Supplementation of two sources and three levels of iodine in the diet of laying hens: effects on performance, egg quality, serum and egg yolk lipids, antioxidant status, and iodine accumulation in eggs

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ABSTRACT
In total, 378 Shaver White layers were allocated into 7 treatments with 6 replicates, from 30 to 42 wk of age, to assess the effects dietary organic (ethylenediamine dihydroiodide [EDDI]) versus inorganic (calcium iodate [CIOD]) iodine in laying hens. A basal diet served as control while the remaining six diets were supplemented with either CIOD (CIOD2, CIOD4, and CIOD8) or EDDI (EDDI2, EDDI4, and EDDI8) to provide 2.0, 4.0, and 8.0 mg of added iodine/kg of diet, respectively. Performance and egg quality were not affected by adding 2.0 or 4.0 mg/kg of iodine to the diets. However, a progressive decline in egg performance and feed intake occurred with EDDI8 and CIOD8 diets. The EDDI8 diet also increased abnormal eggs in parallel with decreasing the eggshell strength and Haugh unit and disturbing the serum and egg yolk lipids. This trend was connected with increase in triiodothyronine (T3) and thyroxine (T4), which led to oxidative stress in serum and egg contents. The iodine levels of eggshell and egg contents were increased by dietary iodine in a dose and time-dependent manner, while the effect of EDDI was higher than CIOD at all levels. To summary, supplementation of diets with 2 or 4 mg/kg of iodine as CIOD and specifically EDDI increased the iodine content of eggs without adverse effect on hen performance and egg quality traits. However, considering the time-dependent nature of this increase, a 12-wk period of supplementation might not be sufficient to achieve a specified level of iodine in the eggs.

HIGHLIGHTS
- Diet supplementation with 2, 4, and 8 mg/kg of iodine as CIOD and EDDI increased iodine content of eggs.
- The highest level of iodine, especially as EDDI, diminished egg performance and egg quality connected with an increase in serum T3 and T4, which led to oxidative stress.
- A 12-wk period of supplementation appears to be not sufficient to achieve a specified level of iodine in the egg.

Introduction
Iodine as a component of thyroid hormones, triiodothyronine (T3) and thyroxine (T4), plays multiple functions as a regulator of cell metabolism and growth and as an essential factor for brain development (Li et al. 2012). Goitre, diminished growth, skeletal deformations, and mental retardation are the major symptoms of a deficient supply of iodine (Kotwal et al. 2007), whereas excess iodine intake reflects in elevated thyroid volume, thyroid autoimmunity, and thyroid dysfunction (Michalaki et al. 2018). Moreover, both deficiency and excess intake of iodine seem to increase the risk of thyroid cancer (Li and Wang 2004; Gärtner et al. 2010).

With the aim of increasing iodine levels in human diets, the application of iodised salt has been widespread. However, the effectiveness of this approach depends largely on the socioeconomic and cultural status of the considered population (Caffagni et al. 2011). Based on the World Health Organisation (WHO...
2019), about 8.5% of the global population has a daily iodine intake below the required level, which is between 65 and 290 µg, depending upon age, gender, and other factors (e.g. pregnancy and lactation). Therefore, alternative iodine delivery strategies are needed to complement the salt iodination programme.

One strategy is to supplement the diet of laying hens with iodine and thus to produce an egg with a higher content of iodine (Kaufmann and Rambeck 1998; Laurberg 2004). Egg is one of the few foods that are used widely worldwide and the per capita egg consumption is expected to touch three-digit marks by 2024 (Omid et al. 2013; Conway 2015). Furthermore, the rate of increase in egg consumption is predicted to be higher in developing countries than in the developed world (Ren 2009). Seizing this opportunity, several recent studies have investigated the transfer of iodine from feed into eggs of laying hens (Dobrzański et al. 2001; Yalçın et al. 2004; Opaliński et al. 2012; Röttger et al. 2012; Saki et al. 2012; Słupczyńska et al. 2014; Bakhshalinejad et al. 2018). However, the reported iodine concentration of eggs varies significantly due to the variation in age of hens, laying rate, and chemical methods used for iodine determination. For example, whereas Dobrzański et al. (2001) reported that supplemental dietary iodine at a level of 2.68 mg/kg increased the iodine content of the whole egg (yolk plus albumen) to about 1.05 µg/g, Słupczyńska et al. (2014) and Bakhshalinejad et al. (2018) reported that such a level of egg iodine was achieved only when the supplementary level of iodine increased to 5 and 13 mg/kg of diet, respectively. Therefore, the available data provide no support for any universal recommendation of a particular level of dietary iodine. This issue is further complicated by the fact that the excess of ingested iodine can cause an excessive amount of iodine in the egg and lead to thyroid dysfunction in human consumers (Flachowsky et al. 2006). It is necessary to note that the ratio of the tolerable upper limit of iodine intake for adults to the recommended level is lower than 5:1, which means that iodine can be toxic at daily intake levels as low as 750 µg (WHO 2019). Moreover, some studies have reported that high dietary iodine is associated with adverse effects upon productive performance, egg quality, and blood cholesterol in laying hens (Lichovnikova et al. 2003; Lewis 2004; Yalçın et al. 2004; Flachowsky et al. 2006; Opaliński et al. 2012; Röttger et al. 2012; Saki et al. 2012). Rodent studies have suggested that the association of excessive dietary iodine and elevated plasma cholesterol and triglycerides levels is mediated by oxidative stress pathways (Venditti et al. 1997; Joanta et al. 2006; Zhao et al. 2011). However, confirmation of this association in poultry studies is still pending.

