

Effect of *in ovo* Injection of Royal Jelly on Post-Hatch Growth Performance and Immune Response in Broiler Chickens Challenged with Newcastle Disease Virus

Research Article

Y. Jafari Ahangari¹, S.R. Hashemi¹, A. Akhlaghi², H. Atashi², Z. Esmaili³, M. Ghorbani⁴, R. Mastani¹, A. Azadegan¹ and H. Davoodi^{5*}

¹ Faculty of Animal Science, Gorgan University of Agricultural Science and Natural Resource, Gorgan, Iran

² Department of Animal Science, Shiraz University, Shiraz, Iran

³ Department of Animal Science, Islamic Azad University, Qaemshahr, Iran

⁴ Faculty of Food Science and Technology, Gorgan University of Agricultural Science and Natural Resource, Gorgan, Iran

⁵ Department of Microbiology and Immunology, Gorgan University of Medical Science, Gorgan, Iran

Received on: 7 Jan 2012

Revised on: 23 Mar 2012

Accepted on: 3 Apr 2012

Online Published on: Mar 2013

*Correspondence E-mail: homdavoodi@yahoo.com

© 2010 Copyright by Islamic Azad University, Rasht Branch, Rasht, Iran

Online version is available on: www.ijas.ir

ABSTRACT

The aim of this experiment was to study the effect of *in ovo* injection of royal jelly (RJ) on growth performance and immune response in broiler chickens challenged with Newcastle disease virus. A total of 200 hatching eggs were divided into four groups of 50 eggs each one using a completely randomized design. The eggs were injected with sterilized sodium bicarbonate buffer (pH=10.4) or RJ (88%) in a 0.3 mL volume into the albumen at d 7 of incubation. A non-injected control and a dry punch control (shell pricked without injecting solution; needle) were included. Chicks hatched from the respective treatment group were reared in floor pens until d 28. The chickens were challenged against Newcastle disease virus on days 7 and 21. On d 21, the chickens emanated from the RJ injected egg had the highest body weight. *In ovo* injection of RJ had a significant effect on feed intake in broiler chickens during starter period (d 1 to 21) and throughout the trial (d 1 to 28; P<0.05). On d 14, serum antibody titer against Newcastle disease virus (NDV) was not significantly different among treatments. On d 14 and 28, heterophil and lymphocyte number and their ratio were affected by *in ovo* injection. In conclusion, the results of the present study suggested that *in ovo* injection of RJ on d 7 of incubation exerted a beneficial effect on growth in starter phase and could stimulate feed intake in broiler chickens challenged with Newcastle disease virus, although the antibody titer against NDV was not influenced.

KEY WORDS broiler, immune system, *in ovo* injection, performance, royal jelly.

INTRODUCTION

Over the past 50 years, a tremendous genetic improvement of both broilers and layer stock has been achieved. Historically, genetic selection has played a significant role in improving production efficiency in poultry which brought about an 85 to 90% increase in broiler growth rate. However, selection for growth rate and efficiency in poultry had resulted in negative complications including ascites, re-

duced reproductive performance, skeletal abnormalities, immune-suppression and susceptibility to infectious disease (Havenstein *et al.* 1994; Emmerson, 1997; Cheema *et al.* 2003). On the other hand, a number of studies indicated that nutrient levels adequate for growth, feed efficiency, egg production and reproduction might not be efficient enough to develop normal immunity and for optimizing the bird's resistance to disease (Ohta *et al.* 1999; Kadam *et al.* 2008). Broiler nutrient needs for immunity may not be parallel to

those for growth. Utilization of immuno-stimulants is one solution to improve the immunity of animals and to decrease their susceptibility to infectious diseases (Liu, 1999).

In ovo feeding of nutrients and natural bioactive compound has been practiced for the manipulating embryonic growth in poultry (Gore and Qureshi, 1997; Johnston *et al.* 1997; Henry and Burke, 1999; Kocamis *et al.* 1999; Ohta *et al.* 1999; Jochemsen and Jeurissen, 2002; Tako *et al.* 2004; Uni *et al.* 2005; Foye *et al.* 2006; Kadam *et al.* 2008; Zhai *et al.* 2008; Keralapurath *et al.* 2010a; Keralapurath *et al.* 2010b). Some experiments using *in ovo* feeding technology have demonstrated that *in ovo* injection of nutrients influences physiological status of broiler embryos pre or post-hatch. Proper *in ovo* injection not only improved hatchability, but also coincided with a superior nutritional status in hatchlings, greater vigour and higher post hatch growth (Bhanja *et al.* 2004; Bhanja and Mandal, 2005).

