Indian Journal of Animal Sciences **82** (9): 993–998, September 2012

Modulation of post hatch-growth and immunocompetence through *in ovo* injection of limiting amino acids in broiler chickens

SUBRAT K BHANJA[1], ASIT BARAN MANDAL[2], SUSHIL K AGARWAL[3] and SAMIR MAJUMDAR[4]

Central Avian Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 16 November 2010; Accepted: 30 January 2012

ABSTRACT

Early post-hatch growth and immunity was assessed through *in ovo* injection of some critical amino acids on 14th d of incubation at the broad end of the egg using 25 mm needle. Percent hatchability in lysine (lys) and arginine (arg) injected groups were better than un-injected control group. Threonine (thr) and methionine (met) injected eggs had higher chick weight and chick to egg weight ratio and met and arg injected chicks had higher fourth and seventh week body weight than un–injected control chicks. No significant difference was recorded for FCR of *in ovo* amino acids injected birds. Met and glycine (gly) injected chicks had higher bursa weight, whereas, those injected with met thr, gly and isoleucine (ile) had higher spleen weight on the day of hatch. Humoral immune response (SRBC titre) was higher in most of the amino acid injected chicks except lys group. Cell mediated immunity (response to PHAP mitogen) was higher in lys, arg, gly and ile injected chicks. The chicks injected with amino acids invariably had higher plasma protein and lower plasma glucose on the day of hatch. It may be concluded that met and thr were critical for the growth of chicken embryo, however, during post–hatch period met and arg played major role. *In ovo* injected amino acids can also act as immunomodulator but their role in gastrointestinal development needs further research.

Key words: Amino acids, Immune response, *In ovo* injection, Gastrointestinal tract, Growth

Egg albumen and yolk contain amino acids for the developing embryo, however, a large fraction of egg protein consists of antibodies, which the hen produces during the immune response experienced at the time of egg laying (Losch *et al*. 1986). Under normal circumstances, these immunoglobulins are fully functional at the time of hatch (Brierley and Hemmings 1956). Gluconeogenesis from protein provides glycogen that fuels hatching activities (Klasings 1998). Thus, early provision of nutrients affects immediate embryo survival and disease resistance and also the ultimate attainment of genetic potential. Efforts were made to achieve higher protein synthesis by injecting AA directly into the egg (Ohta *et al.* 2001, Bhanja *et al.* 2004b). Accelerated enteric development and improved nutritional status afforded by *in ovo* feeding improved hatching weight, growth rate (Al-Murrani 1982, Ohta *et al*. 1999 and Bhanja *et al*. 2004b), immune responses (Konashi *et al.* 2000, Bhanja and Mandal 2005), gastro-intestinal development (Uni and Ferket 2004) and meat yield. *In ovo* supplementation of amino acids during later stage of embryonic development (*in ovo* feeding) may spare those antibodies from utilization as protein source during embryonic and neonate stages. The present investigation was aimed to study the effect of *in ovo* injection of some critical amino acids on embryonic and post–hatch growth performance, immune response and development of digestive organs in broiler chickens.

Present address: [1]Senior Scientist, [3] Head, [4] Principal Scientist, Poultry Housing and Management, (subratcari@gmail.com); [2]Principal Scientist, Division of Avian Nutrition and Feed Technology (abmcari@rediffmail.com).

MATERIALS AND METHODS

In ovo *injection:* Standard size fertile eggs (800), collected from a dam line of a coloured broiler strain, were weighed and distributed into 8 treatment groups– 25 mg each of limiting amino acids viz. lysine (lys), methionine (met), threonine (thr), arginine (arg), glycine (Gly) and isoleucine (ile) dissolved in 0.5 ml of sterile water, a sham control (0.5 ml sterile water) and un- injected control. The treatment solutions or sham control were injected into the yolk of the 14-day-old embryo, through a pinhole made at the broad end of the egg, using a 24G hypodermic needle (25 mm long) as per Bhanja *et al.* (2004a). Prior to *in ovo* injection the injectants were warmed to 30° C. The procedure was carried out under a laminar flow system and the pinhole site was sealed with sterile paraffin wax immediately, and eggs were returned to the incubator. On 18th day of incubation the eggs were transferred to a hatcher and placed in pedigree hatching

