Hendricks and Hanzlick, 1965). In addition, the following identifiable growth landmarks were found very useful for identifying changes in morphological features, embryonic growth, retardations and mortality, for assessing time of embryonic deaths, predicting size and weight of embryos from the incubation age (Fig. 2) bearing in mind the extent of individual variations expected during early development (Pagett and Ivey, 1960; Arora and Kosin, 1966 and Romanoff, 1967; Arora, 2012).

Visible key landmarks used at various incubation periods were as follows, Days 1-2: Growth of blastoderm, formation of area pellucida, primitive streak, neural-tube and blood islands. Measurable traits include: size of blastoderm, primitive streak and somite count, Days 2-3: Development of circulatory system, heartbeat, optical vesicles and head flexion. Measurable traits: size of embryo, area vasculosa, area pellucida and somite count, Days 3-4: Head fully flexed, optic cups, initiation of limb buds and well established omphalomesenteric blood circulation. Measurable traits: size of embryo and somite count, Days 4-5: Midbrain large and protruding, optic vesicles, C-shape body,

Table 1. Embryonic growth parameters as a function of age in Japanese quail (n=30-35 embryos)

Age (days)	Mean Wet Wt. (mg)	Mean Dry Wt. (mg)	Mean water Content %	Progressive Ratios	R-Values (Wet wt.)	Organic content	Inorganic Content	Organic: Inorg Ratios
3	15.72	1.23	91.55	0.08		1.0	0.2	5.7
4	49.81	7.06	89.92	0.14	217.0	2.3	0.4	5.8
5	122.84	8.68	91.68	0.07	147.0	7.1	1.2	5.9
6	175.40	20.37	91.61	0.12	124.0	19.3	2.9	6.6
7	461.86	30.92	92.00	0.07	67.8	22.7	3.3	6.8
8	672.21	50.50	92.00	0.08	45.7	47.3	6.7	7.1
9	969.83	76.00	91.77	0.08	44.3	69.6	8.4	8.3
10	1382.00	121.25	90.04	0.09	41.3	83.0	10.0	8.3
11	2088.22	222.80	88.75	0.11	51.1	217.7	26.3	8.3
12	2565.66	337.50	86.66	0.13	22.8	335.1	34.9	9.6
13	3090.20	528.00	84.15	0.17	20.5	538.6	47.4	11.4
14	3823.43	696.00	80.23	0.18	23.7	640.4	57.6	11.1
15	4972.66	990.25	80.75	0.20	29.9	841.1	73.9	11.4
16	5721.35	1127.80	79.02	0.20	15.0	973.1	81.9	11.9

Table 2: Growth of beak, leg, third toe and metatarsus from d10 to d16 in Japanese quail. (n=25 embryos) Adapted from Arora (2011)

Body part (mm)	10 days Mean SE	12 days Mean SE	14 days Mean SE	16 days Mean SE
Beak	1.59 0.09	2.04 0.07	2.58 0.06	3.40 0.11
Leg	12.72 0.41	19.92 0.46	24.92 0.06	34.10 0.39
Third Toe	4.51 0.16	7.62 0.17	9.26 0.29	12.15 0.15
Metatarsus	5.18 0.30	8.66 0.26	10.45 0.27	14.70 0.18

Correlations and regression equations for different parameters: All of these four structures were highly correlated in all combinations and were used to assess embryonic weight, age, comparative growth rate, retardations and embryonic deaths from d10 to d16. Leg and Wt. of embryo, R^2=0.997 and Y=4.835x+6.616, Leg and Age of embryo, R^2=0.987 and y=3.462x-22.08, Age and Weight of embryo, R^2=0.986 and y=0.714x-5.906, Age and Third Toe, R^2= 0.987 and y=1.228x-7.579, Age and Metatarsus, R^2=0.978 and y=1.518x -9.981, Wt. of Embryo and Third Toe R^2=0.978 and y=1.869x+2.297, Wt. of Embryo and Metatarsus, R^2=0.9936 and y=1.237x-0.628, Third toe and metatarsus measurements were very reliable and they lend themselves to easy and quick measurements