Royal Jelly (RJ) is a honey bee secretion that is used in the nutrition of larvae as well as for feeding the queens. This compound is a rich dietary supplement for humans, containing organic amino acid and B-complex vitamins including pantothenic acid (vitamin B₅) and pyridoxine (vitamin B₆). The RJ is consisted of water (67%), crude protein (12.5%) and simple sugars such as monosaccharides (11%) (Simuth *et al.* 2003). It also contains a relatively high amount of fatty acids (5%), many trace minerals, several enzymes, antibacterial and antibiotic components and trace amounts of vitamin C, but none of the fat-soluble vitamins (Fujii, 1995). The component of RJ that causes a bee to develop into a queen appears to be a single protein known royalactin (Kamakura, 2011). It has been recently demonstrated that this component induces a similar phenotypical change in *Drosophila melanogaster*, marked by an increased body size and ovary development (Kamakura, 2011).

There is a possibility that injecting RJ into the hatching egg may provide some more nutrients, thereby aiding embryogenesis and promoting growth performance and early immunopotency in the broiler chickens. To our knowledge, potentially growth or immune-promoting effect of RJ in poultry has not been evaluated. Therefore, the aim of this experiment was to study the effect of *in ovo* injection of RJ on growth performance and immune response in broiler chickens challenged with Newcastle disease virus.

MATERIALS AND METHODS

Incubation

Hatching eggs (Ross 308; n=200) of the same flock obtained from a commercial hatchery (PAYGIR Chicken Meat Complex, Gorgan, Iran). The eggs were weighed individually and randomly assigned to either of four groups,

50 eggs each. After an 8-h pre-incubation period at 24 °C, the eggs were set on turning trays allotted to same side of trolleys for minimal possible position effect and transferred to the same incubator (Jamesway Incubator Company Inc., Ontario, Canada; specific dry-bulb temperature of 37.8 °C and wet-bulb temperature of 29 °C at 60% of relative humidity for 18 d) to avoid interassay variability. On d 6 of incubation, eggs were candled, unfertilized eggs or those with early embryonic mortality were discarded. The eggs were injected *in ovo* at 7 d of incubation with sterilized sodium bicarbonate buffer (SBB; pH=10.4) or RJ (88%) at 0.3 mL volume. In addition, a non-injected control (regular hatching egg) and a dry punch control (shell pricked without injecting solution) were included. The SBB or RJ injected into the albumen using a 1 mL BD Safety Glide™ insulin syringe with 29 G x 1 / 2 in. The injection site was sealed with sterile paraffin and the eggs were returned into the incubator. At early 19 d, the egg were transferred into the hatcher incubator and kept into pedigree hatching boxes. The hatched chicks were used for a grow-out study.

Birds and housing

One hundred and ninety two hatched chickens from the respective treatment group were weighed and divided into 4 treatments and 4 replicates. Animals were reared in floor pens with wood shavings as litter material. The pens were in a conventional facility with cyclic temperatures (minimum, 24 °C; maximum, 34 °C and 80 to 90% RH). The basal diets (mash form) were formulated to meet or exceed requirements by the NRC (1994) for broiler chickens (Table 1). The starter and the grower diets were provided from d 1 to 21 and from d 22 to 28, respectively. No antimicrobial, anticoccidial drugs or feed enzymes were included in the basal diets. Feed and water were provided *ad libitum* and lighting schedule was continuous (24 h/d).

Newcastle disease virus challenge protocol

The chickens were challenged against Newcastle disease virus (ND; PESTIKAL® LA SOTA SPF, Veterina Ltd., Zagreb, Croatia) on d 7 (eye drop) and on d 21 (nasal route).

The vaccine was packed in vials and each vial recommended for administration to 2500 chickens. The content of the 2500 dose of ND vaccine vial was reconstituted with the following dilution: one vial of vaccine to 7.5 mL of distilled water and each bird was inoculated with 1 drop. Finally, each chicken was received 10 times as high as the normal dosing prescribed for ND vaccine.