Difference in sources of iodine could also contribute to the heterogeneity of the study results. In poultry diets, iodine may be used in the form of inorganic salts such as potassium iodide (KI), potassium iodate (KIO₃), and calcium iodate (Ca(IO₃)₂; CIOD), or organic salts like ethylenediamine dihydroiodide (EDDI) that are being mixed into the mineral mixtures (Miller and Ammerman 1995; European Commission 2016). Słupczyńska et al. (2014) reported that KI was more effective than KIO₃ in accumulation of iodine in the egg contents, whereas Röttger et al. (2012) reported that calcium iodate and KI had the same effects on performance of laying hens and on deposition of iodine in the egg contents. However, since KI is very unstable and spoils quickly with moderate exposure to heat, light, and moisture, CIOD is preferred to KI (Leeson and Summers 2009). Ammerman and Miller (1972) reported that the bioavailability of CIOD is approximately 95% in poultry. In a study with cows, Preston (1994) found that iodine was absorbed equally well from KI and EDDI, while CIOD had a relative bioavailability of 88% when compared with the other two sources, and Herzig et al. (2000) reported similar results in pigs. However, no scientific study has been published on the effect of EDDI in any type of poultry.

In view of these facts, the present study was carried out to evaluate the effects of various levels of CIOD and EDDI as supplemental iodine source in the diet of laying hens on productive performance, egg quality, serum and egg yolk lipids, antioxidant status, and iodine enrichment of eggs.

Materials and methods

Bird husbandry and experimental diets

A total of 378 Shaver White laying hens were used in this study. They were randomly allocated into 7 treatment groups of 54 hens split over 6 replicates of 9 birds each (3 adjacent cages containing 3 hens/40 × 50 × 40 cm). Hens were kept in cleaned and fumigated cages of wire floored batteries (Paravar Company, Sabzevar, Iran) in a closed system house. Each cage was equipped with a rectangular feeder outside the front wall, one nipple drinker on the back wall (36 cm above floor), and a manure collection pen underneath the wire-mesh floor. Feed and water were provided ad libitum and the room temperature was...
maintained at $21 \pm 2$ °C. Daily lighting programme conditions were controlled to 16 h of light and 8 h of dark, as used in commercial operations (Kleyn 2013).

A basal diet was used as the control and the other six diets were supplemented with either calcium iodate ($\text{ClIO}_2$, $\text{ClIO}_4$, and $\text{ClIO}_6$) or ethylenediamine dihydroiodide (EDDI$_2$, EDDI$_4$, and EDDI$_8$) to provide 2.0, 4.0, and 8.0 mg of iodine/kg of diet. CIOD was supplied by Merck (Darmstadt, Germany), while EDDI came from Sigma-Aldrich (Steinheim, Germany). The experimental period started at 30 wk of age and lasted until 42 wk of age. From 28 to 30 wk of age, all hens were fed the control diet to allow them to adapt to their diet and environmental conditions as well as to obtain baseline data (wk 0). The control diet (Table 1) was formulated (expected intake, 105 g/day) to meet or exceed the specifications for Shaver White commercial hen management guidelines (ISA 2011a). Chemical composition of the basal diet was determined in triplicate according to standard methods of AOAC (1995) for dry matter (method 930.15), crude ash (method 942.05), crude protein (method 984.13), and ether extract (method 920.39), and total phosphorus content was analysed by the molybdenum phosphoric acid procedure (method 965.17), and calcium concentration was analysed using atomic absorption spectrophotometry (method 968.08). All experimental procedures were approved by the Animal Care Committee at the Islamic Azad University, Isfahan (Khorasgan) Branch.

### Table 1. Ingredients and chemical composition of the basal diet.

| Item                        | Amount   |
|-----------------------------|----------|
| Ingredients (%)             |          |
| Corn                        | 54.46    |
| Soybean meal (44.0% crude protein) | 18.62   |
| Full fat soybean (35.9% crude protein) | 5.00    |
| Corn gluten meal (35.7% crude protein) | 3.00    |
| Wheat bran                  | 5.72     |
| Soybean oil                 | 1.05     |
| DL-methionine 98.5%         | 0.18     |
| L-lysine-HCl 98.0%          | 0.09     |
| Limestone coarse            | 5.45     |
| Limestone fine              | 4.00     |
| Dicalcium phosphate         | 1.54     |
| Common salt                 | 0.33     |
| Sodium bicarbonate          | 0.06     |
| Vitamin-mineral premix*     | 0.50     |
| Calculated composition (% as-fed basis unless indicated) |            |
| Metabolizable energy (MJ/kg) | 11.30   |
| Crude protein               | 17.40    |
| Methionine + cysteine       | 0.75     |
| Lysine                      | 0.90     |
| Calcium                     | 3.90     |
| Available phosphorus        | 0.36     |
| Analysed composition (% as-fed basis) |          |
| Dry matter                  | 90.00    |
| Crude ash                   | 11.20    |
| Crude protein               | 17.43    |
| Ether extract               | 4.17     |
| Crude fibre                 | 3.29     |
| Calcium                     | 3.96     |
| Total phosphorus            | 0.63     |

*Provided per kilogram of diet: vitamin A (as retinyl acetate), 9000 IU; cholecalciferol, 4000 IU; vitamin E (as dl-α-tocopheryl acetate), 63 mg; vitamin K$_0$, 2.3 mg; thiamine, 2.6 mg; riboflavin, 6.5 mg; niacin, 48 mg; D-calcium pantothenic acid, 15 mg; vitamin B$_6$, 3.0 mg; biotin, 0.2 mg; chlorine chloride, 770 mg; vitamin B$_1$, 0.02 mg; folic acid, 1.7 mg; manganese (as MnSO$_4\cdot$H$_2$O), 105 mg; iron (as FeSO$_4\cdot$H$_2$O), 38 mg; zinc (as ZnO), 126 mg; copper (as CuSO$_4$), 16 mg; selenium (as Na$_2$SeO$_3$), 0.28 mg.