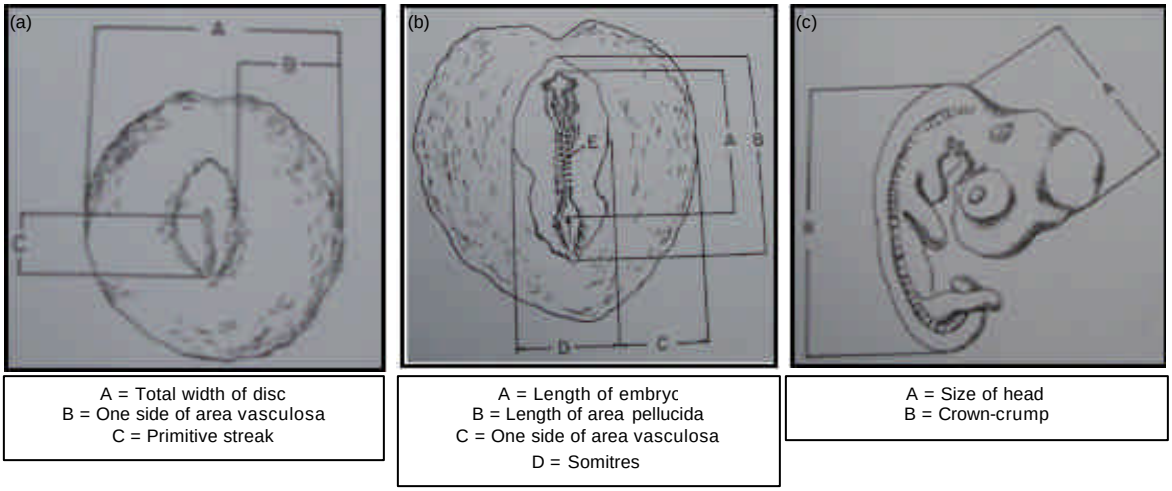


Fig. 1(a-c): Embryonic measurements (drawings) during early embryogenesis. Adapted from Arora, K.L. (1968)

growth of wing and limb buds and auditory meatus. Measurable traits: size of brain, body weight and crown-rump length, Days 6-8: Formation of digits, distinct eye formation, midbrain with protuberance, feather germs appearing in two rows and beak formation. Measurable traits: body weight, crown-rump length, Days 8-10: Feathers germs cover most of the body, protruding eyes, dewclaws, thinly distributed feathers, growth of piping tooth and scales cover most of legs and toes. Measureable traits: beak, leg, third digit, body weight and crown-rump length, Days 11-12: Entire body is covered with feathers, growth of tail feathers, eyes recede into sockets and eye lids look normal. Measurable traits: body weight, size of beak, leg and third digit, Days 13-14: Entire body is covered with long silky feathers in black-brown hue, eyes shut, webbing of toes, receding yolk sac and increased size of egg-tooth. Measureable traits: size of beak, third toe and leg, Days 15-16: Piping on d15 or d16 before hatching, absorption of yolk sac and bulging abdomen and hatch. Measureable traits: body weight, size of beak, leg and third digit.

The data were analyzed using MS Office 2010 (Microsoft Corp., Redmond, US) and SPSS Version 20.0 (Chicago, IL) and are presented in Table 1 and 2 and depicted through Fig. 1 to 10.

RESULTS AND DISCUSSION

The following four embryonic parameters were measured from d3 to d16 (day of hatch): wet weight, dry weight, organic weight (dry wt-minus inorganic wt), inorganic weight (ash) and water content (wet wt. minus dry weight). The dynamics of these constituents were analyzed further for their growth pattern as a function of age. No attempt was made to fit any mathematical growth model to the data as this has already been reported (Ricklefs, 1987).

Growth of embryos as a function of age: A significant increase in wet, dry, organic and inorganic and water content occurred during the course of embryonic development. Following d6, the weights of the embryos grew almost linearly with age. The wet weight increased from 15.7 mg at d3 to 5,721.4mg at d16 at the time of hatching. The dry weight increased from 1.2mg to 1127.8 mg during the same time period (Table 1 and Fig. 3, 4). Wet and dry components were positively correlated with each other and with age particularly