Performance parameters and immune responses

Chickens were weighed individually on d 21 and 28, and feed consumption was recorded on pen basis after a 4 h diet

withdrawal. Feed conversion ratios (FCR) were calculated as the ratio of feed intake to weight gain.

Blood samples were collected on d 14 and 28 from the wing vein (8 birds/treatment), centrifuge (3000 rpm/min), and the sera were decanted and stored at -20 °C await to assays for the antibodies against Newcastle disease virus (NDV), using the standard hemagglutination inhibition (HI) method (Allan and Gough, 1974).

Table 1 Ingredients and nutrient composition of basal diets for broiler chickens

Item	Starter 1 to 21 d	Grower 22 to 28 d
Ingredient (%)		
Corn	53.50	57.79
Soybean meal	37.29	33.00
Soybean oil	5.00	5.00
NaCl	0.45	0.40
Di-calcium phosphate	1.05	1.20
Limestone	1.51	1.51
DL-Methionine	0.20	0.10
Vitamin-mineral premix ¹	1.00	1.00
Nutrient composition²		
Calculated		
ME (kcal/kg)	3058	3100
Crude protein (%)	22.00	19.32
Lysine (%)	1.30	1.15
Methionine + Cysteine (%)	0.90	0.78
Arginine (%)	1.58	1.47
Calcium (%)	1.00	0.93
Available phosphorus (%)	0.45	0.38
Ca/P	2.22	2.44

¹ Supplied per kilogram of diet: vitamin A: 1500 IU; Cholecalciferol: 200 IU; vitamin E: 10 IU; Riboflavin: 3.5 mg; Pantothenic acid: 10 mg; Niacin: 30 mg; Cobalamin: 10 µg; Choline chloride: 1000 mg; Biotin: 0.15 mg; Folic acid: 0.5 mg; Thiamine 1.5 mg; Pyridoxine 3.0 mg; Iron: 80 mg; Zinc: 40 mg; Manganese: 60 mg; Iodine: 0.18 mg; Copper: 8 mg and Selenium: 0.15 mg.

² Based on NRC (1994) feed composition table.

An aliquot of blood in an EDTA-coated tube was used to determine the numbers of heterophil (H) and lymphocyte (L) as well as the H:L ratio. Blood smears were air dried (unfixed) and stained with May-Grünwald-Giemsa method. A minimum of 60 cells per film were examined by using light microscopy (Gross and Siegel, 1983). All leucocytes counts were examined by the same investigator.

Statistical analysis

Data were analyzed using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS, 2005). Duncan's multiple range test was used to compare the means. Statistical significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of *in ovo* injection of RJ or SBB on body weight (BW), feed intake (FI) and FCR in broiler chickens are pre-

sented in Table 2. BW did not significantly differ among treatments on d 1 and 28.

However, significant differences were noted on d 21 ($P < 0.05$) where the highest value was recorded for RJ group (592.35 ± 17.59 g). No significant differences were observed among the other groups ($P > 0.05$). Administrating of RJ increased FI in broiler chickens during starter period (1-21) and all over of the trial ($P < 0.05$). Similarly, the FCR value was the highest for the same group.

Effect of *in ovo* injection of treatments on the numbers of H, L, H:L ratio and antibody titer (AB_{NDV}) against NDV in broiler chickens are presented in Table 3. Although the titer was not affected on d 14, RJ and needle groups showed higher values as compared with SBB group on d 28. However, the observed differences were not statistically significant compared with control group (Table 3).

At 14 and 28 d of the grow-out period, H and L number and the H:L ratio were affected by *in ovo* injection. On d 14, injection of RJ caused a significant increase in the H number and H:L ratio while a significant decrease in the L number compared with other groups. The feature was the same on d 28. The role of RJ in the bee colony is to stimulate and increase the growth of larvae and metabolic processes (Bonomi *et al.* 2001). Effects of administrating RJ in experimental animals were reported after its injection or ingestion, where the most evident consequence was suggested to be the increased BW (Bonomi *et al.* 2001). In his review of biological and pharmacological effects of RJ, Chauvin (1968) found a discrepancy among the available reports. Bioactive components of RJ have not been fully evaluated. However, recent *in vitro* studies have shown that some RJ bioactive components affect a number of crucial physiological processes (Fujiwara *et al.* 1990; Bilikova *et al.* 2002; Bilikova *et al.* 2001). In the current study, broiler chickens in RJ group exhibited the highest feed consumption throughout the trial with the highest BW on d 21. There are several possible explanations for this result. In an animal experiment, RJ increased oxygen metabolism of tissues and activity in mice, due to increased concentration and use of blood glucose (Gonnard and N'guyen, 1957).

