Oral exposure of broiler breeder hens to extra thyroxine modulates early adaptive immune responses in progeny chicks

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ABSTRACT Based on the findings of a recent study suggesting a decreased cold-induced ascites incidence in broiler progeny from hyperthyroid (HYPER) breeder hens, and a controversy on the effects of hyperthyroidism on immunocompetence, the present study was conducted to determine the probable adverse effect of induced maternal hyperthyroidism on immune function in progeny chicks. Breeder hens (n = 88) were randomly allotted to the control or HYPER groups and received common or thyroxine (T_4)-added (1 mg/L) water, respectively. The hens were artificially inseminated, and hatching eggs (n = 924) were incubated. Thereafter, the male hatchlings (n = 288) were reared for 42 d, and several cellular and humoral immune responses were evaluated at standard or low ambient temperature. Prevaccination antibody titers to Newcastle disease, infectious bronchitis, and infectious bursal disease virus were higher in HYPER chicks during 1 wk of age, although not different in their dams. For primary response to SRBC administered at 7 d of age, HYPER chicks recorded higher total, IgM (d 14), and IgG (d 21) anti-SRBC antibody titers. Higher cutaneous basophilic hypersensitivity response in HYPER chicks (d 10) was not observed at 35 d of age. Carbon clearance assay showed no difference, but in vitro lymphoproliferative response to concanavalin A was higher in 19-d-old HYPER chicks, independent of temperature treatment. An increase in lymphocyte percentage coincided with a decreased heterophil percentage and heterophil to lymphocyte ratio (d 14) in the HYPER group. The weight of lymphoid organs in progeny was not influenced by the oral exposure of dams to extra T_4. Independent of T_4 treatment, cold exposure was generally associated with decreased immune functions at early stages. The data suggested that oral exposure of broiler breeder hens to 1 mg/L of T_4 not only had no adverse effect on immune function, but also modulated early adaptive immune responses in progeny chicks for which the causal mechanisms remain to be unraveled.

Key words: maternal hyperthyroidism, thyroxine, immunity, cold stress, broiler

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INTRODUCTION

Proper immune function is influenced by a variety of factors for which the normal levels of thyroid hormones (**TH**) are said to be necessary (Klecha et al., 2006). Although the literature is rather unanimous on depressed immune function during hypothyroidism (Fabris et al., 1995), the effects of hyperthyroidism on cellular and humoral immunity is controversial (Silberman et al., 2002). A stimulatory (Fabris, 1973), an inhibitory (Gupta et al., 1983), or no significant effect (Weetman et al., 1984) on cell-mediated immunity was reported for TH. On the one hand, a direct relationship between TH and antibody response to SRBC, a T cell-dependent antigen, has been suggested to be unlikely in chickens (Martin et al., 1988). On the other hand, several positive immunostimulatory effects of TH have been reported (Glick, 1984), including a positive correlation between TH and peripheral lymphocyte number in chicks (Bachman and Mashaly, 1987) or cytokine secretion in hyperthyroid (**HYPER**) mice (Klecha et

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al., 2006). Therefore, further investigations on immune responses to hyperthyroidism are justified.

As a sequel to our recent study, indicating a lower cold-induced ascites incidence in broiler progeny from HYPER hens (Akhlaghi et al., 2012), the question was raised as to what extent the maternal hyperthyroidism might affect the immune responses in progeny chicks. Practically, a probable impaired immunocompetence in offspring produced from HYPER hens would limit the use of this preventative treatment. A paucity of literature addressing the effect of maternal hyperthyroidism on immune function in their chicks led to the present study to elucidate the effect of oral administration of extra thyroxine (**T_4**) to breeder hens on aspects of cell-mediated and humoral immunity in their progeny chicks. We hypothesized that the administration of T_4 through drinking water in breeder hens would influence the immune function in their offspring by either affecting the maternal circulatory level of specific IgY, direct effect of T_4 on chicks, or both, because both the T_4 (McNabb, 2002) and IgY (Hamal et al., 2006) were suggested that might proportionally be transported from maternal circulation to the yolk. In the present study, the chicks produced from HYPER hens and those from control hens were reared for a 42-d grow-out period. Having a well-established immunosuppressive effect, the chicks were chronically exposed to cold stress (Dohms and Metz, 1991) to unmask possible differences in immune activities between 2 maternal groups at either standard or low ambient temperature. The findings of the current work would also help in better clarifying the contribution of factors causing fluctuations in maternal TH, thereby influencing the immune status in progeny chicks.

MATERIALS AND METHODS

Breeder Flock and Hatchery

Eighty-eight 26-wk-old wing-banded broiler breeder hens (Babolkenar Arian Line Breeding Center, Babolkenar, Iran) were randomly allotted to 2 treatments, control (**CON**) or HYPER (4 replicates of 11 hens each), and housed in trap-nested floor pens covered with wood shavings. The birds were managed in compliance with the Line Breeding Center Animal Care guidelines. Thyroxine was added to the drinking water of the HYPER group (1 mg/L; wk 30 to the end of wk 33), whereas the CON group received the drinking water only. Age-matched male breeders of the same strain (n = 20) were habituated (2 wk) to abdominal massage for semen collection. The hens were artificially inseminated with pooled semen on a weekly basis, and the hatching eggs (n = 924) were collected during the last 14 d of the treatment period, fumigated (20 min), and stored (12.5°C, 75% RH) until transferred to the setter. The birds were maintained under similar management conditions (21°C and a 15L:9D photoschedule) and vaccination program as specified by the breeder company, and fed a standard pelleted diet (2,700 kcal of ME/kg, and 14.0, 2.99, and 0.36% CP, calcium, and phosphorus, respectively). Blood samples (2.5 mL) were obtained weekly from the brachial vein (wk 29 to 35), collected into EDTA-coated tubes, and centrifuged for 12 min (1,800 × *g* at 18°C). The plasma was stored at −20°C until analyzed for total triiodothyronine (**T_3**) and T_4 levels, using commercially available kits (Adaltis, Rome, Italy) validated for chicken plasma (Akhlaghi et al., 2012). The intraassay and interassay CV were 4.7 and 5.5% for T_3, and 4.3 and 7.4% for T_4, respectively. Basal levels of plasma antibodies against SRBC, Newcastle disease (**NDV**), infectious bronchitis (**IBV**), and infectious bursal disease (**IBDV**) virus were also assayed (wk 33 to 35) according to the following procedures described for broiler chicks.