### Measurements, sampling, and analysis of the samples

Feed consumption was recorded at the end of each week of the experimental period. Daily egg production and egg weight were monitored during the trial. The egg production percentage was calculated as the total number of eggs produced divided by the number of birds in each replicate. The rate of production of abnormal eggs such as soft-shelled, cracked, and broken was also assessed daily. The egg mass was calculated by multiplying egg weight by daily egg production, while the feed to egg ratio was calculated by dividing the total feed consumed by total egg mass. Body weights of the birds were also recorded every week. Feed intake, egg production, egg weight, egg mass, feed to egg ratio, body weight changes, and abnormal egg percentage were calculated and analysed every 6 wk. Line graphs with weeks (wk 0–12) as $X$ (abscissa axis) and performance data as $Y$ (ordinate axis) were drawn.

Egg quality assessments were performed 1 wk before the start of the study and then every 3-wk during the treatment phase. All eggs laid in the last 3 d of each period were used for measurements. Weight of each egg sample and the albumen, yolk, and shell weights were measured with a sensitive weighing scale (Radwag PS.R1, Toruńńska 5, 26-600 Radom, Poland) to the nearest 0.01 g. Relative yolk, albumen, and shell weights were expressed as the percentage of egg weight. Egg albumen quality (Haugh units) was evaluated by a P6085 spherometer (tripod micrometer) having an accuracy of 0.01 mm. The yolk index was determined as the ratio of yolk height to yolk width and yolk colour was compared to the Roche yolk colour fan, which ranges from a pale yellow at score 1 to a dark orange at score 15 (Vuilleumier 1969). Eggshell thickness was determined by a micrometer gauge (Ames, Waltham, MA, USA), while eggshell breaking strength was measured by using an eggshell force gauge (Robotmation Co. Ltd., Tokyo, Japan). The shells were ground to pass through a 20-mesh sieve for calcium and phosphorus analysis. The ground eggshell...
samples were ashed at 750 °C for 2 h and then cooled. The ashed samples were slightly moistened by water, and 10 mL 3 N HCl was added. The subsequent procedures were the same as the AOAC methods (AOAC 1995).

After measuring the egg quality, yolk samples from each replicate were separated from the broken eggs and extracted to determine yolk cholesterol and triglycerides. One gram of each egg yolk was homogenised with 19 mL of chloroform-methanol 2:1 (by volume), sonicated, and filtered as detailed elsewhere (Elkin and Rogler 1990). This solution was analysed to determine triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol using enzymatic colorimetric assay kits (Pars Azmun, Tehran, Iran) as per recommended protocols.

At the same week intervals, 5 mL of blood was drawn from the brachial vein of 18 birds per treatment (3 per replicate) into clean sterilised tubes. The serum was obtained by centrifugation of blood at 1500 ×g for 15 min at room temperature and stored at −20 °C until analysis. The serum was analysed for triglycerides, total cholesterol, and lipoprotein cholesterol fractions using the same kits described above for the egg yolk analysis. Serum T3 and T4 concentrations were assessed for antioxidant status using the spectrophotometric methods. The egg contents were homogenised (1:10) in medium containing 120 mM potassium chloride and 30 mM phosphate buffer (pH 7.4) and were centrifuged (800 ×g, 10 min, −4 °C) and then the supernatant fractions and sera were measured for malondialdehyde (MDA) and protein carbonyl (PC) levels and activity of superoxide dismutase (SOD). The MDA levels were measured by the thiobarbital technique (Buege and Aust 1978), whereas the PC levels were determined by measurement of 2,4-dinitrophenylhydrazine derivatives of protein carbonyls (Parissis et al. 2009). The SOD activity was examined by the xanthine oxidase method (Sun et al. 1988), which monitors the inhibition of the reduction of nitroblue tetrazolium and the change of absorbance at 560 nm.

Iodine concentrations in feed and egg compartments (yolk, albumen, and eggshell) were assessed using inductively coupled plasma mass spectrometry (Perkin Elmer, Elan 6000, Toronto, Canada) after digestion with HNO₃-HClO₄ (Benkhedda et al. 2009).

### Statistical analysis

Data analysis was performed by SAS 9.1 (SAS 2003). Assumptions of normal distribution (Shapiro-Wilk’s test) and homogeneity of variance (Bartlett’s test) were checked before analysis. Differences among the treatment groups over time were first tested using multivariate ANOVA (MANOVA) with repeated measures. The MANOVA revealed that either time effects or a time × treatment interaction or both existed in almost all measures, so data were then analysed using the GLM procedure for variance based on each time point of the measurements. Least squares means (LSmeans) were computed for the dietary treatments, and the SLICE and PDIF options within the GLM procedure were used to accurately compare untreated controls to iodine-fed birds, iodine sources (CIOD and EDDI), iodine inclusion levels (2.0, 4.0, and 8.0 mg/kg), and the source × inclusion level interactions within each time period. This analysis was similarly conducted to assess the effect of time on each treatment separately. Differences between treatment and control were assessed by Dunnett’s test, whereas other differences were compared by Tukey’s test. Statistical significance level was set at $p < .05$. The pre-treatment values of each variable were used for covariate adjustment where appropriate. For performance characteristics, the mean of each pen was used as the experimental unit, whereas individual hen data were used for other variables, and the pen was considered a random effect.