Table 1. Composition of starter and finisher diets (NRC 1994 specifications) used for feeding *in ovo* injected and control birds during the expreimental period

	Starter diet (0–4 wk)	Finisher diet (5–7 wk)
Ingredients		
Maize	58.78	66.84
Deoiled ricebran	0.00	2.10
Soybean meal	28.28	19.66
Fish meal	6.00	6.00
Soybean oil	4.42	3.16
Dicalcium phosphate	1.05	0.55
Lime stone powder	0.90	1.05
Min. and vit. mix[1]	0.32	0.32
Salt	0.10	0.10
Lysine	0.00	0.11
Methionine	0.15	0.06
Threonine	0.0	0.05
Chemical composition (*dry matter basis*)*		
ME, Kcal/kg diet	3200	3200
Crude protein (%)	23.00	20.00
Ether extract (%)	7.17	6.18
Crude fiber (%)	2.44	2.55
Lys (%)	1.12	1.00
Met (%)	0.50	0.38
Ca (%)	1.00	0.90
Ava. P (%)	0.45	0.35

[1]Mineral and vitamin premix supplied mg/kg diet: Magnesium, 300 mg; copper, 4 mg; iron, 56 mg; iodine, 1 mg; mangnese, 55 mg, and zinc, 30 mg; Vitamins A, 8250 IU; B_2, 5 mg; D_3, 1200 IU and K, 1 mg. vit. B_1, 8 mg; B_6, 16 mg; B_{12}, 80 mg; E, 40 mg; niacin, 60 mg; calcium pantothenate, 10 mg; choline, 500 mg.* Calculated from the ingredient compositions.

boxes so that chicks coming from a particular egg could be recorded.

Birds and housing: The chicks hatched from the different treatment groups were distributed in four-tier battery brooder cages and kept in a well-lit and ventilated open-sided house. Each treatment group had 4 replicates of 8–10 birds depending upon the hatch size, and chicks were reared up to 49 d of age and provided with a standard broiler ration (National Research Council 1994) and details of the broiler starter and finisher diet are given in Table 1. Food and water were made available *ad lib.* Standard management practices were followed in the test and control group throughout the experiment.

Blood bio-chemicals and lymphoid weights: Eight birds (equal sexes) from each treatment were subjected to 8 h of food withdrawal prior to being killed on day-old and 28th day of age. Blood was collected and allowed to clot at room temperature. Serum was separated and kept at –70°C for blood biochemical analysis. The plasma glucose (Nelson and Somgyl 1957) and plasma protein (Lowry *et al.* 1951) were studied. The weights of the bursa, thymus and spleen were recorded and expressed as gkg^{-1} body weight. The weight of the digestive organs–empty proventriculus, empty gizzard, liver (without gall bladder), pancreas (detached from the duodenal wall) and weight of intestine (duodenum, jejunum and ileum, large intestine and caeca) after removal of chyme were recorded and expressed as gkg^{-1} body weight.

Immune responses

Humoral response: Sheep red blood cells (SRBC) suspended in Alsevar's solution were washed 3- times in isotonic phosphate-buffered saline (PBS: pH 7.2) using centrifugation (700g) and adjusted to provide a 1% suspension (v/v) which was stored at 4°C prior to use. Day-old chicks (28) were injected intravenously with 1 ml of the SRBC suspension. Blood sample (2 ml) was obtained from the jugular vein of each chick 5 days later. Each blood sample was allowed to clot for serum collection and sera were stored at –20°C until analysis. The antibody response to SRBC was determined using a standard haemagglutination assay (Siegel and Gross 1980, Van der Zijpp *et al.* 1983). The reciprocal of highest dilution showing clear agglutination was the end point of titer and the values were expressed as log 2.