After an 8-h preincubation period at 24°C, the eggs were set on turning trays, allotted to the same side of the trolleys for minimal possible positional effect, and transferred to the same incubator (Petersime, Zulte, Belgium; specific dry-bulb temperature of 37.6°C and wet-bulb temperature of 29°C for 18 d). The eggs were then transferred to the hatcher for the remaining 3 d, in such a way that the eggs from each hen were placed under individual pedigree baskets. Blood samples were obtained at the internal pipping (**IP**) stage and hatching (n = 5/replicate, 40 chicks/stage) through cardiac puncture for T_3 and T_4 assay. A total of two hundred eighty-eight 1-d-old vent-sexed high-quality male chicks (n = 144 for CON or HYPER groups) were wing-banded and reared for 42 d.

Broiler Chickens

Housing and Experimental Treatments. Using 2 temperature-controlled rooms (±1°C) and a 23L:1D photoperiod, 1-d-old chicks were raised under standard (**S**; 6 replicates of 9 chicks per maternal treatment group; 108 in total) or ascites-inducing cold (**C**) ambient temperature (10 replicates of 9 chicks per maternal treatment group; 180 in total). Because a higher mortality rate was expected at cold ambient temperature, a higher number of birds was assigned to the cold-exposed groups. The birds were used in a 2 × 2 factorial arrangement with 2 maternal treatment groups (CON or HYPER) and 2 rearing temperatures (S or C) as factors, consisting of the chicks from control hens reared under standard (**CON_S**) or cold (**CON_C**), and those from HYPER hens reared under standard (**$HYPER_S$**) or cold (**$HYPER_C$**) ambient temperatures. Each replicate of birds was housed in a floor pen (1.0 × 0.8 m) covered with wood shavings. The birds in the S group were raised under a standard temperature condition, which was gradually reduced from 32 to 26°C until 3 wk of age and maintained at 22°C for the rest of the study. The temperature in the C group was 32 and 30°C during wk 1 and 2, lowered to 15°C during wk 3, and maintained between 10 and 15°C thereafter (Luger et al., 2002). The chicks were randomly allotted to

each replicate and fed a corn-soybean-based mash diet, meeting or exceeding the NRC (1994) requirements. The diets supplied 2,806, 2,935, and 2,945 kcal of ME/kg, and 21.8, 20.1, and 18.0% CP during starter (0 to 14 d), grower (15 to 28 d), and finisher (29 to 42 d) stages, respectively. Feed and water were provided ad libitum, and the birds were weighed (every other week) and bled (weekly) for TH assay as described previously. Mortalities were recorded daily, and dead birds were necropsied for diagnosis of ascites (Luger et al., 2002).

The attributes used to evaluate the humoral and cell-mediated immune activities, included the cutaneous basophilic hypersensitivity (**CBH**) response, anti-SRBC, and NDV-, IBV-, and IBDV-specific antibodies assay, total and differential leukocyte counts, carbon clearance assay (**CCA**), in vitro lymphoproliferative assay, and lymphoid organ weight. Each bird was considered for a single immune trait, excluding the differential leukocyte count and the CCA, which were evaluated in the same birds such that bleeding for the former followed by injecting colloidal carbon for the latter. Furthermore, 1-d-old male chicks not included in the grow-out period (n = 12/maternal group) were used to collect the data on preimmunization antibody titers and the weight of lymphoid organs at d 1.

CBH Response. The CBH response to phytohemagglutinin-P (**PHA-P**; Sigma Chemical Co., St. Louis, MO) was used to assess in vivo cell-mediated immune response. At 10, 21, and 35 d of age, 10 birds per treatment received 100 μg of PHA-P in 0.1 mL of sterile PBS (0.15 *M* at pH = 7.4) injected intradermally in interdigital skin between the second and third digits of the right foot. The left foot was injected with 0.1 mL of PBS as a sham control. The thickness was measured, using digital calipers, to the nearest 0.05 mm immediately before and at 12, 24, and 48 h postinjection, and the response was evaluated as follows: [(thickness of right toe web postinjection − thickness of right toe web preinjection) − (thickness of left toe web postinjection − thickness of left toe web preinjection)].

