Effects of in ovo injection of threonine on hatchability, intestinal morphology, and somatic attributes in Japanese quail (Coturnix japonica)

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ABSTRACT
A total of 540 Japanese quail eggs were in ovo injected threonine (THR) at day 11 of embryonic development to evaluate the effects of in ovo feeding of THR on the hatchability, somatic attributes, and gut morphology of hatchlings. A completely randomized experimental design with 9 treatments and 4 replicates of 15 eggs per replicate was used. The treatments were non-injected (control) eggs and those receiving in ovo injections of 0.05 or 0.1 ml of saline (diluent) with or without THR (5 mg/ml) in the air sac (IAS) or under the air sac (UAS). Although the number of hatched eggs injected with 0.05 ml diluent both with and without THR under the air sac was not significant in comparison to the control group, it was higher than that of the other injected treatments. Interestingly, the under air sac injection of 0.05 ml THR showed enhanced gut morphology in comparison with the other treatments. The dried yolk-free body weight of all chicks was not statistically significant. Overall, the present results suggest that the injection of 0.05 ml THR under the air sac may improve the intestinal morphology in newly hatched quail while having no negative effects on final yolk-free body weight.

1. Introduction
In recent years, significant research has been devoted to exploring the use of in ovo injection as a means of providing supplemental nutrients to broiler embryos. Such supplemental nutrients have included carbohydrates (Chen et al. 2010; Zhai et al. 2011; Dong et al. 2013), amino acids (Ohta & Kidd 2001; Foye et al. 2007; Kadam et al. 2008; Saki et al. 2013), L-carnitine (Keralapurath et al. 2010), and mannanoligosaccharides (Cheled-Shoval et al. 2011). These studies were conducted based on the assumption that the embryonic development of commercial strain broilers could be compromised by a limited availability of essential nutrients in the egg as a result of intense selection for rapid growth (Foye et al. 2006; Dong et al. 2013).

Several factors influence the successful use of in ovo-injected supplemental nutrients, namely the specific site and day of injection (Wakenell et al. 2002) and the type of nutrient, composition and volume of the carrier solution that is injected (McGruder et al. 2011a,b). As reported by Freeman and Vince (1974), there are four compartments (extra-embryonic coelom, allantoic sac, amnion cavity, and yolk sac) in a fertile broiler breeder egg on day 7 of incubation. In order to determine the best site for amino acid injection in broiler breeder eggs, Ohta and Kidd (2001) injected an amino acid solution into the different compartments of fertile eggs and concluded that an increase in subsequent chick body weight was achieved when the amino acid mixture was injected into the yolk sac and extra-embryonic coelom compared to the other egg compartments.

Despite the fact that moisture and fat levels in eggs are sufficient or in excess of embryonic needs, the levels of available amino acids and proteins are lower than those that are required for optimal growth and development (Ohta et al. 1999, 2001). The results of different experiments have shown that the in ovo injection of amino acids increases chick weight at the time of hatch (Al-Murrani 1982; Ohta et al. 1999).

Threonine (THR) serves a critical role in the physiological and biochemical processes of birds and consequently is the third limiting amino acid in commercial poultry diets. Bhanja and Mandal (2005) reported that THR is important for the growth of the embryo and that the injection of THR + glycine + serine into the fertile eggs of broiler breeders significantly increased the chick weight to egg weight ratio. THR is also important in the synthesis of amylase, mucin, and gamma-globulin as a result of its high inclusion in the structure of these compounds (Kadam et al. 2008). Due to the inclusion of THR in the structures of amylase and mucin, it has been proposed that the in ovo injection of THR may promote the nutrient digestion capability of embryos and post-hatch chicks. Kadam et al. (2008) concluded that yolk sac injections of 20–30 mg of THR promoted post-hatch growth and humoral responses of broiler chicks that were challenged with sheep red blood cells.