Cell-mediated immune response: The cellular immune response was assessed in 28-day-old chicks using the *in vivo* cutaneous basophilic hypersensitivity response to the mitogen phytohaemagglutinin from *Phaseolus vulgaris* (PHA-P). The toe web thickness, between the third and fourth digits, of both the left and right feet was measured using a micrometer. Immediately afterwards 100 µg PHA-P, dissolved in 0.1 mL PBS, was injected into the same inter-digital space of the right foot. The toe web of the left foot was used as the control and injected only with PBS. The inflammatory response was determined 24 h later by measuring the thickness of the respective toe-webs and subtracting the earlier measurements: values for the right toe-web were compared with those for left (control) to determine the response (Corrier and DeLoach 1990).

Battery brooder cages were the experimental unit for analysis of body weight and FCR data, whereas, individual birds were the experimental unit for all immunological and digestive organ study. Data were subjected to statistical analysis using standard procedures (Snedecor and Cochran 1980). Where there was unequal observations LSD (Harvey 1975) else Duncan multiple range test (Duncan 1955) was used for verifying significant difference between treatment means.

RESULTS AND DISCUSSIONS

Chick weight and hatchability in critical AA injected eggs: When injected with specific AA the hatchability was not affected as compared to un-injected control. Instead, in lys or arg injected group, hatchability was higher than in the un-injected control. In thr and gly group the hatchability was comparatively lower, because in those groups more deaths

were recorded while chicks were piping the shell. Though there was no differences in the chick weight among amino acid injected and un-injected control group, but when the ratio of chick weight to corresponding egg weight was compared, the group which received thr or met injection had significantly (P<0.05) higher ratio than sham control and un-injected control group (Table 2). Ohta *et al*. (1999) reported that the proportion of gly and proline (pro) in the embryo increased during incubation. Gly and pro is required for synthesis of collagen, which is required for tissue synthesis. Pro is synthesized from several AA, whereas precursors of gly are limited to only thr and ser. In another study, Bhanja and Mandal (2005) had also reported higher body weight in chicks injected with gly and pro than un-injected control. Conversion of thr to gly, which is also an integral part of uric acid synthesis, might accelerate at later stage of embryonic development, thereby increasing its requirement.

Growth performance in in ovo *AA injected birds:* At fourth week of age, the chicks which received met and arg had significantly (P<0.05) higher body weight than un-injected control chicks. In other amino acid injected groups, except ile all had higher body weight than un-injected control chicks. The similar trend was observed in the body weight of the birds at seventh week of age. During first 4 weeks met and arg injected chicks had 52.8 to 54 4 g higher body weight compared to un-injected control chicks and by seventh week the difference increased to 92.4 to 133.4g. There was no significant difference in the FCR of birds injected with AAs during 0–4wk, 5–7 wk and 0–7 wk period. However, chicks which received lys, met or ile injections had numerically better FCR during 0–4 wk period (Table 3).

Weight gain in AA injected chicks was comparable to that reported by Bhanja and Mandal (2005), where a difference of around 63 g in the body weight of birds (3 weeks) was noticeable in ile + leu + val and gly + pro injected groups. However, in our earlier study (Bhanja *et al.* 2004b) when all 20 AA were injected there was only 37 g higher weight gain during 0–3 weeks period. This could be attributed to lower doses of amino acids used in the injection compared to present study. The body weight gain in the present study was also comparable to that obtained by Al-Murrani (1982), who

Table 2. Effect of *in ovo* injection of limiting AA at 14th day of incubation on the hatchability parameters