Anti-SRBC Antibody Assay. As a T cell-dependent antigen, 1 mL of a 7% SRBC suspension in PBS (pH = 7.5) was injected intravenously to 10 chicks per treatment at 7 d of age. The birds were then given a booster injection at 21 d of age to determine the secondary antibody response. The chicks were bled from the brachial vein (2 mL) at 7 (preimmunization), 14, 21, 28, and 35 d of age, and the blood samples were centrifuged for 12 min (1,800 × *g* at 18°C) to separate the sera. The serum samples were heat inactivated (56°C for 30 min) and then assessed for total, 2-mercaptoethanol (**2-ME**)-resistant (**MER**, presumably IgG), and 2-ME-sensitive (**MES**, presumably IgM) anti-SRBC titers, using microhemagglutination assay in 96-well microplates (Qureshi and Havenstein, 1994). To determine the total anti-SRBC antibody titer, 50 μL of PBS and 50 μL of serum sample were placed in the first row and incubated at 37°C for 30 min. Then, 50 μL of PBS was added to the remaining wells to make a 2-fold serial dilution for each sample on successive rows. Each well then received 50 μL of a 2% SRBC suspension, and the plate was reincubated (37°C for 30 min) and subjected to a hemagglutination test. Antibody titers were expressed as $\log_2$ of the reciprocal of the highest dilution giving visible agglutination. The sera were also tested for MER and MES titers, using the same procedure as for the total titers, except that 50 μL of 0.2 *M* 2-ME was added to the first row of wells. The difference between total and MER titers was considered as the MES titer.

NDV-, IBV-, and IBDV-Specific Antibody Assay. Twelve birds per maternal treatment group were vaccinated with live virus vaccines against IBV (d 8, intranasal), NDV (d 8 and 23, ocular), and IBDV (d 10 and 29, oral) according to the manufacturer's recommendations (Intervet International B.V. Co., the Netherlands). Serum samples were prepared at 3 and 7 d of age from all birds for preimmunization analysis and then at 14, 21, and 42 d of age to evaluate the humoral immune response to vaccinations.

A hemagglutination inhibition test was used according to Singh et al. (2006). The serum (25 μL) was added to a microplate containing normal saline (25 μL/well) to make 2-fold serial dilutions. Four hemagglutination (**HA**) units of NDV were added (25 μL/well) and 1 HA unit of the virus was determined as the highest dilution causing agglutination of a chicken RBC suspension (1:100 dilution in normal saline) at 25°C. The chicken RBC suspension was then added (25 μL/well) and incubated at 37°C for 45 min. The hemagglutination inhibition titer was expressed as the $\log_2$ of the reciprocal of the highest serum dilution inhibiting HA activity.

The IBV- and IBDV-specific antibodies were assayed using commercially available ELISA kits (ProFlok Plus, Synbiotics Corporation, San Diego, CA) following the manufacturer's directions. Briefly, the serum samples were diluted in the dilution buffer (1:50) and incubated (30 min at room temperature), using the corresponding antigen-coated 96-well microtiter plate. After washing, the goat anti-chicken IgG (H^+L)-peroxidase conjugate was added to each well before reincubation (30 min at room temperature). The plate was then washed and incubated (15 min) after adding the substrate-chromogen solution. The reaction was terminated by adding a stop solution. The optical density (**OD**) values were determined at 405 nm by a microplate reader (Anthos 2020, Salzburg, Austria). The sample to positive (**SP**) ratio was calculated using a positive control serum and a normal control serum supplied with the kit. The difference between the average normal control OD and average positive OD, known as corrected positive control OD, was used in an equation in which SP = (sample OD − average normal control OD) was divided by corrected positive control OD. The $\log_{10}$ titers for IBV- and IBDV-specific antibodies were respectively calculated according to the equations [(1.172 × $\log_{10}$SP) +

3.614] and [(1.642 × $\log_{10}$SP) + 3.568], and the antilog of the $\log_{10}$ value was expressed as the titer.

Total and Differential Leukocyte Counts. At 1, 7, 14, 21, and 41 d of age, blood samples (0.5 mL) were collected into EDTA-coated tubes (6 birds/treatment) to enumerate the total leukocytes, using the Natt-Herrick's solution and a hemacytometer (Buitenhuis et al., 2006). To determine the percentages of lymphocytes, monocytes, heterophils, eosinophils, basophils, and heterophil to lymphocyte (**H:L**) ratio, duplicate blood smears were air-dried, stained with Wright's-Giemsa (Saikin Kagaku Institute Co. Ltd., Sendai, Japan), and counted to a total of 100 cells per slide, using a Zeiss (Jena, Germany) compound light microscope (× 1,000 magnification).

Carbon Clearance Assay. Mononuclear phagocytic system function was assessed by CCA in a timely manner as a reliable procedure in broilers (M. A. Qureshi, USDA-National Institute of Food and Agriculture, Washington, DC, personal communication). Colloidal carbon (Pelikan Co., Tehran, Iran) was injected into the brachial vein of 12 chicks/maternal treatment (100 μL/bird) at 13 and 14 d of age, and the birds were bled 5 and 20 min postinjection. Preinjection blood samples (0 min) were drawn from the opposite wing. The samples were immediately transferred into 1.5 mL of 1.0% sodium citrate and centrifuged for 4 min (300 × *g* at 18°C), using a rotating bench-top centrifuge (International Equipment Co., Needham Heights, MA). The concentration of carbon particles in supernatant was measured spectrophotometrically with a microplate reader (Anthos 2020) at 675 nm, and the OD values were obtained. For each bird, the OD value at 0 min was subtracted from those at 5 and 20 min, and the increase in OD was reported for each time.