Growing interest and subsequent research concerning the in ovo injection of nutrients into the fertile eggs of different species have emerged (Foye et al. 2007; Kadam et al. 2008;
Bottje et al. 2010; Cheled-Shoval et al. 2011). However, to our knowledge, there has been little, if any, research into the effects of the in ovo injection of nutrients in quail eggs. The objective of this study, therefore, was to evaluate the effects of THR injections on the subsequent hatchability, intestinal morphology, and somatic attributes of Japanese quail eggs.

### 2. Materials and methods

#### 2.1. Incubation, injection method, and treatments

This experiment consisted of two parts: (1) from embryonic day 0 until hatch day, and (2) from hatch day until day 10 post-hatch. Japanese quail eggs were set in a single-stage incubator (Jamesway Incubator Company Inc., Ontario, Canada). The relative humidity and temperature in the incubator from 0 to 14 days were 68% and 37.8°C, respectively, and from 15 to 17 days they were 78% and 36.8°C, respectively.

Preliminary tests were performed in our laboratory to define the proper position for injection, the optimum volume of injecting solutions and dose of THR. We observed that among different selected depths of injection (1, 3, 5, 8, and 11 mm), 5 and 8 mm were the most suitable depths for the injections. Subsequently, different serum solutions with different THR doses were used, and resulted data (unpublished data) showed that among different serum solutions (0, 0.05, 0.1, and 0.2 ml) and doses of THR (0, 5, and 10 mg/ml) only lower volumes (0, 0.05, and 0.1 ml) and doses (0, 5, and 10 mg/ml) had no detrimental effects on hatchability.

After validating the best volume, doses, and injection depths, the primary experiment was conducted with a total of 540 Japanese quail eggs. On embryonic day 11 (E11), eggs were injected with two volumes of solutions (0.05 and 0.1 ml) containing physiological saline with or without THR (5 mg/ml). All injections were administered with the use of a 31-gauge needle in the air sac (IAS; depth of injection: 5 mm) or under the air sac (UAS; depth of injection: 8 mm) of quail eggs. The experiment was conducted as a completely randomized design with 9 treatments and 4 replicates of 15 eggs each. Treatments were (Table 1): (1) non-injected (control) group; (2) IAS injection of 0.05 ml saline (containing 0.9 g salt/litre of distilled water) (5SIAS); (3) UAS injection of 0.05 ml saline (5SUAS); (4) IAS injection of 0.1 ml saline (1SIAS); (5) UAS injection of 0.1 ml saline (1SUAS); (6) IAS injection of 0.05 ml saline containing 5 mg/ml of THR (5TIAS); (7) UAS injection of 0.05 ml saline containing 5 mg/ml of THR (5TUAS); (8) IAS injection of 0.1 ml saline containing 5 mg/ml of THR (1TIAS); and (9) UAS injection of 0.1 ml saline containing 5 mg/ml of THR (1TUAS). The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Iran.

#### 2.2. Gut morphology

On the hatch day, after measuring quails’ weights, three quails from each replicate (totally 12 per treatment) were euthanized by CO2 asphyxiation. The intestines were washed with distilled water and jejunum segments (from Meckel’s diverticulum to ileocecal junction) were removed for the morphological analysis. Samples were first fixed in 10% formalin and then stained with hematoxilin–eosin, as described by Khodambashi Emami et al. (2012).

Each jejunal sample from each quail was cross-sectioned four times and 10 measurements per cross-section (for a total of 480 measurements per treatment) were performed. The numbers of goblet cells were numerated under a light microscope after undergoing the acid-Schiff staining method, as described by Smirnov et al. (2006).

#### 2.3. Embryo and hatching parameters

Two days (E13) after the in ovo injection of solutions, two eggs from each replicate were broken, and body weight (BW), yolk sac weight (YSW), yolk-free body weight (YFBW), dry body weight (DBW), and dry yolk-free body weight (DYFBW) were measured. In addition, the ratio of BW was calculated as a percentage of set egg weight (BW/SEW).