	Egg wt (g)	Chick wt (g)	Chick wt to egg wt ratio(%)	Hatchability on fertile egg set basis (%)	Dead after injection (%)	Dead in shell (%)
L-lysine	69.25	48.21	69.64^{ab}	94.44	5.56	0.00
DL-methionine	70.88	50.11	70.64^{bc}	87.88	12.12	0.00
L-threonine	68.89	48.92	71.04^{c}	81.48	7.41	11.11
L-arginine	68.41	48.09	70.26^{abc}	91.67	4.17	4.17
Glycine	68.13	47.48	69.92^{ab}	87.50	3.13	9.38
Isoleucine	68.54	47.89	70.06^{abc}	89.47	7.89	2.63
Sham control	70.21	48.73	69.38^{a}	94.29	5.71	0.00
Un-injected control	70.02	48.58	69.31^{a}	90.32	0.00	9.68
SEM	0.39	0.31	0.15	ND	ND	ND
Significance	NS	NS	P<0.05	ND	ND	ND

NS, nonsignificant (P>0.05); ND, not determined as single hatch was taken; Means bearing different superscript within a column vary significantly (P<0.05).

Table 3. Effect of *in ovo* injection of limiting AA on the post-hatch body weight (g/bird) and FCR of broiler chickens

Attributes	Body weight (g)		Weight gain (g)		FCR		
	4th wk	7th wk	0–4 wks	5–7 wks	0–4 WK	4–7 WK	0–7 WK
L-Lysine	507.3^{ab}	1073.2^{a}	448.1	561.5^{a}	2.13	3.32	2.79
DL-Methionine	539.9^{b}	1180.5^{bc}	488.9	640.5^{bc}	2.24	3.42	2.92
L-Threonine	514.9^{ab}	1090.0^{a}	465.9	575.1^{ab}	2.38	3.30	2.77
L-Arginine	534.3^{ab}	1221.5^{c}	487.3	687.2^{c}	2.34	3.10	2.78
Glycine	515.9^{ab}	1138.1^{abc}	468.1	622.2^{bc}	2.35	3.25	2.86
Isoleucine	483.4^{a}	1116.6^{ab}	436.2	633.2^{bc}	2.27	3.07	2.74
Sham control	511.8^{ab}	1131.7^{ab}	462.1	616.8^{abc}	2.17	3.33	2.81
Un-injected control	485.2^{a}	1088.1^{a}	434.5	602.8^{ab}	2.38	3.31	2.90
SEM	7.00	11.79	7.19	8.85	0.03	0.04	0.02
Significance	P<0.05	P<0.01	NS	P<0.05	NS	NS	NS

NS, nonsignificant (P>0.05). Means bearing different superscript within a column vary significantly (P<0.05).

Table 4. Effect of *in ovo* injection of limiting AA on the development of digestive tract and lymphoid organs in broiler chicks at day-old

	Liver ($g.kg^{-1}BW$)	Gizzard and proventriculus ($g.kg^{-1}BW$)	Small intestine weight ($g.kg^{-1}BW$)	Large intestine weight ($g.kg^{-1}BW$)	Yolk sac ($g.kg^{-1}BW$)	Bursa of Fabricius ($g.kg^{-1}BW$)	Spleen ($g.kg^{-1}BW$)
L-lysine	70.1	27.33	34.03	8.90	120.1	0.134^{ab}	0.280^{a}
DL-methionine	70.1	29.93	29.76	10.40	88.8	0.145^{b}	0.510^{b}
L-threonine	70.4	24.47	33.93	11.06	137.6	0.117^{a}	0.553^{b}
L-arginine	69.8	25.67	31.33	10.16	145.6	0.103^{a}	0.478^{ab}
Glycine	69.3	27.13	30.40	8.10	65.4	0.179^{c}	0.578^{b}
Isoleucine	67.5	26.87	28.00	13.83	132.2	0.115^{a}	0.561^{b}
Sham control	70.6	29.07	31.43	10.77	114.4	0.124^{a}	0.556^{b}
Un injected control	67.3	26.83	30.27	10.50	122.0	0.131^{ab}	0.395^{a}
SEM	1.25	0.71	0.65	0.50	7.60	0.005	0.003
Significance	NS	NS	NS	NS	NS	P<0.01	P<0.05