Royal jelly increases also tissue oxygen consumption and thus increases performance and endurance (Krylov and Sokolskii, 2000). This effect has been attributed to promoting effect of RJ on respiration and oxidative phosphorylation (Krylov and Sokolskii, 2000). An antioxidant property of RJ has also been demonstrated (Krylov and Sokolskii, 2000). Alternatively, there are a great number of dietary proteins with wide range of functional and biological properties, some of which are attributed to biologically active peptides (2 to 20 amino acid residues), which are showed to be inactive within the sequence of the food protein but are activated when digested *in vivo* (Cheison and Wang, 2003).

Table 2 Effect of *in ovo* injection of treatments on body weight (BW), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens challenged with Newcastle disease virus (Mean±SEM)¹

	Treatments			
	Control	Needle	SBB ²	RJ ³
BW (g)				
D 1	42.91±0.43	43.15±0.48	42.72±0.53	43.96±0.60
D 21	534.00±14.53 ^b	575.33±19.97 ^{ab}	538.90±13.22 ^b	592.35±17.59 ^a
D 28	825.43±35.47	791.56±38.88	838.66±25.30	849.16±30.04
FI (g/bird)				
1-21 d	853.80±31.29 ^c	973.60±55.43 ^b	956.00±21.32 ^b	1184.41±26.43 ^a
1-28 d	1962.80±52.13 ^b	1906.60±77.80 ^b	1823.00±41.08 ^b	2261.41±72.46 ^a
FCR (feed/gain)				
1-21 d	1.76±0.05 ^c	1.87±0.07 ^{bc}	1.97±0.05 ^b	2.19±0.07 ^a
1-28 d	2.55±0.11 ^{ab}	2.39±0.08 ^b	2.31±0.07 ^b	2.75±0.07 ^a

¹ The means within the same row with at least one common letter, do not have significant difference (P<0.05).

² SBB: sodium bicarbonate buffer.

³ RJ: royal jelly.

Table 3 Effect of *in ovo* injection of Royal jelly (RJ) on numbers of heterophil (H), lymphocyte (L), heterophil to lymphocyte ratios (H/L) and antibody titer against Newcastle disease virus (AB_{NDV}) in broiler chickens (Mean±SEM)¹

Parameters	Treatments			
	Control	Needle	SBB ²	RJ ³
	d 14 of experiment			
AB_{NDV}	0.87±0.18	1.00±0.18	1.00±0.37	0.87±0.29
H	11.78±6.19 ^b	11.62±5.70 ^b	11.16±4.04 ^b	24.62±7.93 ^a
L	48.22±6.19 ^a	48.38±5.70 ^a	48.84±4.04 ^a	35.38±7.93 ^b
H/L	0.26±0.15 ^b	0.25±0.17 ^b	0.23±0.10 ^b	0.79±0.46 ^a
	d 28 of experiment			
AB_{NDV}	0.66±0.49 ^{ab}	1.20±0.96 ^a	0.40±0.24 ^b	1.40±0.24 ^a
H	9.50±4.99 ^b	12.30±6.51 ^{ab}	15.60±9.60 ^{ab}	18.60±11.69 ^a
L	50.50±4.99 ^a	47.70±6.51 ^{ab}	44.70±9.60 ^{ab}	41.40±11.69 ^b
H/L	0.19±0.12 ^b	0.28±0.19 ^{ab}	0.41±0.30 ^{ab}	0.63±0.79 ^a

¹ The means within the same row with at least one common letter, do not have significant difference (P<0.05).

² SBB: sodium bicarbonate buffer.

³ RJ: royal jelly.