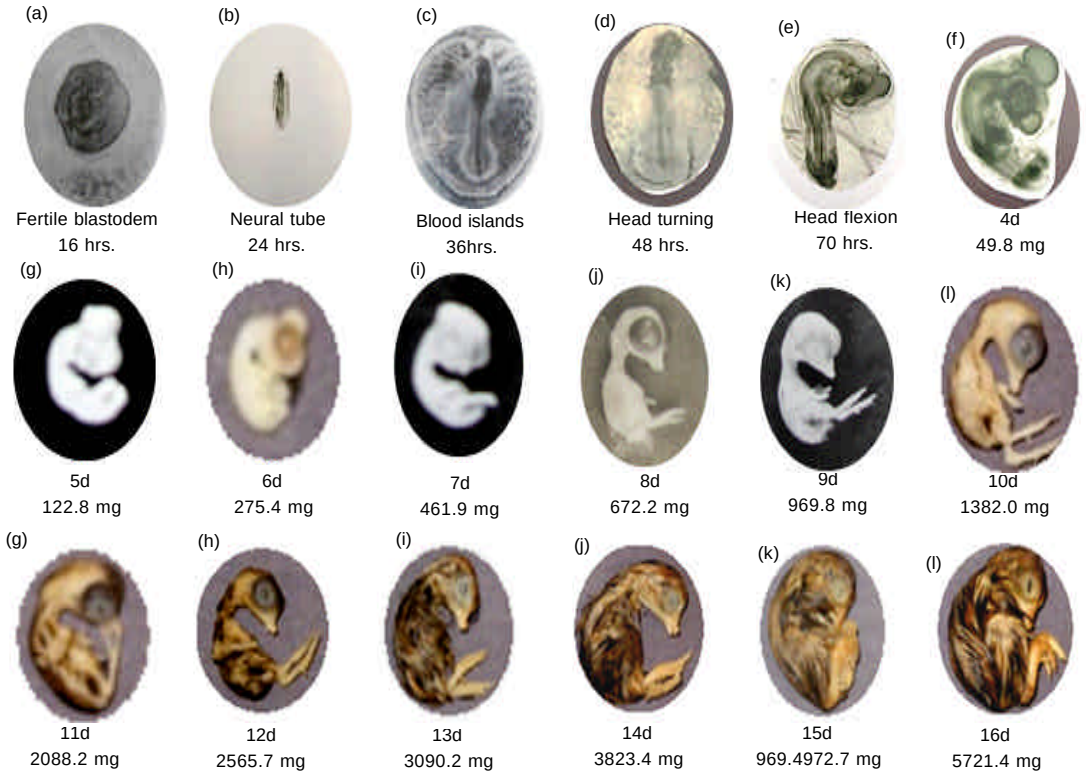
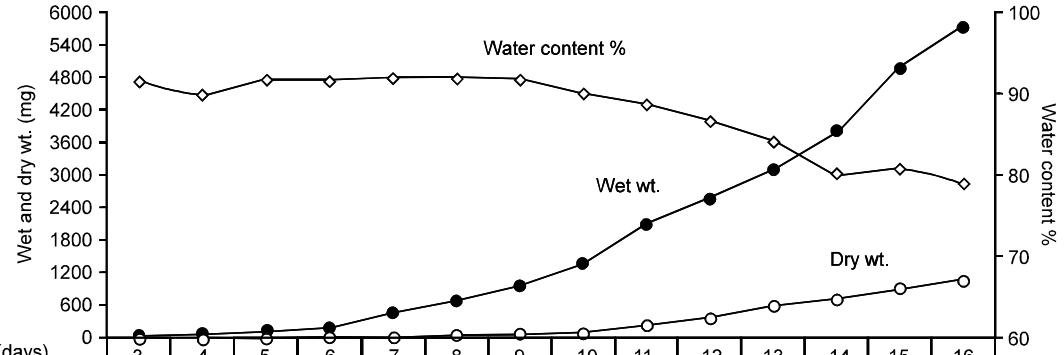


Fig. 2(a-l): Day-to-day growth of Japanese quail embryos presenting important age-specific morphological features

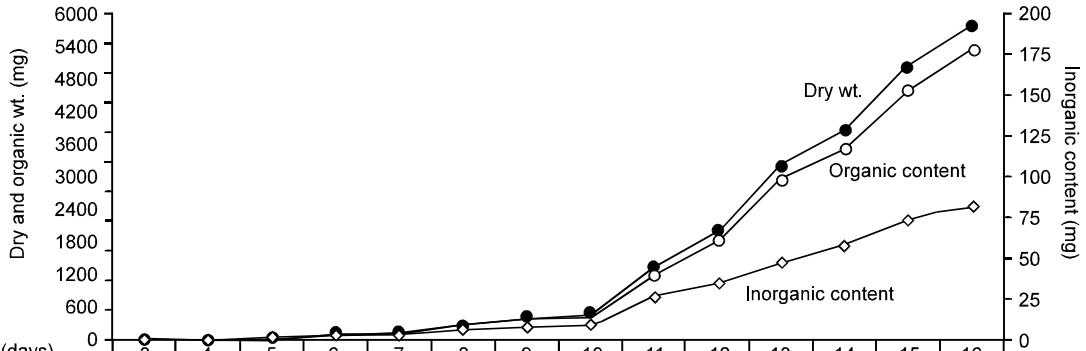


Age (days)	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Mean wet. wt. (mg)	15.7	49.8	122.8	175.4	461.9	672.2	969.8	1382.0	2088.2	2565.7	3090.2	3823.4	4972.7	5721.4
Mean dry wt. (mg)	1.2	2.7	8.3	22.2	26.0	54.0	78.0	93.0	244.0	370.0	586.0	708.0	915.0	1055.0
Mean water content %	91.6	89.9	91.7	91.6	92.0	92.0	91.8	90.0	88.8	86.7	84.2	80.2	80.8	79.0