In Vitro Lymphoproliferative Assay. Heparinized blood samples were collected at 19 and 41 d of age (10 birds/treatment) and used for lymphoproliferative response to stimulation with the T-cell mitogen, concanavalin A (**ConA**; Sigma Chemical Co., St. Louis, MO), according to Guo et al. (2006) with minor modifications. Briefly, each sample was subjected to Ficoll density gradient centrifugation (1,500 × *g*; 25 min, 18°C), and the peripheral blood mononuclear leukocytes (**PBMC**) were collected from the interphase. The cells were washed twice with RPMI-1640 (Invitrogen Co., Grand Island, NY) and the suspension was adjusted to 4×10^6 PBMC/mL. The cell suspensions were then cultured in 96-well culture plates (0.1 mL/well) with 10 μg/mL of ConA dissolved in RPMI-1640 (ConA-stimulated cultures) or with 10 μg of RPMI-1640 (unstimulated cultures) and incubated in a humidified incubator at 5% CO_2 (39°C for 60 h). Fifteen microliters of 5 mg/mL of 4,5-dimethylthiazole-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) was then added to each well, and the plates were reincubated for 4 h after which 10% sodium dodecyl sulfate with 0.01 *N* HCl (100 μL/well) was added to lyze the cells. After 2 h, the culture plates were allowed to equilibrate for 5 min at 25°C, and the OD was determined for each sample at 570 nm (reference wavelength of 630 nm), using an automated microplate reader (Anthos 2020). The differences between the OD values in ConA-stimulated and unstimulated cultures were then determined.

Lymphoid Organs. At 1, 14, 21, and 42 d of age, 6 chicks per treatment were humanely decapitated to weigh fat-trimmed thymus, bursa of Fabricius, and spleen and the values were adjusted for BW.

Statistical Analysis. The data were subjected to the PROC MIXED (SAS, 2002), and BW was included as a covariate in the ANOVA. The effects of maternal treatment group, ambient temperature, time, and their interactions were included in the model. The means were compared by the least squares means adjusted for the Tukey's test. Nonnormally distributed data were analyzed by the GENMOD procedure, using the logistic regression model (SAS, 2002), based on the Wald chi-squared values. When no difference was found between the maternal treatment groups or the ambient temperatures, the data were pooled accordingly. The level of significance was set at $P \leq 0.05$.

RESULTS

TH, BW, and Mortality

Hyperthyroidism induced by adding T_4 in drinking water increased the plasma concentration of T_4 in HYPER hens (36.1 ± 3.75 vs. 11.9 ± 0.77 ng/mL for HYPER and CON hens, respectively) and embryos at the IP stage (7.81 ± 0.42 vs. 5.33 ± 0.29 ng/mL for HYPER and CON groups, respectively). However, T_3 concentrations in HYPER and CON hens (1.62 vs. 1.60 ng/mL, respectively) or in their embryos at the IP stage (2.79 vs. 2.93 ng/mL, respectively) were not affected by maternal hyperthyroidism.

Table 1 shows the plasma concentration of TH and BW gain in broilers emanating from HYPER and CON hens. Maternal hyperthyroidism, irrespective of the rearing temperature, did not affect the plasma TH concentrations in broilers ($P > 0.05$); however, the higher T_3 values ($P = 0.0001$) coincided with the lower ones for T_4 ($P = 0.005$) in cold-exposed birds, compared with the broilers reared under standard temperature. Oral exposure to extra T_4 did not influence the BW in breeder hens (3,361 ± 21 in HYPER vs. 3,335 ± 21 g in the CON group), and no mortality was recorded during the laying period. The BW gain in broilers was not affected by maternal hyperthyroidism (Table 1); however, the overall weight and BW gain during the last 2 wk of the 42-d period were lower in cold-exposed broilers. Maternal hyperthyroidism was associated with a decreased ascites mortality rate in cold-exposed chickens (7.8 vs. 26.7% for $HYPER_C$ and CON_C, respectively); however, the mortality rate under standard temperature was not different between the 2 groups (3.7 and 5.7% for $HYPER_S$ and CON_S, respectively).

Table 1. Effect of induced maternal hyperthyroidism on weekly plasma levels of triiodothyronine (T_3), thyroxine (T_4), and BW gain in broiler chickens reared under standard (S) or cold (C) ambient temperature[1]

Item	CON_S	$HYPER_S$	CON_C	$HYPER_C$	SEM
T_3, ng/mL					
1 wk of age	2.35	2.41	2.32	2.37	0.19
2 wk of age	1.09	1.11	1.11	1.17	0.23
3 wk of age	1.43[b]	1.39[b]	1.93[a]*	1.89[a]*	0.26
4 wk of age	1.71[b]	1.76[b]	2.29[a]*	2.38[a]*	0.21
5 wk of age	0.89[b]	0.92[b]	1.84[a]*	1.69[a]*	0.27
6 wk of age	0.86[b]	0.84[b]	1.85[a]*	1.76[a]*	0.23
T_4, ng/mL					
1 wk of age	7.9	8.0	7.9	8.1	0.21
2 wk of age	8.8	8.3	8.3	8.5	0.23
3 wk of age	5.7[a]	5.9[a]	4.3[b]*	4.3[b]*	0.22
4 wk of age	5.2[a]	5.1[a]	3.1[b]*	3.2[b]*	0.21
5 wk of age	7.0[a]	7.1[a]	4.5[b]*	4.6[b]*	0.22
6 wk of age	7.6[a]	7.6[a]	5.7[b]*	5.4[b]*	0.24
BW gain, g/bird					
1 to 2 wk of age	391	384	396	390	9.1
3 to 4 wk of age	872	881	836*	847*	24.1
5 to 6 wk of age	1,271[a]	1,263[a]	900[b]*	889[b]*	32.4
1 to 6 wk of age	2,534[a]	2,528[a]	2,132[b]	2,126[b]	49.0

[a,b]Within each row, values with different superscripts differ significantly ($P \leq 0.05$).

[1]n = 10 for T_3 and T_4 assay in broilers emanating from the control (CON) or hyperthyroid (HYPER) hens, which were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature. The birds at cold ambient temperature were exposed to cold stress during 14 to 42 d of age.