Once they are liberated in the body, bioactive peptides may act as regulatory compounds with hormone-like activity (Cheison and Wang, 2003). Bioactive peptides originating from RJ should be taken into account as potential modulators of various regulatory processes in the body (Simuth *et al.* 2003). There is growing scientific evidence confirming the concept that RJ proteins and peptide might act as a revitalization factor (Simuth *et al.* 2003). The proteins secreted by honeybee into its products have different functions in establishment of optimal development of honeybee colony (Hanes and Simúth, 1992). Hypopharyngeal, mandibular and head salivary glands are the sources of the most important honeybee proteins. These glands synthesize hundreds of various proteins and peptides which have an irreplaceable role in nutrition of the brood and its differentiation (Simuth *et al.* 2003). It has been shown that a 57-kDa protein in RJ, previously designated as royalactin, induces the differentiation of honeybee larvae into queens (Kamakura, 2011). Royalactin increased body size and ovary development and shortened developmental time in honeybees (Kamakura, 2011).

Furthermore, free amino acids contents of RJ normally range from 0.6 to 1.5%, the majority of which belongs to the L-series. The most representative are proline and lysine (Serra-Bonvehi, 1990; Oselli *et al.* 2003). Amino acids are known to influence the responses of poultry to disease challenge (Maroufyan *et al.* 2010a; Maroufyan *et al.* 2010b). During such a challenge, body proteins are normally broken down and amino acids are shunted away from growth, exploiting by specific cells to synthesize critical proteins which allow the bird to mount a successful immune response to a particular disease challenge (Maroufyan *et al.* 2010a). The NRC (1994) have lowered the recommended level of dietary lysine for 1 to 21 d-old chicks from 1.20 to 1.10% of diet (Kidd *et al.* 1997). Lysine is the second limiting amino acid and the reference amino acid for the ideal protein concept (Baker, 1994). However, commercial diets may utilize lysine levels above that considered adequate by the NRC (1994). These factors may explain the relatively good correlation between free amino acids in RJ and increasing performance in challenged broiler chickens in our works.

The present results also revealed that RJ injection decreased the number of L and increased that of H count as well as their ratio as compared to the control group. It has been reported that the number of lymphocytes in chicken peripheral blood decreases and the number of heterophils increases in response to stressors and to increasing levels of corticosterone (Gross and Siegel, 1983). This means that in *ovo* RJ injection could not help the chickens facing stressful condition.

The highest BW and increased H number and H:L ratio in the current study are consistent with those of Kowalski *et al.* (2002) and Huff *et al.* (2005) who found that the fast-growing poultry was more sensitive to stress and showed a much larger increase in corticosterone and the H:L ratio when exposed to stressful condition than did the slower-growing poultry (Kowalski *et al.* 2002; Huff *et al.* 2005). However, measurements of H:L ratios or corticosteroid levels cannot always be acceptable parameters as indicators of stress in poultry (Maxwell, 1993).

It appears that more reliable methods for measurements of immune system suppression, such as numbers and proportions of leukocytes, different immunological functions and lymphoid organs size are needed (Siegel, 1985; Freeman, 1987). Further studies are required to elucidate the detailed modulatory effects of both *in vitro* and *in vivo* administration of RJ to reveal potential effects of RJ in enhancement of different immune functions and disease resistance.

CONCLUSION

In conclusion, the results of the present study suggested that *in ovo* injection of RJ on d 7 of incubation exerted a beneficial effect on growth performance in starter phase and could stimulate feed intake in broiler chickens challenged with Newcastle disease virus, although the AB_{NDV} responses was not influenced.