Fig. 3: Wet weight, dry weight and water content of embryos as a function of age

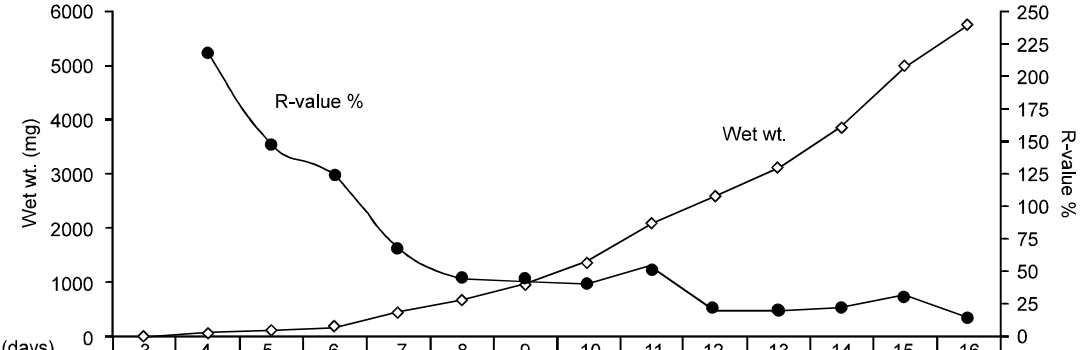
during d10 to d16 with regression values of 2115.1 ln (age) + 801.66 with R^2 = 0.8609; p = 0.05 for wet wt and 491.73 ln (age) - 31.585 with R^2 = 0.9105 at p = 0.01 for dry weight. Log values of wet wt. and organic content as a function of age were highly correlated with each other and with age, however, the wet weight was more related with age (R = 0.8121) than organic weight (R = 0.7641) (Fig. 10). Relative growth rate (R-values) of wet weight decreased progressively up to d10 and then exhibited a small spike on d11 and d15 decreasing on d16 (Fig. 5). This reduction in growth rate of embryos with age was probably associated with a decrease in metabolism and limitation on the availability of raw materials (Romanoff, 1967).

Daily increment of wet weights reflected rapid growth with age and spikes on d11, d13 and d15 followed by an abrupt decrease on d16 (Fig.7). Daily increments of wet wt. and dry wt. was significantly associated with age:



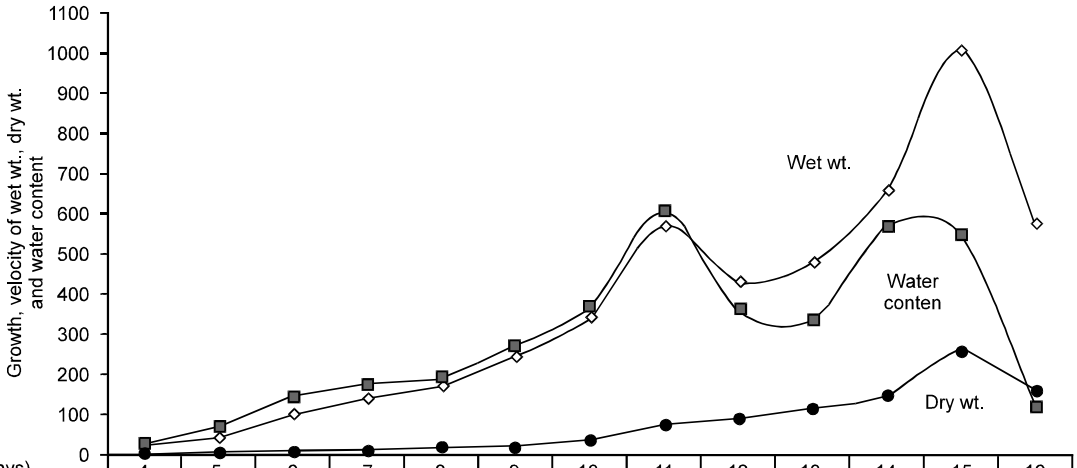
Age (days)	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Organic content (mg)	1.0	2.3	7.1	19.3	22.7	47.3	69.6	83.0	217.7	335.1	538.6	640.4	841.1	973.1
Mean dry wt. (mg)	1.2	2.7	8.3	22.2	26.0	54.0	78.0	93.0	244.0	370.0	586.0	708.0	915.0	1055.0
Inorganic content %	0.2	0.4	1.2	2.9	3.3	6.7	8.4	10.0	26.3	34.9	47.4	57.6	73.9	81.9