NS, nonsignificant (P>0.05); means bearing different superscript within a column vary significantly (P<0.05).

reported 11% higher body weight at eighth week of age in amino acid injected birds. Uni *et al.* (2005) reported that *in ovo* feeding to the late term embryos increased the hatching weight by 5 to 6% over controls. Ohta *et al*. (2001) and Bhanja *et al*. (2004a) reported that *in ovo* administration of all 20 AA increased the chick weight by 3.6% and 2.1%, respectively. It is postulated that *in ovo* administration of AA might have stimulated the AA utilization and concomitant decrease in AA degradation by the embryo. Free AAs also decreased protein degradation in the hepatocytes of rats (Venerando *et al*. 1994).

Enteric development in amino acid injected birds: In the present study there was no significant difference in the weight of digestive organs and intestine at day old age. However, the weight of proventriculus and gizzard in met and weight of small intestine in lys and thr treated chicks were apparently higher compared to un-injected control chicks. Weight of bursa of Fabricius was significantly higher (P<0.01) in met and gly injected chicks compared to sham control chicks. Weight of spleen was significantly higher (P<0.05) in met, thr, gly and ile injected chicks compared to un-injected control chicks (Table 4). Similar to that of present finding, Bhanja and Mandal (2005) had also reported that *in ovo* injection of thr + gly + ser, lys + met and ile + leu + val had apparently higher bursa and spleen weight at 21 days of age. In another study, Bhanja *et al.* (2004b) reported that thymus and spleen weights were comparatively higher in AA injected group than control birds.

Immune response in in ovo *injected chicks:* In the present study *in ovo* injection of met, thr, arg, gly and ile had significantly higher (P<0.01) HA titer value (Log 2) against sheep RBC compared to un-injected control chicks, surprisingly lys injection had no effect on SRBC response. Lotan *et al*. (1980) reported that lys did not have significant effect on antibody titer in rats. Our observation on met was similar with the findings of Tsiagbe *et al*. (1987), they reported that met supplementation resulted in significant dose related increase in total antibody, IgG and response to PHA-P, but not in IgM. They suggested that met was required for some components of the antibody response and might be required for thymus derived (T) cell helper function. Swain and Johri (2000) also found that met supplementation had higher leucocyte migration inhibition value and enhanced antibody titer of Newcastle Disease (ND) virus.

Table 5. Effect of *in ovo* injection of limiting AA on the humoral and cell mediated immune response of post hatch broiler chickens

	HA titre against SRBC ($\log_2$)	Foot web index (*in vivo* PHAP response, mm)
L-lysine	8.3^{b}	0.551^{b}
DL-methionine	9.5^{cde}	0.350^{ab}
L-threonine	9.0^{cd}	0.287^{a}
L-arginine	10.2^{e}	0.410^{b}
Glycine	10.0^{de}	0.386^{b}
Isoleucine	9.9^{cde}	0.385^{a}
Sham control	7.0^{a}	0.356^{ab}
Un-injected control	8.5^{b}	0.313^{a}
SEM	0.20	0.021
Significance	P<0.01	P<0.05

Means bearing different superscript within a column vary significantly (P<0.05).