REFERENCES

- Allan W.H. and Gough R.E. (1974). A standard haemagglutination inhibition test for Newcastle disease. A comparison of macro and micro methods. *Vet. Rec.* **95**, 120-123.
- Baker D.H. (1994). Ideal amino acid profile for maximal protein secretion and minimal nitrogen excretion in swine and poultry. Pp. 134-139 in Proc. Cornell Nutr. Conf., Ithaca, NY.
- Bhanja S.K., Mandal A.B. and Goswami T.K. (2004). Effect of *in ovo* injection of amino acids on growth, immune response, development of digestive organs and carcass yields of broilers. *Indian J. Poult. Sci.* **39**, 212-218.
- 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 Aus. J. Anim. Sci.* **18**, 524-531.
- Bilikova K., Hanes J., Nordhoff E., Saenger W., Klaudiny J. and Simuth J. (2002). Apisimin, a new serine-valine-rich peptide from honeybee (*Apis mellifera*) royal jelly: purification and molecular characterization. *Febs Lett.* **528**, 125-129.
- Bilikova K., Wu G. and Simuth J. (2001). Isolation of a peptide fraction from honeybee royal jelly as a potential antifouling factor. *Apidol.* **32**, 275-283.
- Bonomi A., Bonomi B.M. and Quarantelli A. (2001). Royal jelly in turkey feeding; Royal jelly in guinea-fowl feeding. *Riv. Sci. Anim.* **30**, 49-60.
- Chauvin R. (1968). Action physiologique et therapeutique des produits de la ruche. Traite de biologie de l'abeille. *Masson. Paris.* **13**, 116-154.
- Cheema M.A., Qureshi M.A. and Havenstein G.B. (2003). A comparison of the immune response of a 2001 commercial broiler with a 1957 random bred broiler strain when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* **82**, 1519-1529.
- Cheison S.C. and Wang Z. (2003). Bioactive milk peptides: Redefining the food-drug interphase. A review. *Afr. J. Food Nutr. Sci. Agric. Dev.* **3**, 29-38.
- Emmerson D.A. (1997). Commercial approaches to genetic selection for growth and feed conversion in domestic poultry. *Poult. Sci.* **76**, 1121-1125.
- Foye O.T., Uni Z. and Ferket P.R. (2006). Effect of *in ovo* feeding egg white protein, beta-hydroxy-beta-methylbutyrate and carbohydrates on glycogen status and neonatal growth of turkeys. *Poult. Sci.* **85**, 1185-1192.
- Freeman B.M. (1987). The stress syndrome. *World's Poult. Sci. J.* **43**, 15-19.
- Fujii A. (1995). Pharmacological effects of royal jelly. *Honeybee Sci.* **16**, 97-104.
- Fujiwara S., Imai J., Fujiwara M., Yaeshima T., Kawashima T. and Kobayashi K. (1990). A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. *J. Biol. Chem.* **265**, 11333-11337.
- Gonnard P. and N'guyen C.C. (1957). Action of royal jelly on oxygen consumption in tissues *in vitro*. *Ann. Pharmacol. Fr.* **15**, 383-393.
- Gore A.B. and Qureshi M.A. (1997). Enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. *Poult. Sci.* **76**, 984-991.
- Gross W.B. and Siegel H.S. (1983). Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis.* **27**, 972-979.
- Hanes J. and Simuth J. (1992). Identification and partial characterization of the major royal jelly protein of the honey bee (*Apis mellifera*). *J. Apic. Res.* **31**, 22-26.
- Havenstein G.B., Ferket P.R., Scheideler S.E. and Larson B.T. (1994). Growth, livability and feed conversion of 1957 vs. 1991 broilers when fed 'typical' 1957 and 1991 broiler. *Poult. Sci.* **73**, 1785-1794.
- Henry M.H. and Burke W.H. (1999). The effects of *in ovo* administration of testosterone or an antiandrogen on growth of chick embryos and embryonic muscle characteristics. *Poult. Sci.* **78**, 1006-1013.
- Huff G.R., Huff W.E., Balog J.M., Rath N.C., Anthony N.B. and Nestor K.E. (2005). Stress response differences and disease susceptibility reflected by heterophil to lymphocyte ratio in