Fig. 4: Dry, organic and inorganic (ash) contents of embryos as a function of age



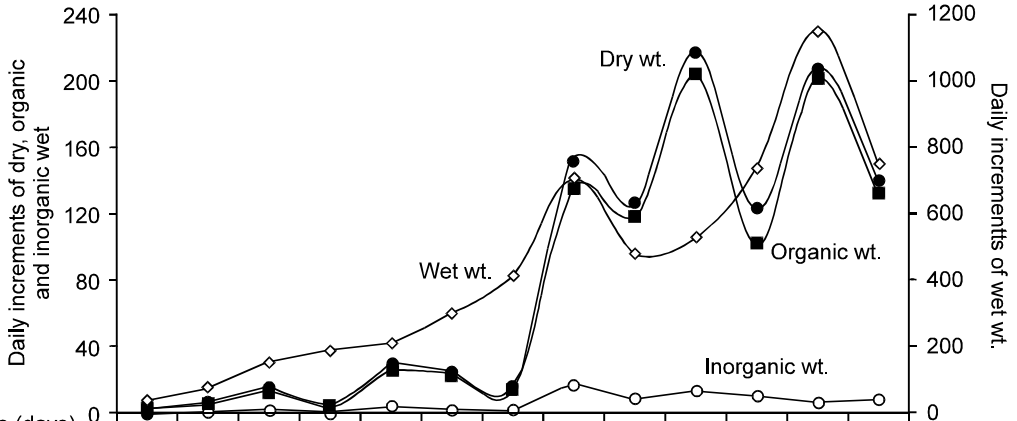
Age (days)	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Mean wet wt. (mg)	15.7	49.8	122.8	175.4	461.9	672.2	969.8	1382.	2088.	2565.	3090.	3823.	4972.	5721.
R-value (%)		217.0	1470	124.0	67.8	45.7	44.3	41.3	51.1	22.8	20.5	23.7	29.9	15.0

Fig. 5: Wet weight and R-values (%) of embryos according to age



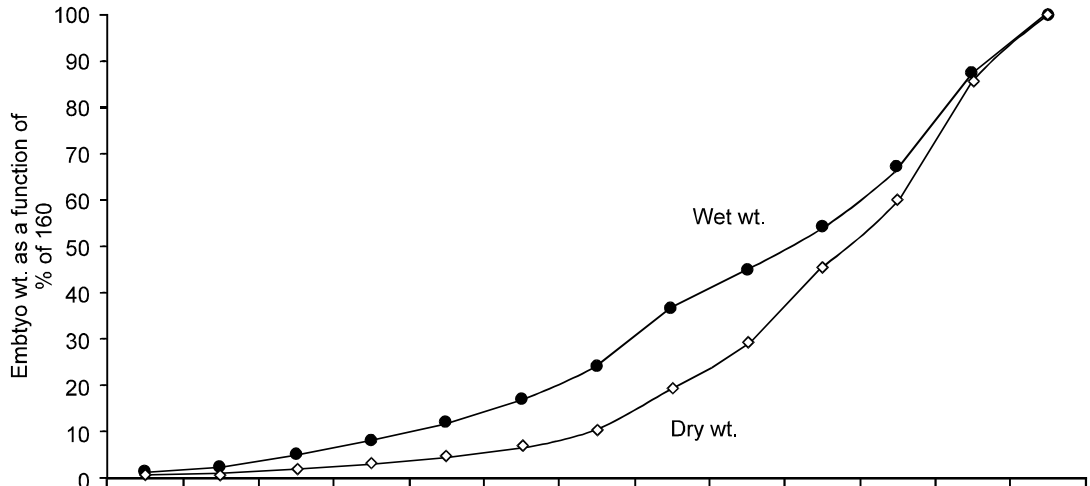
Age (days)	4	5	6	7	8	9	10	11	12	13	14	15	16
Wet wt. (dw/dt)	18.5	44.5	99.5	142.5	172.8	246.5	343.9	568.2	432.1	478.1	658.0	1004.4	578.5
Water content (mg)	30.2	69.4	140.9	175.9	190.8	272.1	367.0	604.7	362.1	334.7	656.2	546.4	118.9
Dry wt. (dw/dt)	1.7	2.7	8.3	8.5	15.2	20.6	35.6	73.6	88.0	113.2	146.0	241.9	156.6