### Results

#### Iodine content in the diets

Feed iodine analyses indicated values slightly above the expected levels (Table 2), which can be explained based on iodine present naturally in the feed matrix, as determined in the control diet. The results did not indicate significant differences between treatments of

| Iodine source | Theoretical iodine supplementation (mg/kg of feed) | Analysed iodine level (mg/kg of feed) |
|---------------|---------------------------------------------------|-------------------------------------|
| Control       | 0                                                 | 0.87                                |
| CIOD †        | 2.0                                               | 2.85                                |
|               | 4.0                                               | 4.85                                |
|               | 8.0                                               | 8.84                                |
| EDDI ‡        | 2.0                                               | 2.89                                |
|               | 4.0                                               | 4.81                                |
|               | 8.0                                               | 8.81                                |

*Values are based on triplicate determinations and presented as means (as-fed basis).
†CIOD: calcium iodate; ‡EDDI: ethylenediamine dihydroiodide.
the same targeted iodine level for different iodine sources.

**Hen productivity**

A summary of performance data is depicted in Figure 1, and the results of the statistical analysis of the data are given in Table 3. As Figure 1 illustrates, all performance variables were similar at the beginning of the experiment. Egg production in the control group showed a slight increase up to 5 wk, but then decreased slowly during the rest of the treatment period (Figure 1(A)). No change was recorded in egg production when supplemental iodine amounts of 2.0 and 4.0 mg/kg were added to the diet as either CIOD (ClO\textsubscript{2}I and ClO\textsubscript{4}I) or EDDI (EDDI\textsubscript{2} and EDDI\textsubscript{4}). Hens receiving the CIOD\textsubscript{8} diet also gave an egg production pattern similar to the control until 5 wk after treatment, but then showed a decrease below the control afterward, so that a marked reduction (about 4.5%) in egg production was observed in the second 6-wk period of the study (p < .05). However, when the EDDI\textsubscript{8} diet was offered to the birds, the egg production decreased more rapidly, starting at wk 2, and was consistently lower than the control by about 4 and 10% during the first and second 6-wk periods, respectively (p < .05). These differences were reflected in significant interactions between level and source of iodine at both the first and second 6-wk periods (p < .05). The production of abnormal eggs increased during the second 6-wk period (p < .05), but this increase was about twice as great in birds fed the EDDI\textsubscript{8} diet as in those fed the control or other iodine-supplemented diets (p < .05).

Egg weight increased gradually over time (p < .05; Figure 1(B)), which was the same in all treatments. Thus, the egg mass followed the same trend as the egg production (Figure 1(C)). Feed intake increased as birds grow older (p < .05; Figure 1(D)), except when birds provided with the highest level of iodine. However, there was an interaction between iodine level and source on feed intake (p < .05). Hens receiving the EDDI\textsubscript{8} diet showed an increasing decline in their consumption from wk 3 until the end of the study, with less than a 2% drop than the control in the first 6-wk as compared with a greater than 7% drop in the second 6-wk of the treatment (p < .05). In birds receiving the CIOD\textsubscript{8} diet feed intake was relatively constant in the second 6-wk and remained less (2.3%) than those of the control (p < .05). Feed to egg ratio showed an opposite tendency to those observed for egg production and feed intake in different treatment groups (Figure 1(E)). Regardless of dietary iodine, the mean body weight showed an age-related increase, but this increase was less pronounced in the second 6-wk period (p < .05; Figure 1(F)).

**Table 3. Effects of dietary treatments on performance variables in laying hens (30–42 weeks of age).**

| Item                                | Main effects |
|-------------------------------------|--------------|
|                                    |              |
|                                     | Iodine as CIOD (mg/kg) | Iodine as EDDI (mg/kg) | Iodine Source | Iodine level (mg/kg) | Pooled SEM\# |
|                                     | Control 2.0 4.0 8.0 | Control 2.0 4.0 8.0 | CIOD | EDDI | CIOD | EDDI |
| EGG production (%)                  |              |
| 1–6 wk                              | 96.69 96.70 96.52 96.01 96.59 96.70 92.60 | 96.18 96.21 96.62 | 96.41 96.30 96.64 96.61 | 0.036 |
| 7–12 wk                             | 96.40 96.24 96.25 92.12 96.18 96.21 86.62 | 94.87 93.00 96.21 | 96.23 89.37 |              |
| Egg weight (g)                      |              |
| 1–6 wk                              | 60.22 60.07 60.02 60.06 60.22 60.17 59.77 | 60.05 60.06 60.15 60.10 59.92 | 0.105 |
| 7–12 wk                             | 61.20 61.22 61.12 61.06 61.22 61.22 60.89 | 61.13 61.11 61.22 61.17 60.97 |              |
| Egg mass (g/ hen/d)                 |              |
| 1–6 wk                              | 58.22 58.09 57.94 56.66 58.17 58.18 55.36 | 57.90 57.24 58.13 58.06 56.51 | 0.210 |
| 7–12 wk                             | 59.00 58.91 58.83 56.24 58.88 58.90 52.75 | 57.99 56.84 58.90 58.87 54.50 |              |
| Feed intake (g/hen/d)               |              |
| 1–6 wk                              | 104.92 104.89 104.62 104.87 104.79 104.80 102.58 | 104.79 104.06 104.84 104.74 103.73 | 0.226 |
| 7–12 wk                             | 106.65 105.98 106.07 104.20 106.02 105.85 98.96 | 104.52 103.62 106.00 105.96 101.59 |              |
| Feed to egg ratio (g/g)             |              |
| 1–6 wk                              | 1.80 1.81 1.81 1.82 1.80 1.80 1.85 | 1.81 1.82 1.80 1.80 1.84 | 0.004 |
| 7–12 wk                             | 1.81 1.80 1.80 1.85 1.80 1.80 1.88 | 1.82 1.83 1.80 1.80 1.87 |              |
| Body weight change (g)              |              |
| 1–6 wk                              | 42.50 45.17 40.67 41.17 43.17 42.83 42.33 | 42.33 42.78 44.17 41.75 41.75 1.698 |
| 7–12 wk                             | 22.50 22.50 23.15 21.67 22.67 22.50 24.33 | 22.44 22.83 22.08 22.82 23.00 |              |
| Abnormal eggs (g)                   |              |
| 1–6 wk                              | 0.25 0.19 0.14 0.22 0.28 0.16 0.30 | 0.18 0.25 0.24 0.15 0.26 0.024 |
| 7–12 wk                             | 0.47 0.36 0.34 0.40 0.47 0.32 0.94 | 0.37 0.58 0.41 0.33 0.67 |              |