*The values with asterisks represent data collected during the cold stress.

CBH Response

Table 2 shows the CBH response in 10- and 35-d-old broiler chicks. On d 10, a higher value was recorded for the HYPER group only at 12 h following the PHA-P injection ($P = 0.001$). Regardless of the maternal group, cold exposure was associated with a decreased CBH response at 12 and 24 h postinjection on d 35. The response at 21 d was not influenced by either the maternal hyperthyroidism or cold exposure (data not shown).

Anti-SRBC Antibody Assay

The primary and secondary responses to SRBC in broilers are presented in Table 3. Compared with their respective control, the chicks in the HYPER group had higher total ($P = 0.007$) and IgM ($P = 0.006$) anti-SRBC antibody titers at 7 d and higher total ($P = 0.02$) and IgG ($P = 0.03$) at 14 d after the primary immunization. Maternal hyperthyroidism, however, did not affect the early or late secondary response in progeny chicks. Cold-exposed chicks recorded lower titers at 14 d postprimary ($P = 0.02$) and at 7 d postsecondary ($P = 0.001$) injection. No differences were found in antibody titers between HYPER and CON breeder hens during the period of egg collection (data not shown).

NDV-, IBV-, and IBDV-Specific Antibody Assay

Serum levels of specific antibody titers between the CON and HYPER hens were not different [8.11 vs. 8.23 (±0.33), 6,125 vs. 6,389 (±720), and 5,518 vs. 5,745 (±610) for NDV-, IBV-, and IBDV-antibody titers in CON and HYPER hens, respectively]. Effect of induced maternal hyperthyroidism on antibody titers against NDV, IBV, and IBDV at 3, 7, and 14 d of age in the chicks is shown in Table 4. Interestingly, 3-d-old chicks from HYPER dams showed higher antibody titers against all 3 viruses compared with the values in their age-matched control chicks. This was also true for anti-

Table 2. Effect of induced maternal hyperthyroidism on cutaneous basophilic hypersensitivity response in interdigital web (mm) to phytohemagglutinin-P (PHA-P) at 12, 24, and 48 h postinjection in 10- and 35-d-old cold-exposed progeny chicks[1]

Item	12 h	24 h	48 h
Web thickness, mm (d 10)			
CON	1.12[b]	0.93	0.81
HYPER	1.31[a]	0.97	0.97
SEM	0.07	0.10	0.08
Web thickness, mm (d 35)			
CON_S	1.81[a]	1.76[a]	1.52
$HYPER_S$	1.89[a]	1.70[a]	1.44
CON_C	1.53[b]*	1.41[b]*	1.30*
$HYPER_C$	1.46[b]*	1.38[b]*	1.33*
SEM	0.14	0.11	0.18

[a,b]Within each column and for each age group, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age, n = 10/treatment). The birds were injected with 100 μg of PHA-P in 0.1 mL of PBS intradermally in the right foot. The left foot was injected with 0.1 mL of PBS only. The values were obtained as follows: [(thickness of right toe web postinjection − thickness of right toe web preinjection) − (thickness of left toe web postinjection − thickness of left toe web preinjection)].

*The values with asterisks represent data collected during the cold stress.

Table 3. Effect of induced maternal hyperthyroidism on primary and secondary total, IgM, and IgG anti-SRBC antibody titers (log_2) in cold-exposed progeny chicks[1]

	Age[2]			
Item	d 14	d 21	d 28	d 35
Total				
CON_S	2.3[b]	1.1[b]	5.7[a]	3.2
$HYPER_S$	3.4[a]	1.6[a]	5.5[a]	2.9
CON_C	2.3[b]	0.6[c]*	4.0[b]*	2.6*
$HYPER_C$	3.4[a]	1.1[b]*	4.3[b]*	2.7*
SEM	0.33	0.15	0.40	0.25
IgM				
CON_S	2.2[b]	0.2	1.6	0.8
$HYPER_S$	3.4[a]	0.4	1.4	0.8
CON_C	2.2[b]	0.2*	1.0*	0.4*
$HYPER_C$	3.4[a]	0.3*	1.1*	0.4*
SEM	0.31	0.13	0.35	0.25
IgG				
CON_S	0.1	0.9[b]	4.1[a]	2.4
$HYPER_S$	0.0	1.2[a]	4.1[a]	2.1
CON_C	0.1	0.4[c]*	3.0[b]*	2.2*
$HYPER_C$	0.0	0.8[b]*	3.2[b]*	2.3*
SEM	0.31	0.11	0.35	0.35

[a–c]Within each column and for each trait, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age, n = 10/treatment).

[2]The birds received the primary (at 7 d of age) and secondary (at 21 d of age) intravenous injections with 1 mL of a 7% SRBC suspension.

*The values with asterisks represent data collected during the cold stress.

IBDV titer at 7 d of age, but not for NDV- and IBV-specific antibody titers at the same age. The chicks in the HYPER group recorded a lower titer against IBDV at d 14 ($P = 0.001$). Although the antibody titers were not influenced by maternal hyperthyroidism on d 21, a decrease was observed for all 3 antibody titers measured in cold-stressed birds (Table 5). The antibody values at 42 d of age were not affected by either the maternal treatment or cold exposure (data not tabulated).