- turkeys selected for increased body weight. *Poult. Sci.* **84**, 709-717.
- Jochemsen P. and Jeurissen S.H. (2002). The localization and uptake of *in ovo* injected soluble and particulate substances in the chicken. *Poult. Sci.* **81**, 1811-1817.
- Johnston P.A., Liu H., O'Connell T., Phelps P., Bland M., Tyczkowski J., Kemper A., Harding T., Avakian A., Haddad E., Whitfill C., Gildersleeve R. and Ricks C.A. (1997). Applications in *in ovo* technology. *Poult. Sci.* **76**, 165-178.
- Kadam M.M., Bhanja S.K., Mandal A.B., Thakur R., Vasani P., Bhattacharyya A. and Tyagi J.S. (2008). Effect of *in ovo* threonine supplementation on early growth, immunological responses and digestive enzyme activities in broiler chickens. *Br. Poult. Sci.* **49**, 736-741.
- Kamakura M. (2011). Royalactin induces queen differentiation in honeybees. *Nature*. **473**, 478-483.
- Keralapurath M.M., Corzo A., Pulikanti R., Zhai W. and Peebles E.D. (2010a). Effects of *in ovo* injection of L-carnitine on hatchability and subsequent broiler performance and slaughter yield. *Poult. Sci.* **89**, 1497-1501.
- Keralapurath M.M., Keirs R.W., Corzo A., Bennett L.W., Pulikanti R. and Peebles E.D. (2010b). Effects of *in ovo* injection of L-carnitine on subsequent broiler chick tissue nutrient profiles. *Poult. Sci.* **89**, 335-341.
- Kidd M.T., Kerr B.J. and Anthony N.B. (1997). Dietary interactions between lysine and threonine in broilers. *Poult. Sci.* **76**, 608-614.
- Kocamis H., Yeni Y.N., Kirkpatrick-Keller D.C. and Killefer J. (1999). Postnatal growth of broilers in response to *in ovo* administration of chicken growth hormone. *Poult. Sci.* **78**, 1219-1226.
- Kowalski A., Mormede P., Jakubowski K. and Jedlinska-Krakowska M. (2002). Comparison of susceptibility to stress in two genetic lines of turkey broilers BUT-9 and Big-6. *Pol. J. Vet. Sci.* **5**, 145-150.
- Krylov V. and Sokolskii C. (2000). Royal jelly (in Russian). *Agroprompoligrafist Krasnodar*. Pp. 214.
- Liu X.Y. (1999). Stress and immunity. Pp. 230-252 in *Poultry Immunology*. Yin, T.B. Ed. China Agriculture Press, Beijing, China.
- Maroufyan E., Kasim A., Hashemi S.R., Loh T.C., Hair Bejo M. and Davoodi H. (2010a). The effect of methionine and threonine supplementations on immune responses of broiler chickens challenged with infectious bursal disease. *Am. J. Appl. Sci.* **7**, 44-50.
- Maroufyan E., Kasim A., Hashemi S.R., Loh T.C. and Hair Bejo M. (2010b). Responses of performance and differential leukocyte count to methionine and threonine supplementations on broiler chickens challenged with infectious bursal disease in tropical condition. *Asian J. Biol. Sci.* **3**, 68-76.
- Maxwell M.A. (1993). Avian blood leucocyte responses to stress. *World's Poult. Sci. J.* **39**, 34-43.
- NRC. (1994). Nutrient requirements of poultry. 9th rev. Ed. National Academy Press, Washington D.C. USA.
- 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. *Poult. Sci.* **78**, 1493-1498.
- Oselli E., Caboni M.F., Sabatini A.G., Marazzan G.L. and Lercker G. (2003). Determination and changes of free amino acids in royal jelly during storage. *Apidol.* **34**, 129-137.
- SAS Institute. (2005). SAS Proprietary Software Release 9.1. SAS Inst. Inc., Cary, NC.
- Serra-Bonvehi J. (1990). Studies on the proteins and free amino acids of royal jelly. *Anal. Bromatol.* **42**, 353-365.
- Siegel H.S. (1985). Immunological responses as indicators of stress. *World's Poult. Sci. J.* **41**, 36-44.
- Simuth J., Bilikova K. and Kovacora E. (2003). Royal Jelly proteins as a tool for development of functional ingredients for health. Pp. 312-340 in Proc. 38th Congress of Apimondia, Ljubljana, Slovenia.
- Tako E., Ferket P.R. and Uni Z. (2004). Effects of *in ovo* feeding of carbohydrates and β -hydroxy- β -methylbutyrate on the development of chicken intestine. *Poult. Sci.* **83**, 2023-2028.
- Uni Z., Ferket P.R., Tako E. and Kedar O. (2005). *In ovo* feeding improves energy status of late-term chicken embryos. *Poult. Sci.* **84**, 764-770.
- Zhai W., Neuman S., Latour M.A. and Hester P.Y. (2008). The effect of *in ovo* injection of L-carnitine on hatchability of White Leghorns. *Poult. Sci.* **87**, 569-572.