Fig. 6: Velocity of growth (dw/dt) of wet and dry weights of embryos. Daily water contents of embryos are also depicted



Age intervals (days)	4	5	6	7	8	9	10	11	12	13	14	15	16
Dry wt. (mg)	1.5	5.6	13.9	3.8	28.0	24.0	15.0	151.0	126.0	216.0	122.0	207.0	140.0
Inorganic wt. (mg)	0.2	0.8	1.7	0.4	3.4	1.7	1.6	16.3	8.6	12.5	10.2	6.3	8.0
Organic content (mg)	1.3	4.8	12.2	3.4	24.6	22.3	13.4	134.7	117.4	203.5	101.8	200.7	132.0
Wet wt. (mg)	34.7	73.0	152.6	186.4	210.4	297.6	412.2	706.2	476.8	525.2	733.2	1148.6	749.3

Fig. 7: Daily increments in the wet, dry, organic and inorganic weights of embryos

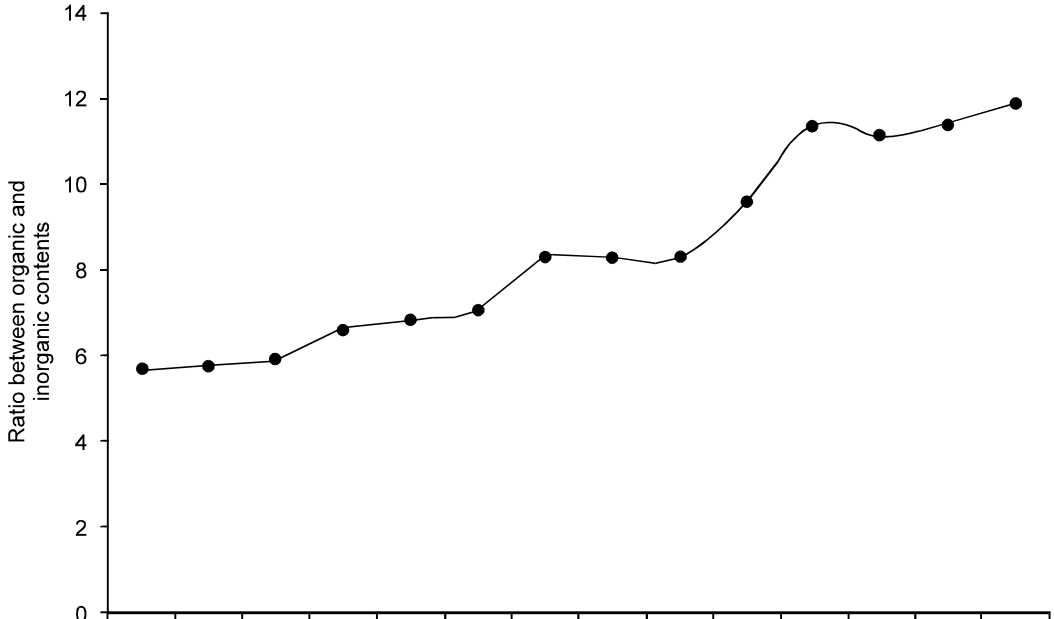


Age (days)	4	5	6	7	8	9	10	11	12	13	14	15	16
Wet wt. %	0.9	2.2	4.8	8.1	11.8	17.0	24.2	36.5	44.8	54.0	66.8	86.9	100.0
Dry wt. %	0.4	0.7	1.7	2.6	4.3	6.5	10.4	19.1	28.9	45.2	59.6	85.5	100.0

Fig. 8: Embryonic wet and dry mass as a percentage of their weights at hatching

357.18 ln (age)-180.69 with R^2 = 0.6992 for wet wt. and 94.697 ln (age)-71.48 with R^2 = 0.5619 at p = 0.01 for dry wt. Both wet and dry weights when expressed as a percent of d16 weights reflected a linear and comparable relationship with age; the wet values were larger than corresponding dry values and the difference among them was wider between d8 to d13 due to rapid growth of wet weight of embryos. The percent values were identical on d15 and d16 (Fig. 8). The velocity of growth (dw/dt) of wet and dry weights and water contents increased progressively in a linear fashion spiking on d11, falling on d12, spiking again on d15 and dropping on d16. The velocity of dry weight reflected a progressive growth from d9 onwards spiking on d15 and falling on d16 similar to wet weight and water content (Fig. 6). A significant association of wet wt. with embryonic age was 314.43 ln (age) – 177.19 with R^2 = 0.7001 and 76.645 ln (age)-62.04 with R^2 = 0.5739 at p = 0.01 with dry weight at p0.01 and 0.05, respectively.