Foot web index (response to PHAP, mitogen) was significantly higher in chicks injected with lys, arg, gly and ile compared to un-injected control chicks. Amino acids like arg, gly and ile influenced both homoral and cell mediated immunity in chicks (Table 5). Konashi *et al*. (2000) reported that the branch chain amino acids (ile, leu and val) affect the humoral and cell mediated immune response by affecting antibody production. Our result also supported findings of Bhanja and mandal (2005) where *in ovo* injection of thr + gly + ser and ile + leu + val resulted in higher antibody titer against SRBC and response to PHA-P. An analysis of the

Table 6. Effect of *in ovo* injection of limiting AA on the plasma protein and glucose level at 0 and 28 d of age

	Plasma glucose (mg/dl)		Plasma protein (g/dl)	
	Day old	28th d	Day old	28th d
L-lysine	229.99^{bc}	218.89^{a}	2.50^{d}	2.08^{a}
DL-methionine	210.37^{a}	237.82^{b}	2.48^{d}	2.20^{b}
L-threonine	222.16^{b}	263.98^{de}	1.71^{a}	1.94^{a}
L-arginine	234.45^{cd}	256.25^{cd}	2.63^{d}	2.15^{ab}
Glycine	241.39^{d}	264.38^{de}	2.48^{d}	2.60^{e}
Isoleucine	252.48^{e}	269.33^{e}	2.28^{c}	2.40^{cd}
Sham control	231.28^{bc}	255.66^{cd}	2.25^{c}	2.47^{d}
Un injected control	253.67^{e}	247.33^{bc}	1.96^{b}	2.34^{bc}
SEM	2.28	2.61	0.064	0.046
Level of significance	P<0.01	P<0.01	P<0.01	P<0.01

Means bearing different superscript within a column vary significantly (P<0.05).

AA composition of immunoglobulin, found that thr, leu and val content was comparatively higher in immunoglobulin (Tenenhouse and Deutsch 1966). Higher production of antibody in those groups might have been due to higher supply of corresponding substrate through *in ovo* injection. This also supported the findings of Bhargav *et al*. (1970, 1971) where they observed that a deficiency of val and thr reduced the antibody production against ND virus in chicken.

Blood biochemical parameters: Most of the amino acid injected chicks except ile group had significantly lower (P<0.01) plasma glucose level on day of hatch compared to un-injected control chicks. Whereas, on 28 days of age the chicks which received thr, gly and ile injection had significantly higher (P<0.01) plasma glucose compared to control chicks. The chicks injected with amino acids invariably had higher plasma protein level on the day of hatch, however, only gly injected chicks had higher plasma protein level on 28 day of age (Table 6).

The amount of carbohydrates in the egg is very low, so gluconeogenesis from protein is the source of glucose for the accumulation of glycogen that eventually fuels hatching activities (Klasings 1998). As free amino acids were injected that might have reduced the gluconeogenesis resulting in lower plasma glucose, but higher plasma protein level on the day of hatch.

It may be concluded that met and thr were critical for the growth of chicken embryo and additional requirement arg during post–hatch period could not be ruled out. *In ovo* injected amino acids may act as immunomodulators and their role in gastrointestinal development needs further research. This preliminary study may open a window for future research on this aspect.

REFERENCES

Al- Murrani W K. 1982. Effect of injecting amino acids into the egg on embryonic and subsequent growth in the domestic fowl. *British Poultry Sci*ence **23**: 171–74.

Bhanja S K and Mandal A B. 2005. Effect of *in ovo* injection of critical amino acids on pre and post hatch growth, immunocompetence and development of digestive organs in broiler chickens. *Asian-Australasian Journal of Animal Science* **18**: 524–31.

Bhanja S K, Mandal A B and Goswami T K. 2004b. Effect of in ovo injection of amino acids on growth, immune response, development of digestive organs and carcass yield of broiler. *Indian Journal of Poultry Science* **39** (1): 212–18.

Bhanja S K, Mandal A B and Johri T S. 2004a. Standardization of injection site, needle length, embryonic age and concentration of amino acids for *in ovo* injection in broiler breeder eggs. *Indian Journal of Poultry Science* **39** (2): 105–11.

Bhargava K K, Hanson R P and Sunde M L. 1970. Effect of methionine and valine on antibody production in chickens infected with Newcastle disease virus. *Journal of Nutrition* **100**: 241–48.