\*In each row within each time period LSmeans with an asterisk indicates a significant difference from control (p < .05); CIOD: calcium iodate; EDDI: ethylenediamine dihydroiodide; SEM: standard error of LSmeans; Abnormal eggs: the percentage of all cracked, broken soft-shelled, and shellless eggs of total laid eggs; wk: week.
Egg quality

Data on external egg quality traits are shown in Table 4, while Table 5 summarises the results of internal egg quality traits. The values of all measured variables were similar for all treatment groups at the beginning of the experiment. Eggshell thickness decreased parallel with the reduction of the shell as a percentage of egg weight as the birds advanced in age \((p < .05)\), while percentage contents of calcium and phosphorus remained relatively constant, without any difference among the experimental treatments. Eggshell strength showed a steady state in all groups \((p < .05)\), except in group receiving the EDDI \(_8\) diet, which revealed a decreasing trend in eggshell strength from the 6 wk \((p < .05)\). This trend led to significant differences between the controls versus the EDDI \(_8\) treatment as well as significant differences between the EDDI \(_8\) and other iodine-supplemented treatments at the 9 and 12 wk stages \((p < .05)\).

Yolk weight percentage increased steadily, while albumen weight percentage dropped considerably as the hens aged \((p < .05)\), whereas no change from the control levels occurred in the other groups. No statistically significant difference in the yolk index or yolk colour resulted from the addition of iodine to the diet. All treatment groups showed a declining trend in Haugh unit as the birds became older, but the most
A reduction occurred with the EDDI8 diet that reached a statistical significance than all other groups at the 12 wk stage (p < .05; EDDI8 versus control and iodine source x iodine level interaction).

**Serum and egg yolk lipids**

The serum and egg yolk lipid contents are listed in Tables 6 and 7, respectively. The serum and egg yolk lipids (triglycerides, total, HDL, and LDL cholesterol) were not different at the beginning of the experiment. However, up to the 6 wk sampling, the increasing trend of each variable was greatly affected by its initial level (p < .05). Hens treated with the EDDI8 diet exhibited much more increase in the levels of triglycerides, total cholesterol, and LDL-cholesterol that accompanied by a much less increase in the level of HDL cholesterol, though the pattern of treatment differences indicated significance only at the 12 wk stage (p < .05; EDDI8 versus control and iodine source x iodine level interaction).

**T3 and T4 levels and antioxidant status**

Table 8 shows the serum concentrations of T3 and T4 and the results of antioxidant activity can be seen in Table 9. The values of these parameters were not different at the baseline. The serum T4 concentration showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05). This was confirmed by significant interactions between source and level of iodine (p < .05). The serum concentration of T3 showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05). This was confirmed by significant interactions between source and level of iodine (p < .05). The serum concentration of T3 showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05). This was confirmed by significant interactions between source and level of iodine (p < .05). The serum concentration of T3 showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05). This was confirmed by significant interactions between source and level of iodine (p < .05). The serum concentration of T3 showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05). This was confirmed by significant interactions between source and level of iodine (p < .05). The serum concentration of T3 showed, respectively, about 17 and 30% increase in T4 concentrations during this period (p < .05).
about 12 and 71% and 24 and 108% in the former and latter groups, respectively, and reflected in an interaction between the source and level of iodine as well (p < 0.05). Both groups also represented a parallel increase in serum and egg SOD activities (p < 0.05), while an increase in MDA and PC contents could only be found in that receiving the EDDI8 diet (p < 0.05).

**Iodine accumulation in eggs**

The concentrations of iodine in egg yolk, egg albumen, and eggshells are shown in Table 10. The initial iodine levels were not different at the beginning of the study. The control group revealed a slight (non-significant) decrease in iodine levels as progressed in age, while all egg compartments showed gradually increases in iodine contents as the level of iodine increased in the diet (p < 0.05). The effect of EDDI, however, was superior to that of CIOD at all tested levels, as indicated by significant source x level interactions during the treatment period (p < 0.05).

**Discussion**

The productivity of the control hens was acceptable for this age (ISA 2011b), and none of the production traits were influenced by dietary supplementation of 2 or 4 mg/kg of iodine, indicating that the iodine content in the basal diet (0.87 mg/kg) might be to meet the iodine requirement for laying hens. Röttger et al. (2012) also reported that dietary iodine levels of 0.4–5.0 mg/kg (as CIOD or KI) had no significant effect on egg production, egg weight, and egg mass. This result is consistent with the recommendation given by the Gesellschaft für Ernährungsphysiologie (GfE 1999) that a 0.5 mg/kg of dietary iodine is adequate for maximal productivity in laying hens. In contrast, Damaziak et al. (2018) found that the dietary addition of 10 mg/kg of iodine (as CIOD) caused beneficial effect on laying performance of hens in terms of egg production, egg weight, and feed conversion efficiency. This is while, in the present study, a progressive decline in egg production was noticed when the supplementation level of iodine increased to 8 mg/kg. Also, this reduction began earlier and proceeded to a greater
Table 6. Effects of dietary treatments on serum triglyceride and cholesterol levels (29–42 wk of age).