Organic and Inorganic (ash) contents: Dry, organic and inorganic weights of embryos increased linearly with age particularly after d10 with increasing embryonic mass. They were correlated positively with each other



Age (dyas)	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Org: inorg ratio	5.7	5.8	5.9	6.6	6.8	7.1	8.3	8.3	8.3	9.6	11.4	11.1	11.4	11.9

Fig. 9: Daily variations in the ratio between organic with inorganic contents according to age

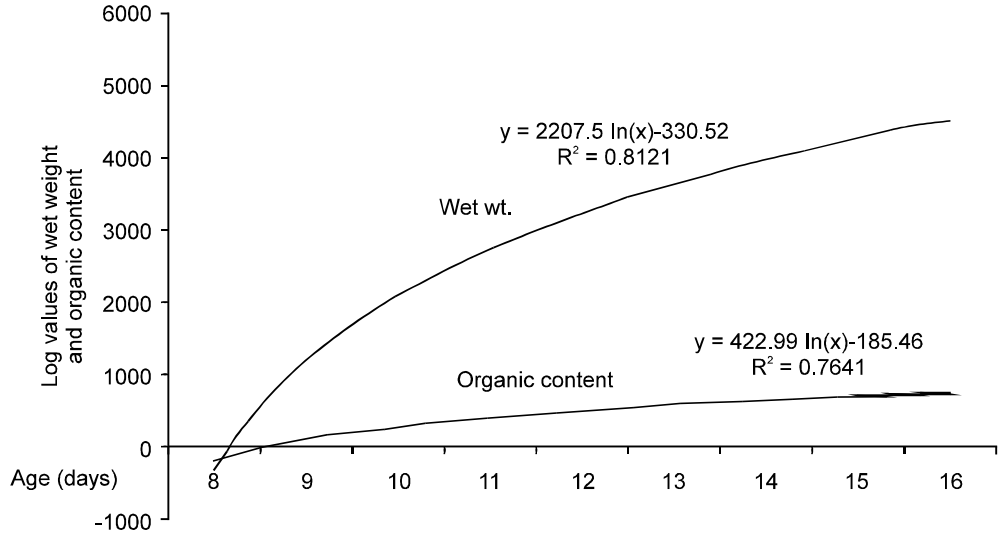


Fig. 10: Log values in relation to wet weight and organic content with embryonic age

and with age (Fig. 4). Association between age and organic wt. during d10 to d16 was 453.94 ln (age) - 34.413 with R^2 = 0.9063; the association between age and inorganic wt. was 36.402 ln (age)+3.0951 with R^2 = 0.9329 and the association between age and dry wt. was 520.92 ln (age)-51.014 with R^2 = 0.8794; all at p = 0.01 level. When expressed on the basis of daily increments, both dry and organic weights exhibited a similar growth pattern with spikes on d6, d8, d9, d11, d13 and d15, ultimately falling on d16. The wet weight increased linearly up to d11, fell on d12, increased rapidly following d12 reaching a peak spike on d15 and falling on d16. Conversely, the increase in inorganic weight was evident only after d9, spiked on d11 and d13 decreasing gradually until hatching (Fig. 3 and 4). The ratios between organic and inorganic values were linear with spikes on d9 and d13, rising slightly up to d16, the time of hatching (Fig. 9). Quantitatively, inorganic content increased to the highest level on d11 and then decreased gradually until time of hatching as body

weight increased. Inorganic components are necessary for carrying out various biochemical processes and for maintaining electrolyte balance. Log values of organic and wet weights with respect to age reflected very high correlation and high predictability (Fig. 10).