On the hatch day, and in addition to the above-mentioned parameters (BW, YSW, YFBW, DBW, and DYFBW), the hatchability of settled eggs, intestine weight, and intestine weight to body weight ratio of hatched chicks were measured.

All weights were measured using a digital scale. For measuring DBW, and after measuring YSW and intestine weight, the carcasses of quail chicks were placed in an oven (60°C) for 48 h.

#### 2.4. Statistical analysis

All data were analysed using SAS statistical software (2004), and significant differences between means (P < .05) were determined by Tukey’s Honestly Significant Difference (HSD).

### 3. Results

#### 3.1. Gut morphology

The results of gut morphology are presented in Table 2. It was observed that neither the injected solution (THR or saline) nor site of injection (IAS or UAS) had any effect (P > .05) on villus
Table 2. Effect of in ovo injection of THR on morphological indices of jejunal villi in quail eggs on day of hatch.

| Treatments | Villus height (μm) | Villus width (μm) | Villus surface area (μm²) | Crypt depth (μm) | Villus height/crypt depth | Muscular layer (mm) | Goblet cells (n/villus) |
|------------|-------------------|------------------|---------------------------|-----------------|---------------------------|-------------------|----------------------|
| THR 0.05 IAS | 184.73bc | 40.92 | 27802ab | 29.95 | 6.54ab | 33.63 | 7.70ab |
| THR 0.05 UAS | 190.84abc | 43.89 | 31315a | 31.43 | 7.24c | 35.90 | 8.50a |
| THR 0.1 IAS | 182.66bc | 40.52 | 25461bc | 34.25 | 5.86b | 35.05 | 6.30c |
| THR 0.1 UAS | 186.08bc | 47.33 | 25131bc | 29.13 | 5.83b | 34.40 | 6.20c |
| Serum 0.05 IAS | 185.97bc | 41.89 | 28433ab | 30.56 | 5.80ab | 32.63 | 7.00bc |
| Serum 0.05 UAS | 187.11bc | 45.89 | 28236ab | 31.69 | 6.57ab | 32.08 | 7.60ab |
| Serum 0.1 IAS | 182.66bc | 51.07 | 25730bc | 31.31 | 5.87bc | 33.87 | 6.60bc |
| Serum 0.1 UAS | 181.82bc | 44.77 | 25530bc | 31.74 | 5.81b | 32.26 | 6.10c |
| Control | 180.42bc | 43.07 | 28561bc | 32.83 | 6.51ab | 33.51 | 7.10bc |

a,bValues within a column with different letters differ significantly (P ≤ .05).

4. Discussion

The results of gut morphology were in favour of the 5TUAS treatment and indicated a better or higher THR utilization by the embryo compared with those in which THR was injected IAS area. In the previous study, Ohta and Kidd (2001) observed that at the time of amino acid in ovo injection into the broiler breeder eggs, best results were obtained when the amino acid was injected into the yolk sac or extra-amniotic fluid. As tested, the area referred to as ‘under air sac’ in the current study is equivalent to the extra-amniotic fluid explained by Ohta and Kidd (2001).

An increased number of goblet cells per villi seen in 5TUAS were the most notable result obtained in the present study. The effect of THR on gut morphology has been previously well documented. Kaddam et al. (2008) reported that the injection of THR into the broiler breeder eggs could increase mucin secretion. Mucin is secreted by goblet cells, so an increase in the amount of mucin might be due to the higher rate of secretion by goblet cells or, as shown in this experiment, due to the higher number of goblet cells per villi.

Mucin secretion could be changed by proteins and some specific amino acids. Therefore, these nutrients might alter mucin production directly through interaction with goblet cells or the enteric nervous system (Azzam et al. 2011, 2012). For example, in rat models it has been reported that restricted amounts of THR in the diet decrease the rate of mucin synthesis (Faure et al. 2005) and that increasing the amount of different amino acids (THR, serine, proline, and cysteine) could improve the population of commensal bacteria in rats during colitis (Faure et al. 2006). In a more recent study, increased expression of mucin2 mRNA in the jejunum and ileum could improve the population of commensal bacteria in rats during colitis (Faure et al. 2006). In a more recent study, increased expression of mucin2 mRNA in the jejunum and ileum could improve the population of commensal bacteria in rats during colitis (Faure et al. 2006).
higher rate of enterocytes maturity and functional capacity (Chee et al. 2010). In addition, higher villus height and villus surface area might provide more surface area for nutrient absorption.