| Item                  | Control | Iodine as CIOD | Iodine as EDDI | Pooled SEM |
|-----------------------|---------|----------------|---------------|------------|
| Triglycerides (mg/dL) |         |                |               |            |
| 0 wk                  | 0.053   | 0.046          | 0.050         | 0.060      |
| 3 wk                  | 0.052   | 0.046          | 0.050         | 0.060      |
| 6 wk                  | 0.051   | 0.046          | 0.050         | 0.060      |
| 9 wk                  | 0.050   | 0.046          | 0.050         | 0.060      |
| 12 wk                 | 0.049   | 0.046          | 0.050         | 0.060      |
| Total cholesterol (mg/dL) |        |                |               |            |
| 0 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 3 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 6 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 9 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 12 wk                 | 20.07   | 20.10          | 20.09         | 20.08      |
| LDL cholesterol (mg/dL) |        |                |               |            |
| 0 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 3 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 6 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 9 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 12 wk                 | 3.62    | 3.62           | 3.62          | 3.62       |
| HDL cholesterol (mg/dL) |        |                |               |            |
| 0 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 3 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 6 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 9 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 12 wk                 | 44.81   | 44.74          | 44.77         | 44.74      |

Table 7. Effects of dietary treatments on egg yolk triglyceride and cholesterol levels (29–42 wk of age).

| Item                  | Control | Iodine as CIOD | Iodine as EDDI | Pooled SEM |
|-----------------------|---------|----------------|---------------|------------|
| Triglycerides (mg/g)  |         |                |               |            |
| 0 wk                  | 0.053   | 0.046          | 0.050         | 0.060      |
| 3 wk                  | 0.052   | 0.046          | 0.050         | 0.060      |
| 6 wk                  | 0.051   | 0.046          | 0.050         | 0.060      |
| 9 wk                  | 0.050   | 0.046          | 0.050         | 0.060      |
| 12 wk                 | 0.049   | 0.046          | 0.050         | 0.060      |
| Total cholesterol (mg/g) |        |                |               |            |
| 0 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 3 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 6 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 9 wk                  | 20.07   | 20.10          | 20.09         | 20.08      |
| 12 wk                 | 20.07   | 20.10          | 20.09         | 20.08      |
| LDL cholesterol (mg/g) |        |                |               |            |
| 0 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 3 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 6 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 9 wk                  | 3.62    | 3.62           | 3.62          | 3.62       |
| 12 wk                 | 3.62    | 3.62           | 3.62          | 3.62       |
| HDL cholesterol (mg/g) |        |                |               |            |
| 0 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 3 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 6 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 9 wk                  | 44.81   | 44.74          | 44.77         | 44.74      |
| 12 wk                 | 44.81   | 44.74          | 44.77         | 44.74      |
Table 8. Effects of dietary treatments on serum triiodothyronine (T3) and thyroxine (T4) concentrations (29–42 wk of age).

| Item | T3 (ng/mL) | T4 (ng/mL) |
|------|------------|------------|
|      | Control    |            |
| 0 wk | 1.45  1.44 1.42 1.44² |            |
| 3 wk | 1.46  1.45 1.43 1.46² |            |
| 6 wk | 1.36  1.32 1.37 1.35³ |            |
| 9 wk | 1.46  1.47bcc 1.44bc 1.63bc,²,³ |            |
| 12 wk | 1.48  1.45c 1.48c 2.49bc,²,³ |            |

Table 9. Effects of dietary treatments on antioxidant measurements in serum and egg contents (29–42 wk of age).

| Item | Serum SOD (U/mL) | Egg SOD (U/mg) |
|------|-----------------|----------------|
|      | Control         |                |
| 0 wk | 139.61          | 48.27          |
| 3 wk | 141.90          | 48.37          |
| 6 wk | 142.00          | 48.47          |
| 9 wk | 140.42          | 48.57          |
| 12 wk | 143.00        | 48.67          |

| Item | Iodine as ClOD³ (mg/kg) | Iodine as EDDI² (mg/kg) | Iodine source | Iodine level (mg/kg) |
|------|------------------------|------------------------|---------------|---------------------|
|      | Control                 |                        |               |                     |
| 0 wk | 1.45  1.44 1.42 1.44³ | 1.46  1.43 1.41³      | 1.43          | 1.45  1.43 1.42     |
| 3 wk | 1.46  1.45 1.43 1.44³ | 1.49  1.42 1.46³      | 1.44          | 1.46  1.42 1.45     |
| 6 wk | 1.36  1.32 1.37 1.35³ | 1.31  1.37 1.35³      | 1.35          | 1.32  1.37 1.35     |
| 9 wk | 1.46  1.47bcc 1.44bc 1.63bc,²,³ | 1.42bc 1.41î 1.78bc,²,³ | 1.51  1.53 | 1.4b  1.4b 1.71³     |
| 12 wk | 1.48  1.45c 1.48c 2.49bc,²,³ | 1.45c 1.44î 3.01î,²,³ | 1.81î 1.97³ | 1.45î 1.46î 2.75³     |