Total and Differential Leukocyte Counts

Table 6 presents the total and differential leukocyte counts at 14 and 21 d of age. An increase in lymphocyte percentage ($P = 0.02$) coincided with a decrease in heterophil percentage ($P = 0.008$) and H:L ratio ($P = 0.001$) in 14-d-old broilers produced from HYPER hens, compared with the CON group. At 21 d, however, the differences disappeared; meanwhile, the birds at low ambient temperature experienced a lower lymphocyte percentage ($P = 0.02$) with an increased heterophil percentage ($P = 0.001$) and H:L ratio ($P = 0.0001$). No differences were observed at 1, 7, and 42 d of age among the experimental groups (data not shown).

Carbon Clearance Assay

Data on CCA before cold exposure suggested that the mononuclear phagocytic system function in progeny chicks was not affected by exposing the dams orally to extra T_4. There were no differences in OD_{675} values between HYPER and CON at 5 min (148.3 ± 24.69 and 131.7 ± 23.09% for CON and HYPER group, respectively) and 20 min (31.3 ± 5.09 and 23.4 ± 4.83% for CON and HYPER group, respectively) postcarbon colloid injection.

In Vitro Lymphoproliferative Assay

Maternal hyperthyroidism, irrespective of the rearing temperature, was associated with an increased in vitro lymphoproliferation to ConA on d 19 of age ($P = 0.0001$; Table 7). At the same time, cold stress was associated with a decreased proliferation response in both

Table 4. Effect of induced maternal hyperthyroidism on antibody titers against Newcastle disease (NDV), infectious bronchitis (IBV), and infectious bursal disease (IBDV) virus in 3-, 7-, and 14-d-old progeny chicks[1]

Item	d 3 of age	d 7 of age	d 14 of age	Age at vaccination, d
NDV, log_2				
CON	6.41[b]	5.96	7.49*	8
HYPER	7.33[a]	6.57	7.23*	8
SEM	0.33	0.27	0.20	
IBV				
CON	1,870[b]	1,421	2,043*	8
HYPER	2,550[a]	1,591	2,123*	8
SEM	270	240	217	
IBDV				
CON	1,771[b]	1,221[b]	850[a]*	10
HYPER	2,103[a]	1,534[a]	447[b]*	10
SEM	117	132	79	

[a,b]Within each column and for each antibody titer, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were vaccinated against NDV and IBV on d 8 and against IBDV on d 10 of age (n = 12/treatment). The titers of anti-NDV were determined by a hemagglutination inhibition test and those of anti-IBV and anti-IBDV by an ELISA test.

*The values with asterisks represent data collected postvaccination.

Table 5. Effect of induced maternal hyperthyroidism on antibody titers against Newcastle disease (NDV), infectious bronchitis (IBV), and infectious bursal disease (IBDV) virus in 21-d-old cold-exposed progeny chicks

Treatment[1]	NDV	IBV	IBDV
CON_S	3.83[a]	2,379[a]	543[a]
$HYPER_S$	3.91[a]	2,301[a]	385[b]
CON_C	3.09[b]*	1,608[b]*	318[b]*
$HYPER_C$	3.17[b]*	1,751[b]*	124[c]*
SEM	0.17	201	41

[a–c]Within each column, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age, n = 6/treatment). The birds were vaccinated against NDV and IBV on d 8 and against IBDV on d 10 of age. The titers of anti-NDV were determined by a hemagglutination inhibition test (log_2) and those of anti-IBV and anti-IBDV by an ELISA test.

*The values with asterisks represent data collected during the cold stress.

HYPER and CON groups ($P = 0.002$). Neither the maternal treatment nor the cold temperature influenced the response at 41 d (Table 7).

Lymphoid Organs

The relative weight of the lymphoid organs was not affected by maternal hyperthyroidism (Table 8); however, exposure to cold temperature decreased the values recorded for the bursa of Fabricius ($P = 0.001$) and thymus ($P = 0.03$) at 21 d, and for all 3 organs at 42 d of age.

DISCUSSION

In an earlier work, we found a decreased incidence of ascites in cold-exposed broilers emanating from HYPER hens (Akhlaghi et al., 2012), which led to the present study to determine the effect of hyperthyroidism, if any, on immune function as well as gaining more insight into the interrelations of TH and immune system. The present findings indicated that the maternal hyperthyroidism induced by T_4-added drinking water (1 mg/L) not only had no adverse effect on immune responses studied, but also was, interestingly, associated with some improvements in early immune function in progeny chicks.

Induced maternal hyperthyroidism resulted in a higher plasma T_4, but not T_3, level in HYPER hens and their embryos at the IP stage, although no maternally derived differences were found thereafter. The higher T_4 level at IP reaffirmed the concept of maternal TH transfer into eggs (McNabb, 2002). Similar to the findings of Luger et al. (2002), cold exposure of broilers resulted in a decreased plasma T_4 level, but the reverse was true for T_3, suggesting the extrathyroidal deiodination of T_4 (Goodman, 2003). Although the broiler BW gain was not affected by maternal thyroidal state, cold-exposed birds recorded a lower BW gain, mostly due to a higher energy demand for thermoregulation under cold temperature (Hangalapura et al., 2003).

Data on CBH response to PHA-P and on in vitro ConA-stimulated lymphoproliferation in broilers indicated a more potent early immune response in the HYPER group at 10 and 19 d of age, although the response disappeared thereafter. At low ambient temperature, however, the decreased CBH response at 35 d was not associated with a change in lymphoproliferative response at 41 d compared with those in age-matched birds at the standard temperature. Although the CCA and lymphoid organ weights showed no difference between the CON and HYPER progenies, cold stress generally decreased the weight of lymphoid organs. An increase in lymphocyte percentage with a decreased heterophil percentage and H:L ratio were observed in the HYPER group (d 14), but the reverse was true for cold-exposed birds at 21 d of age.