As stated by Chee et al. (2010), THR might also be directly involved in the synthesis of mucosa and mucin in the intestine and could thus have a direct effect on intestinal morphology. The same results have been shown by researchers who reported that THR deficiency has a detrimental effect on villus height (Hamard et al. 2007; Chee et al. 2010).

In agreement with the results of the current work, Zaefarian et al. (2008) found that villus height, crypt depth, epithelial thickness, and goblet cells number were all affected by THR supplementation (THR levels were 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, and 1.1%). Improved intestinal morphology (villus height, villus width, and crypt depth) was also reported by supplementing the broiler diet with 7.5 g/kg L-threonine (Rezaipour et al. 2012).

However, it should be noted that some reports have indicated no positive effect of THR supplementation on the intestinal morphology in poultry. For example, in the study by Azzam et al. (2012), intestinal villus height and mucosal thickness of laying hens was not affected by dietary THR supplementation (THR levels were 0.2% or 0.3%). In addition, dietary THR supplementation (0.7, 1.0, and 1.3 of National Research Council 1994, recommendations) had no significant effect on intestinal crypt depth and muscularity thickness of chickens (Chee et al. 2010).

Despite the effect of the 0.05 ml THR injection on gut morphology, it is not completely clear why injecting 0.1 ml of THR solution did not likewise affect gut morphology. One reason might be that the volume of 0.1 ml would be very high for injection into quail eggs. Another explanation might be that an amino acid imbalance occurred at the time of injecting the 0.1 ml THR solution. Kadam et al. (2008) observed that chick weight tended to be lower when broiler breeder eggs in ovo were injected with 40 mg of THR, and they concluded that the higher dose could have caused an amino acid imbalance. THR requires a special mechanism for its catabolism, and the activity of this pathway might not yet be developed well in the embryo (Kadam et al. 2008).

Similar hatchability and higher significant somatic attributes between THR-injected eggs and the control group have been observed, and it could be speculated that the higher BW at E13 and hatch day in 1T1AS, 1TUAS, 1S1AS, and 1S1US groups might be due to the higher SEW ratio in these groups rather than the possible effects of the injected solution.

In contrast with the results of this study, the injection of 2% and 3% L-arginine into eggs of 0-day-old quail embryos resulted in a significant improvement in the hatchability rate, initial body weight, and final body weight (Al-Daraji et al. 2012). In addition, improved chick weight to SEW ratio by in ovo injection of THR or Thr + Gly + Ser has been reported by Kadam et al. (2008) and Bhanja and Mandal (2005), respectively. Ohata et al. (1999) administered 53 mg of specific amino acids into Cobb strain broiler breeder eggs on day 7 of incubation and showed that the ratio of body weight of hatched chicks to egg weight improved without any effect on hatchability (Ohata & Kidd 2001). As stated by Ohata et al. (2001), the in ovo injection of amino acids might cause an increase in chick weight by increasing amino acid yolk content or by increasing amino acid utilization in the embryo.

The results of this study are in agreement with those of a previous work showing that the administration of disaccharide + hydroxy methyl butyrate to chick embryos significantly increased hatch weight and body weight at day 10 (Tako et al. 2004). In addition, it has been shown that THR could influence apparent metabolizable energy corrected for nitrogen and growth performance through extensive involvement in the intestinal mucosa and digestive enzymes (Rezaeipour et al. 2012).