Water content as a function of age: Water weight, as a percent (%) of body weight, decreased progressively after d10 reaching lowest level from d14 to d16, the day of hatching. As the body continued to gain mass (solid materials), the water content continued to decrease correspondingly with age. The water content of the embryos decreased from 90.00% at d10 to 79.00% at the time of hatching, similar to that which was reported for chicken embryos (Romanoff, 1967). When expressed in terms of velocity of growth (dw/dt), both water content and wet wt. increased up to d11, spiked on d14 and d15 along with wet weight, falling drastically on d16, the day of hatching. There was a similar relationship between velocity of water content and dry weight, although to a lesser degree with a spike on d15 and decreasing on d16 (Fig. 3 and 6).

Variation in embryonic growth and mortality: Pertinent literature on embryonic mortality in chickens were reviewed by Landauer (1951) and Romanoff (1967, 1972). Variability in avian embryonic growth particularly during the early stages of development is well recognized (Padgett and Ivey, 1960; Abbott, 1967; and Romanoff, 1967); some embryos lag behind others making it difficult for accurate staging of embryos. Variability in the degree of development of blastoderms at oviposition is well established (Romanoff, 1967; Abbott, 1967; Sellier *et al*., 2006; Ainsworth *et al*., 2010). This could be a result of the length of time the egg spends in the oviduct, the time of egg collection, the length of preincubation holding of egg, physical factors during incubator with respect to temperature, humidity and turning, individual genetic differences, age of the hens, weight of egg, porosity and color of shell and maternal diet among others. One or more factors could influence growth rate and cause variability in growth. The germ discs at oviposition can be identified macroscopically as fertile or infertile. The fertile blastoderm is 3-4 mm in size located on top of the yolk, lightly whitish in color compared to the surrounding yolk, opaque in appearance, invariably circular in shape and devoid of vacuoles. Conversely, the infertile blastodiscs are relatively smaller, asymmetrical and irregular in shape and vacuolated with the presence of condensed whitish material within the disc. Staining discs with neutral red in situ is helpful (Arora, 1968 and Rugh, 1971) after making a window in the shell or after breaking open the egg carefully into a bowl and separating tissues from the white of the egg using a pair of forceps and a dissecting needle. Generally, early blastodermal deaths are difficult to distinguish from infertile discs. In our experience, the breeding stock with a high degree of fertility is invariably associated with higher levels of embryonic viability and hatchability given an optimal incubator environment. However, health problems may occur as a result of illness in the flock, incubation mismanagement and from extraneous bacterial and viral contaminations. During the course of this experiment, the mortality rate was 3.3% during d1 to d4 of incubation and 2.5% during d15 to d16 of incubation. Middle stage deaths can be identified by the presence or absence of various morphological structures which are expected to appear at specific developmental stages. Taha (2011) also evaluated embryonic mortality in Japanese quail and reported mortality at both ends of embryonic growth. During late mortality, the development of the following parameters should be evaluated: size of beak, third digit and metatarsus (Table 2), eyes, live or dead piping and unthrifty appearance of embryos. In the event early incubation mortality occurs prior to the formation of blood islands, the embryonic disc will appear enlarged, fragmented and vacuolated with little or no differentiation of area pellucida, primitive streak and neural tube (Arora and Kosin, 1966). The embryonic tissues can be stained, in situ, or after opening the eggs into a dish and separating the tissues from the light yellow and tainted egg fluid and transferring to the glass slide and staining with neutral red for accurate evaluation. In the event mortality occurs after the formation of blood vessels (3 to 4 days of incubation), the embryos will sink in the sub-embryonic fluid and blood rings will appear adhering to the internal surface of shell. Candling is not practical for colored Japanese quail eggs and the egg breakout procedure is recommended to determine infertility, retardations, mortality, deformities and malpositions.

Conclusion: Embryonic growth is very rapid and organogenesis was almost complete by d10 and, thereafter, the degree of growth is attributed to an increase in weight and the size and shape of the differentially growing organs until hatching. The percentage of growth rate (R-values) decreased with advancing age. Throughout development, the wet and dry weights were closely related with respect to growth and growth spikes were observed on d11, d13 and d15. The embryos gained approximately 46.0, 53.2, 44.6 and 4.2% of wet, dry, organic and inorganic contents, respectively, during the last four days of hatching. Water content (%) decreased gradually as the embryos gained weight towards maturity. The incubation process could potentially disturb or interfere with the sequence of complex morphogenetic, physical and biochemical processes which embryos undergo during normal growth. Any deviation from these processes can result in malformations, deaths and experimentally and environmentally-induced alterations during the course of development and possibly affecting the post-natal performance of chicks as well. Various important

morphological features were found to be very useful in studying growth rate. Staging and predicting the age of dead embryos were presented here. Proper control of potential sources of variability in embryonic growth is paramount for obtaining accurate and reliable data.

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