Possessing nuclear receptors for TH, leukocytes are directly affected by these hormones (Bachman and Mashaly, 1987). Reports inconsistently showed that

Table 6. Effect of induced maternal hyperthyroidism on total ($\times 10^3/mm^3$) and differential (%) leukocyte count and on heterophil to lymphocyte (H:L) ratio in 14- and 21-d-old cold-exposed progeny chicks

Treatment[1]	Total leukocyte	Heterophil	Lymphocyte	Monocyte	Eosinophil	Basophil	H:L ratio
d 14							
CON_S	38.43	25.9[a]	65.1[b]	4.2	1.6	3.2	0.40[a]
$HYPER_S$	38.01	17.3[b]	73.6[a]	4.3	1.6	3.2	0.23[b]
CON_C	37.14	28.0[a]	63.2[b]	3.9	1.8	3.1	0.44[a]
$HYPER_C$	37.73	18.6[b]	72.5[a]	4.1	1.5	3.3	0.26[b]
SEM	0.65	2.11	3.17	0.38	0.21	0.39	0.03
d 21							
CON_S	33.74	34.6[b]	57.9[a]	3.2	1.6	2.7	0.60[b]
$HYPER_S$	31.92	32.9[b]	59.5[a]	3.5	1.6	2.5	0.55[b]
CON_C	32.18*	47.0[a]*	46.1[b]*	2.8*	1.7*	2.4*	1.02[a]*
$HYPER_C$	32.77*	45.1[a]*	47.4[b]*	3.6*	1.5*	2.4*	0.95[a]*
SEM	0.93	2.21	3.08	0.41	0.19	0.42	0.05

[a,b]Within each column and for each day, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age, n = 6/treatment).

*The values with asterisks represent data collected during the cold stress.

Table 7. Effect of induced maternal hyperthyroidism on in vitro lymphoproliferative response to concanavalin A in 19- and 41-d-old cold-exposed progeny chicks

Treatment[1]	d 19	d 41
CON_S	0.156[b]	0.351
$HYPER_S$	0.211[a]	0.372
CON_C	0.121[c]*	0.340*
$HYPER_C$	0.149[b]*	0.329*
SEM	0.009	0.017

[a–c]Within each column, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age, n = 10/treatment). The values are the difference between the optical densities at 570 nm in concanavalin A-stimulated and unstimulated cultures.

*The values with asterisks represent data collected during the cold stress.

Table 8. Effect of induced maternal hyperthyroidism on the relative weight (g/100 g of BW) of the lymphoid organs in 1-, 14-, 21-, and 42-d-old cold-exposed progeny chicks[1]

Item	Bursa of Fabricius	Thymus	Spleen
d 1			
CON	0.100	0.220	0.040
HYPER	0.096	0.232	0.043
SEM	0.010	0.050	0.004
d 14			
CON	0.183	0.522	0.073
HYPER	0.198	0.539	0.069
SEM	0.012	0.026	0.004
d 21			
CON_S	0.227[a]	0.376[a]	0.079
$HYPER_S$	0.241[a]	0.354[a]	0.088
CON_C	0.167[b]*	0.242[b]*	0.064*
$HYPER_C$	0.171[b]*	0.239[b]*	0.061*
SEM	0.024	0.042	0.014
d 42			
CON_S	0.186[a]	0.305[a]	0.118[a]
$HYPER_S$	0.195[a]	0.317[a]	0.131[a]
CON_C	0.123[b]*	0.183[b]*	0.063[b]*
$HYPER_C$	0.137[b]*	0.177[b]*	0.069[b]*
SEM	0.019	0.036	0.021

[a,b]Within each column and for each day, means with different superscripts differ significantly ($P \leq 0.05$).

[1]The broilers emanating from the control (CON) or hyperthyroid (HYPER) hens were reared under standard (CON_S and $HYPER_S$, respectively) or cold (CON_C and $HYPER_C$, respectively) ambient temperature (14 to 42 d of age). Data for d 1 and 14 are before cold exposure (n = 6/treatment).

*The values with asterisks represent data collected during the cold stress.

TH exerted either a stimulatory (Fabris, 1973), an inhibitory (Gupta et al., 1983), or no effect (Weetman et al., 1984) on cell-mediated immunity. Although a clear decrease in mitogen-induced lymphoproliferation response in hypothyroid animals has been reported (Fabris et al., 1995), effect of hyperthyroidism on cellular and humoral immunity is still controversial (Silberman et al., 2002). In their study on relationship between circulating TH and cell-mediated immunity in 3-wk-old Single Comb White Leghorn male chicks, Bachman and Mashaly (1987) found positive correlations between TH and the weight of thymus and spleen and also between T_3 and the number of peripheral lymphocytes; however, the PHA-P response was not affected. On the other hand, Hangalapura et al. (2004a) found no correlation between TH and proliferative responses in 3- to 4-wk-old chicks. An increase in IL-2 (crucial for lymphocyte proliferation and activation) and interferon-γ (a Th_1 cytokine important for cellular immunity) secretion has been reported in the lymphocytes from the HYPER mice (Klecha et al., 2006). Interpreting the present data would be rather different because hyperthyroidism was induced maternally. However, higher T_4 level at IP may be an indication of late immune responses to increased T_4 at IP, possibly through affecting the differentiation and maturation process of immature progenitor cells, a mechanism that has been suggested to be involved in immune dysfunction in the offspring of aflatoxin-exposed dams (Qureshi et al., 1998). The permissive role of TH for optimal effects of other hormones (Goodman, 2003), including growth hormone function whose receptors are also found on leukocytes (Griffin, 1989), makes an indirect effect of TH a possibility. Although Hangalapura et al. (2004b) found an improved in vitro cellular immune response to ConA following a 7-d-long cold exposure in 26-d-old chicks, the literature generally indicates an immunosuppressive effect of cold stress (Dohms and Metz, 1991). This concept is confirmed by the current study; however, the present data suggested some degree of adaptability to low ambient temperature, associated with a recovery in responses late in the grow-out period, which might also be the case for humoral immunity responses.