Contrary to the results of the current study, previous studies have indicated that the effects of in ovo injection of nutrients are limited and that positive effects disappear after the chicks gain access to feed post-hatch. Research conducted by Chen et al. (2010) on in ovo injection of disaccharides combined with glutamine or hydroxy methyl butyrate at day 25 of ducklings’ eggs incubation indicated that the development and growth rate were, to some extent, attenuated after the ducklings had access to feed post-hatch.

### Table 4. Effect of in ovo injection of THR on performance parameters at day of hatch.

| Treatments       | Hatchability (%) | BW (g) | BW/SEW (%) | YSW (g) | YFBW (g) | DBW (g) | DYFBW (g) | Intestine (g) | Intestine/BW (%) |
|------------------|------------------|--------|------------|---------|----------|---------|-----------|---------------|------------------|
| THR 0.05 IAS     | 77.08ab          | 6.04d  | 76.27      | 0.148e  | 5.89c    | 2.064   | 1.258     | 0.475bc       | 7.15c            |
| THR 0.05 UAS     | 81.82a           | 5.45s  | 75.80      | 0.147e  | 5.30d    | 2.077   | 1.266     | 0.506a        | 7.93ab           |
| THR 0.1 IAS      | 68.75b           | 7.85s  | 76.45      | 0.414d  | 7.49b    | 2.059   | 1.255     | 0.468b        | 5.50d            |
| THR 0.1 UAS      | 68.75b           | 7.92s  | 75.96      | 0.427e  | 7.49b    | 2.072   | 1.263     | 0.475bc       | 5.45d            |
| Serum 0.05 IAS   | 72.73s           | 6.09s  | 76.29      | 0.148e  | 5.94c    | 2.064   | 1.258     | 0.465cd       | 7.09c            |
| Serum 0.05 UAS   | 81.82a           | 5.49s  | 75.81      | 0.148e  | 5.34d    | 2.076   | 1.265     | 0.459def       | 7.84b            |
| Serum 0.1 IAS    | 68.75b           | 7.82s  | 76.53      | 0.433b  | 7.39b    | 2.057   | 1.254     | 0.465def       | 5.52d            |
| Serum 0.1 UAS    | 68.18b           | 7.85s  | 76.03      | 0.446a  | 7.41b    | 2.070   | 1.262     | 0.456b        | 5.50d            |
| Control          | 83.33a           | 5.41i  | 75.74      | 0.146e  | 5.57b    | 2.079   | 1.267     | 0.475b        | 7.97a            |
| SEM              | 1.331            | 0.095  | 0.131      | 0.012   | 0.083    | 0.003   | 0.002     | 0.001         | 0.094            |
| P-value          | <0.0001          | <0.0001| <0.0001    | <0.0001 | <0.0001  | <0.0001 | <0.0001 | <0.0001       | <0.0001          |

**Note:** Means within a parameter with no common superscript differ (P ≤ 0.05).

**a**BW = body weight; BW/SEW = BW relative to set egg weight; YSW = yolk sac weight; YFBW = yolk-free BW; DBW = dry BW; DYFBW = dry yolk-free BW; Intestine/BW = intestine weight relative to BW.

**b**THR: threonine, IAS: in the air sac.

**c**THR: threonine, UAS: under the air sac.

**d**SEM: standard errors of means (results are given as means (n = 12) for all treatments).

5. Conclusion

The results of this study showed that the in ovo injection of THR in quail eggs can improve intestinal morphology by hatch day, although in ovo injection had no effects on somatic attributes.
An increase in villus height and goblet cells in in ovo chicks injected with 0.05 ml THR under the air sac (5TUAS) demonstrated this improvement, while the same results were not observed in other THR in ovo-injected groups. The present results also showed that quail eggs are satisfactory for testing in ovo feeding, and that it is possible to feed and support quail embryos’ growth through in ovo injection of critical nutrients. However, more research is required to determine the ultimate efficacy of in ovo injection as well as applicability of such method to the poultry industry.

Note

1. Day 13 of embryo.

Disclosure statement

No potential conflict of interest was reported by the authors.

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