Note: All values are mean ± SEM.
extent with the EDDI8 diet than with the CIOD8 diet. A corresponding decrease in egg mass was also evident, because egg weight did not respond to dietary treatments. Such a difference is rather surprising taking into account the similarity of our findings to those of previous published reports. Lichovnikova et al. (2003) reported an age-dependent decrease in egg production and egg mass when the iodine level of diet increased from 3.57 to 6.07 mg/kg by using a higher level of KI. Likewise, in his review of literature, Lewis (2004) found that birds tend generally, but not always, to adjust their egg production rather than egg weight to adjust their egg production rather than egg weight when excessive levels of dietary iodine are utilised. Arrington et al. (1967) indicated that the reason for the prevention of follicular development (Marcilese et al. 2001), and because of the major accumulation of iodine in the thyroid gland and its indispensable role in T3 and T4 synthesis, it is highly probable that the effects of excess iodine on egg production are mediated by changes in thyroid hormone release (Travnicek et al. 1999). Sechman (2013) showed that T3 treatment resulted in lower concentrations of LH (luteinizing hormone) and sex-steroid hormone in blood circulation, which in turn, led to atresia of preovulatory follicles and stoppage of egg-laying. In support of this view is our observation that the serum concentrations of T3 and T4 increased while the percentage of abnormal (soft-shelled, cracked, and broken) eggs occurred, in line with this result is the finding that irrespective of the same source of supplement, Bakhshalinejad et al. (2013) showed that T3 treatment resulted in lower concentrations of LH (luteinizing hormone) and sex-steroid hormone in blood circulation, which in turn, led to atresia of preovulatory follicles and stoppage of egg-laying. Moreover, during the second 6-wk period of the present study, an increase in the percentage of abnormal (soft-shelled, cracked, and broken) eggs occurred, which was about twice as high in birds fed the EDDI8 diet. However, using the CIOD8 diet. This increase well corresponded with the decrease in eggshell strength resulted in a reduction of eggshell strength that was independent of weight and thickness of the eggshell. Therefore, the detrimental effect of the

| Item | Control | 2.0 | 4.0 | 8.0 | SEM |
|------|---------|-----|-----|-----|-----|
| Yolk iodine (µg/g) | 0 wk | 0.507 | 0.499 | 0.500 | 0.506 | 0.510 | 0.532 | 0.521 | 0.502 | 0.521 | 0.505 | 0.516 | 0.513 | 0.0162 |
| | 3 wk | 0.493 | 0.524 | 0.606 | 0.804 | 0.596 | 0.683 | 0.965 | 0.643 | 0.750 | 0.560 | 0.647 | 0.884 | |
| | 6 wk | 0.472 | 0.649 | 0.751 | 0.949 | 0.704 | 0.850 | 1.174 | 0.777 | 0.909 | 0.672 | 0.800 | 1.057 | |
| | 9 wk | 0.449 | 0.685 | 0.819 | 1.311 | 0.765 | 1.078 | 1.477 | 0.940 | 1.107 | 0.727 | 0.949 | 1.394 | |
| | 12 wk | 0.404 | 0.733 | 0.968 | 1.559 | 0.903 | 1.163 | 1.676 | 1.087 | 1.248 | 0.818 | 1.065 | 1.618 | |
| Albumen iodine (µg/g) | 0 wk | 0.104 | 0.100 | 0.103 | 0.103 | 0.102 | 0.104 | 0.099 | 0.101 | 0.102 | 0.101 | 0.103 | 0.101 | 0.0024 |
| | 3 wk | 0.103 | 0.100 | 0.109 | 0.120 | 0.108 | 0.114 | 0.143 | 0.114 | 0.122 | 0.107 | 0.112 | 0.136 | |
| | 6 wk | 0.101 | 0.112 | 0.124 | 0.170 | 0.121 | 0.138 | 0.210 | 0.135 | 0.156 | 0.117 | 0.131 | 0.190 | |
| | 9 wk | 0.100 | 0.121 | 0.135 | 0.227 | 0.131 | 0.152 | 0.250 | 0.161 | 0.180 | 0.127 | 0.144 | 0.241 | |
| | 12 wk | 0.098 | 0.126 | 0.155 | 0.255 | 0.147 | 0.173 | 0.297 | 0.179 | 0.206 | 0.137 | 0.164 | 0.276 | |
| Shell iodine (µg/g) | 0 wk | 16.468 | 16.487 | 16.500 | 16.451 | 16.251 | 16.035 | 16.602 | 16.479 | 16.296 | 16.369 | 16.268 | 16.527 | 0.4514 |
| | 3 wk | 16.289 | 18.479 | 21.750 | 20.612 | 20.489 | 22.530 | 33.592 | 23.614 | 26.204 | 19.484 | 23.140 | 32.102 | |
| | 6 wk | 16.093 | 21.551 | 25.503 | 25.374 | 24.706 | 38.899 | 30.594 | 23.128 | 26.842 | 23.128 | 26.842 | 37.187 | |
| | 9 wk | 15.953 | 23.530 | 27.659 | 39.545 | 26.250 | 31.107 | 42.427 | 30.244 | 33.264 | 24.895 | 29.383 | 40.986 | |
| | 12 wk | 15.714 | 25.512 | 30.644 | 43.849 | 29.900 | 34.067 | 48.034 | 33.335 | 37.434 | 27.706 | 32.509 | 45.941 | |
EDDI$_8$ diet on the incidence of abnormal eggs and eggshell strength seems likely to result from a higher level of bioavailable iodine. Nevertheless, this information does not specify how the excess iodine affects eggshell structure and strength. Previous studies (Christensen and Ort 1990; Christensen et al. 1991) have shown that dietary T4 elevates plasma T4 fivefold with no effect on functional characteristics of eggshell. Thus, the data suggest that the mechanism may not involve only the plasma concentrations of thyroid hormones.