Bhargava K K, Hanson R P and Sunde M L. 1971. Effect of threonine on growth and antibody production in chickens infected with Newcastle disease virus. *Poultry Science* **50**: 710–13.

Brierley J and Hemmings W A. 1956. The selective transport of antibodies from the yolk sac to the circulation of the chick. *Journal of Embryology and Experimental Morphology* **4**: 34–41.

Corrier, D E and Deloach J R. 1990. Evaluation of cell mediated, cutaneous basophil hypersensitivity in young chickens by interdigital skin test. *Poultry Science* **69**: 403–08.

Duncan D B. 1955. Multiple range and multiple F tests. *Biometrics* **11**: 1–42.

Harvey W R. 1975. Least square analysis of data with unequal subclass numbers. Agric Research Service, United State, Department of Agriculture.

Klasing K C. 1998. Carbohydrates. *Comparative Avian Nutrition*. Pages 201–209. CAB International, New York.

Konashi S, Takahashi K and Akiba Y. 2000. Effect of dietary essential amino acid deficiency on immunological variables in broiler chickens. *British Journal of Nutrition* **83**: 449–56.

Losch U, Schranner I, Wanke R and Jurgens L. 1986. The chicken egg, an antibody source. *Journal of Veterinary Medicine* **33**: 609–19.

Lotan R, Mokady S and Horenstein L. 1980. The effect of lysine and threonine supplementation on the immune response of growing rat fed wheat gluten diets. *Nutrition Report International* **22**: 313–18.

Lowry O H, Rosebrough N J, Farr A L and Randall R J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–75.

National Research Council. 1994. *Nutrients Requirements of Poultry*. 8th edn. Washington, DC, National Academy Press, USA.

Nelson N and Somogyi M. 1957. *Methods in Enzymology.* (Eds) Colowick S P and Kaplan, Vol. **3**, p. 85.

Ohta Y, Tsushima N, Koide K, Kidd M T and Ishibashi T. 1999. Effect of amino acid injection in broiler breeder eggs on embryonic growth and hatchability of chicks. *Poultry Science* **78**: 1493–98.

Ohta Y, Kidd M T and Ishibashi T. 2001. Embryo growth and amino

acid concentration profiles of broiler breeder eggs, embryos and chicks after *in ovo* administration of amino acids. *Poultry Science* **80**: 1430–36.

Siegel P B and Gross W B. 1980. Production and persistency of antibodies in chickens to sheep erythrocytes. 1. Directional selection. *Poultry Science* **59**: 1–5.

Snedecor G W and Cochran WG. 1980. *Statistical Methods*. 6th edn. Iowa State Univ Press, Ames, Iowa.

Swain B K and Johri T S. 2000. Effect of supplemental methionine, choline and their combination on the performance and immune response of broilers. *British Poultry Science* **41**: 83–88.

Tenenhouse H W and Deutsch H F. 1966. Some physical – chemical properties of chicken gamma globulins and their pepsin and papain digestion products. *Immunochemistry* **3**: 11–20.

Tsiagbe V K, Good M E, Harper A E and Sunde M L. 1987. Enhanced immune response in broiler chickens fed methionine supplemented diets. *Poultry Science* **66**: 1147–54.

Uni Z and Ferket R P. 2004. Methods for early nutrition and their potential. World's *Poultry Science Journal* **60**: 101–11.

Uni Z, Ferket P R, Tako E and Kedar O. 2005. *In ovo* feeding Improves energy status of the late-term chicken embryos. *Poultry Science* **84**: 764–70.

Van der Zijpp A J. 1983. The effect of genetic origin, source of antigen and dose of antigen on the immune response of cockerels. *Poultry Science* **62**: 205–11.

Venerando R, Mitto G, Kadowaski M, Siliprandi N and Mortimore G E. 1994. Multiphasic control of proteolysis by leucine and alanine in isolated rat hepatocyte. *American Journal of Physiology* **266**: C455–61.