To our knowledge, this is the first report indicating an enhanced primary anti-SRBC response in chicks produced from HYPER hens. However, the responses were adversely influenced during the first 2 wk of cold exposure, with unaffected titers thereafter. Additionally, preimmunization assays also revealed the higher specific antibody levels at 3 (to NDV, IBV, and IBDV) and 7 (to IBDV) d of age in the HYPER group. On the other hand, cold-exposed birds recorded lower titers at 21 d, but not at 42 d. As the basal levels of anti-SRBC antibodies between the maternal groups or between their preimmunized offspring were similar, factor(s) contributing to the higher primary response to SRBC in HYPER group might have acted after immunizing the progeny chicks with SRBC. The relationship between plasma TH and antibody response to SRBC has not been consistent. Comparing dwarf and normal chickens, Martin et al. (1988) suggested that a direct relationship between TH and anti-SRBC response was unlikely. They cited several works indicating either higher, lower, or unchanged anti-SRBC titers in hypothyroid chicks fed an antithyroidal agent, thiouracil. The relationship of anti-SRBC antibodies with thymic and bursal weight has also been equivocal (Ubosi et al., 1985). Accordingly, the lack of difference in the weight of lymphoid organs between HYPER and CON groups

is not surprising because the organ size may not necessarily be associated with antibody titers (Li et al., 2001). Involvement of early increase in peripheral lymphocyte percentage found in the HYPER group or of cytokine secretion, as reported for IL-2 in lymphocytes from HYPER mice (Klecha et al., 2006) in high specific antibody titers in the HYPER group, warrants further study. A precedent increase in IgM followed by a higher IgG (IgY) level reemphasized IgM as the predominant isoform in primary humoral response and the predominance of IgG in secondary one (Davison et al., 2008). The lower anti-SRBC response due to cold stress has been related to immunosuppressive effects of corticosterone (Dohms and Metz, 1991). This effect, however, did not last up to d 42, suggesting an adaptation to cold stress.

The higher titers of NDV-, IBV-, and IBDV-specific antibodies at 3 d posthatch found in the HYPER group could not be related to maternal antibody levels because no differences were found in Ig titers between CON and HYPER hens. Maternal antibodies are transferred from hens to chicks via the egg (Hamal et al., 2006). Theoretically, different maternal IgY levels may not necessarily imply the comparable capability of Ig production in CON and HYPER hens. In other words, the broad-spectrum effects of TH (Goodman, 2003) lead to the hypothesis that the IgY transfer from the dam's blood to the yolk via its receptors on ovarian follicles (Loeken and Roth, 1983) might be potentiated by the higher blood levels of T_4 which may influence the Ig production or its half life as a result of hyperthyroidism, a concept that has not been adequately addressed. Measuring the IgY content in eggs obtained from HYPER hens may help to clarify this possibility. Alternatively, higher specific IgY titers during the first week posthatch might be due to events occurring during late incubation to early postemergence life. The higher T_4 level at IP coincided with the higher levels of antibodies at 3 d posthatch. Considering the concept that the yolk residue retraction continues during the early posthatch period (Koutsos and Arias, 2006), it is speculated that the higher antibody levels during early posthatch life might be a response to higher T_4 level at IP, due either to a more efficient absorption of maternal antibodies via a selective receptor-mediated mechanism (Loeken and Roth, 1983) or to the half life of IgY in the embryo. The explanation would be more plausible because the speed of yolk sac absorption has been reported to be affected by TH (Torres, 2006). The contribution of such mechanisms cannot be discerned from the present data. Further works are needed to test these hypotheses and unravel the causal mechanism(s) for higher early specific IgY titers in the chicks produced from HYPER dams. Contrary to NDV- and IBV-specific antibody titers, the anti-IBDV antibody level remained higher at 7 d posthatch, most likely due to its longer half life (6.7 d; Fahey et al., 1987) compared with those of anti-NDV (5.2 d; Kaleta et al., 1977), and anti-IBV (5 to 6 d; Darbyshire and Peters, 1985) specific antibodies. Higher anti-IBDV antibody titer at 7 d of age in the HYPER group, in turn, could be a reason why a lower anti-IBDV titer was recorded at 14 d in the same group compared with CON chicks, possibly through interfering with the development of specific humoral responses to IBDV by neutralizing the antigen load of the vaccine (d 10 of age).

Overall, the present study suggested that induced maternal hyperthyroidism did not impair immune function in progeny chicks, but stimulated the early immune responses to some extent. The underlying causal mechanism(s) for these consequences remain(s) to be clarified. The current preliminary evidence shed more light on the thyroid-immune cross-talk, suggesting the modulatory effects of TH on the immune system. In practical terms, the treatment may help the newly hatched chicks, without a fully functional immune system, to better withstand early immunological challenges. However, a higher level of maternally derived antigen-specific IgY would make reprogramming the vaccination a necessity. Due to the sex differences in immune responses, further studies are warranted to elucidate the effects of such a treatment on female progeny chicks. Essentially, unmasking the consequences of long-term exposure of breeder hens to T_4, including those on reproductive performance and immune functions, must not be ruled out.

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