The EDDI$_8$ diet also exacerbated the age-dependent decrease of Haugh unit, while no changes in yolk index or yolk colour could be noticed according to dietary iodine or age. Besides, with increasing age, there tended to be an increase in the percentage of yolk weight and a decrease in the percentage of albumen weight, which was not altered by dietary iodine. In agreement with our results, Christensen and Ort (1990), Bakhshalinejad et al. (2018), and Damaziak et al. (2018) reported that dietary inorganic iodine (2.1–12.9 mg/kg as CIOD or KI) had no significant effects on egg yolk and albumen percentages, yolk colour, or Haugh unit. However, Yalcın et al. (2004) found a linear decrease in albumen index and Haugh unit when the level of dietary iodine increased to 24 mg/kg by using CIOD as the additive, and Lichovnikova et al. (2003) reported that even a level of 6.1 mg/kg of iodine could have deleterious effect on Haugh unit in long-term period of treatment (30 wk). Therefore, our results indicate that EDDI exerts its harmful impact on Haugh unit at a much lower level and in a shorter period than have been obtained for inorganic sources of iodine. The mechanism of these findings remain to be more precisely examined, but might be linked to the inducing effect of excess iodine on oxidative stress, which has been reported to correlate negatively with Haugh unit score (Seven 2008; Zhang et al. 2012; Liu et al. 2014; Ma et al. 2014; Wang et al. 2016).

As the study progressed, we recognised that the EDDI$_8$ diet increased the serum and egg contents of MDA and PC, which are products of lipid and protein oxidation, respectively (Wang et al. 2018). The phenomenon in which excess iodine could exert oxidative stress in some target tissues of the thyroid hormones has been reported by Joanta et al. (2006), and they found that Wistar rats supplemented with a high-iodine diet (1 μg/100 g body weight/daily) for 10 d showed a marked increase in MDA levels in the liver as compared with the control group. These increases are probably related to the increase of thyroid hormone production. Indeed, the synthesis of thyroid hormones crucially depends on H$_2$O$_2$, which works as a donor of oxidative equivalents for thyroperoxidase (Corvilain et al. 1991). Because of its high toxicity, H$_2$O$_2$ synthesis must always remain in adequation with the hormonal synthesis and strictly contained at the apical pole of the cell. Thyrocytes possess various enzymatic systems, such as SOD, glutathione peroxidase, catalase, and peroxiredoxins that contribute to limit cellular injuries when H$_2$O$_2$ or other reactive oxygen species are produced in excess (Kim et al. 2000; Mutaku et al. 2002; Poncin et al. 2008). These results are confirmed by our findings, which showed a parallel increase in serum and egg yolk SOD activities as the level of serum T3 and T4 increased in birds by feeding the CIOD$_8$ and EDDI$_8$ diets. However, in the latter group, this increase was not sufficient to prevent the oxidative damage. Along with this modification, this group of hens also exhibited a much more increase in serum and egg yolk levels of triglycerides, total cholesterol, and LDL cholesterol that accompanied by a much less increase in contents of HDL cholesterol as the study progressed. These findings support the results of previous studies which showed that T3 increased lipogenesis and lipogenic enzyme activities in relation or secondary to oxidative stress (Venditti et al. 1997; Rosebrough 1999; Rosebrough and McMurtry 2000). Rodent studies have confirmed that excessive iodine can induce similar effects (Zhao et al. 2011; Sarkar et al. 2018). Some studies reported that iodine supplementation had no significant effect on lipid indices such as triglycerides or cholesterol fractions (Rys et al. 1997; Yalcın et al. 2004; Saki et al. 2012; Słupczyńska et al. 2014). A regression of data from Perry et al. (1989, 1990) showed that plasma cholesterol concentration increases by 0.3 mM/L for each 100 mg/kg increase in dietary iodine concentration (Lewis 2004). However, our data suggest that this estimation cannot be extended to organic sources of iodine.

In this study, the transfer of iodine into the egg contents and eggshells increased significantly as the level of iodine increased in the diet, which is consistent with the other studies (Dobrzański et al. 2001; Yalcın et al. 2004; Röttger et al. 2012; Saki et al. 2012; Słupczyńska et al. 2014; Bakhshalinejad et al. 2018). Moreover, in agreement with the literature (Opaliński et al. 2012), EDDI as the organic form of iodine was found to be more effective than CIOD in increasing the level of iodine in the egg compartments. However, whereas in most of the previous studies the iodine level of the eggs has been reported at the end
of the treatment period (Dobrzański et al. 2001; Yalçın et al. 2004; Opaliński et al. 2012; Röttger et al. 2012; Saki et al. 2012; Bakhshalinejad et al. 2018), the most interesting feature revealed by the present study was that the iodine levels continued to increase over the time course of the experiment. This means that a 12-wk period of iodine supplementation in laying hens might not be sufficient to achieve a specified level of iodine in the eggs. These findings add to the complexities that are involved in the production of iodine-enriched eggs. Iodine content in eggs depends on many factors, including the source and level of iodine supplements (Marcilese et al. 1968; Perry et al. 1989; Röttger et al. 2012; Saki et al. 2012), the type of diet (Ślupczyńska et al. 2014), genetic characteristics of birds (Knapp et al. 1998), bird age, laying rate, as well as the chemical methods used for the determination of iodine content in the biological matter (Gasior and Szczypula 2010). Determining the appropriate level of iodine from a given source, thus, still requires further investigation in more specifically designed studies. The presence of variable amounts of iodine in egg contents makes it difficult to control the dose of mineral that the consumer receives and represents a risk factor, especially in those countries, populations, and individuals that are already at risk of excessive iodine consumption. Moreover, considering the fact that eggshells are a potential substrate for producing food supplements (Opaliński et al. 2012), the variable iodine concentration in eggshells should also be taken into account.

Conclusions

The results of this study indicate that dietary supplementation of 2 or 4 mg/kg of iodine as CIOD or especially EDDI could be used to increase the iodine content of eggs without any adverse effect on performance, egg quality, and health of laying hens. However, due to the increasing accumulation of iodine in the eggs, further studies are necessary to evaluate the safety of egg fortification with iodine and to determine the optimal iodine intake of laying hens for the enrichment of their eggs.

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Ethical approval

All experimental procedures were approved by the Animal Care Committee at the Islamic Azad University, Isfahan (Khorasgan) Branch.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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