

Effects of ammonium perchlorate exposure on the thyroid function and the expression of thyroid-responsive genes in Japanese quail embryos and post hatch chicks

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ABSTRACT

Perchlorate ion interferes with thyroid function by competitively inhibiting the sodium-iodide symporter, thus blocking iodide uptake into the thyroid gland. In this study, the effect of perchlorate exposure on thyroid function and thyroid-responsive gene expression were examined in (1) embryos from eggs laid by perchlorate-treated Japanese quail hens and (2) perchlorate-treated young Japanese quail. I hypothesized that perchlorate exposure would decrease thyroid function and that the consequent hypothyroidism would alter the expression of thyroid sensitive genes. Laying Japanese quail hens were treated with 2000 mg/l and 4000 mg/l ammonium perchlorate in drinking water. Eggs from these hens were incubated. Embryos, exposed to perchlorate in the egg, were sacrificed at day 14 of the 16.5 day incubation period. Japanese quail chicks, 4-5 days old, were treated with 2000 mg/l ammonium perchlorate in drinking water for 2 and 7.5 weeks. Thyroid status was evaluated by measuring plasma thyroid hormone concentrations, thyroid gland weight and thyroidal thyroid hormone storage. Expression of thyroid-responsive genes was evaluated by measuring the mRNA levels of Type 2 deiodinase (D2) in the brain and liver, RC3/neurogranin mRNA level in the brain and Spot 14 mRNA level in the liver. Maternal perchlorate exposure led to embryonic hypothyroidism, demonstrated by thyroid hypertrophy and very low embryonic thyroidal TH storage. Embryonic hypothyroidism decreased body growth and increased D2 mRNA level in the liver (a presumed compensatory response to hypothyroidism) but did not affect the mRNA levels of D2 and RC3 in the brain. Spot 14 mRNA was not detected in embryonic liver.

In the second part of the study, quail chicks showed early signs of hypothyroidism after two weeks of 2000 mg/l ammonium perchlorate exposure; plasma concentration and thyroid gland stores of both T_4 and T_3 were significantly decreased. After 7.5 weeks of perchlorate exposure, all thyroid variables measured indicated that the chicks had become overtly hypothyroid. D2 mRNA level was increased, a compensatory response to hypothyroidism, and spot 14 mRNA level was decreased, a substrate-driven response in the liver of quail chicks after two weeks of perchlorate exposure. However, no difference was observed in the mRNA levels of D2 and spot 14 in the liver after 7.5 weeks of perchlorate exposure, suggesting there was some adaptation to the hypothyroid condition. The mRNA level of D2 and RC3 in the brain was not affected by perchlorate-induced hypothyroidism in quail chicks after either 2 or 7.5 weeks of perchlorate exposure. As in the embryos, this suggests the brain of chicks was “protected” from the hypothyroid body conditions.

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Abbreviations

AP	Ammonium perchlorate
CNS	Central nervous system
DES	Diethylstilbestrol
D1	Type 1 deiodinase
D2	Type 2 deiodinase
D3	Type 3 deiodinase
HPT axis	hypothalamus-pituitary-thyroid axis
PCBs	Polychlorinated biphenyl
RC3	RC3/neurogranin
SP14	Spot 14

Chapter 1 Introduction

Thyroid hormones control metabolic rate to maintain temperature homeostasis of homeothermic vertebrates and also are critical for the growth and development of various organs and tissues in all classes of vertebrates. Hypothyroidism during development can lead to severe morphological and physiological defects in young animals. Thyroid hormones regulate thyroid responsive genes in the brain during development and are therefore required for the normal development of central nervous system (CNS). Hypothyroidism during development is found to be associated with various degrees of neurological deficiency and may be responsible for irreversible developmental defects in the brain such as asymmetries, hypomyelination and defects in cell migration and differentiation in the brain.

Ammonium perchlorate (AP) is a known thyroid disruptor in that the perchlorate ion competitively inhibits the iodide uptake into the thyroid gland for thyroid hormone synthesis. The use of AP as an oxidizer in rocket fuel and fireworks has led to contamination of water and soil in many states in the United States. Ground dwelling birds in contaminated areas may develop hypothyroidism due to perchlorate exposure. Perchlorate-induced hypothyroidism in young birds may interfere with development. Moreover, recent evidence shows maternal exposure to perchlorate led to perchlorate deposition in eggs, exposing the embryos to perchlorate before hatching. Hypothyroidism resulting from perchlorate exposure at such early development stages may have significant effect on the development of many thyroid-responsive tissues such as CNS.

In the present study, the effects of both maternal perchlorate exposure on Japanese quail embryos and perchlorate exposure of young quail chicks were evaluated. Expression of thyroid-responsive genes in brain and liver of perchlorate-exposed birds were also measured to study the effect of perchlorate-induced hypothyroidism in target organs.

Project objectives

Objective 1: To investigate the effects of maternal perchlorate exposure on embryonic thyroid function and the embryonic brain and liver. I have studied the thyroid status and thyroid-responsive gene expressions in the brain and liver in Japanese quail embryos from eggs of perchlorate exposed hens. Laying hens were treated with AP solutions in drinking water; eggs from exposed hens were incubated and sacrificed on day 14 of the 16.5 day incubation period. Body weight, circulating thyroid hormone concentrations, thyroid gland weight and thyroid gland thyroid hormone content were measured to assess the embryonic thyroid status. The mRNA levels of RC3 in brain during embryonic development were described. The effects of maternal perchlorate exposure on gene expression were investigated for D2 and RC3 in brain and D2 in liver of embryos.

Objective 2: To investigate the effects of perchlorate exposure on thyroid function and expression of thyroid responsive genes in brain and liver of post-hatch Japanese quail and. I have studied the effect of perchlorate exposure in young Japanese quail chicks after two and 7.5 weeks of perchlorate exposure. Chicks of 4-5 days old were treated with AP solutions in drinking water for two and 7.5 weeks before sacrifice. Body weight, circulating thyroid hormone concentrations, thyroidal thyroid hormone storage were measured to evaluate thyroid status in these birds. The mRNA levels of D2 and RC3 in the brain total RNA of the chicks were measured to determine the effect of perchlorate exposure on the expression of thyroid-responsive genes in the brain. The mRNA levels of D2 and spot 14 were measured in the liver total RNA of the chicks to evaluate the effect of perchlorate exposure on peripheral organs.

Chapter 2

Literature review

Endocrine disruptors are defined as exogenous chemicals (usually environmental contaminants) that alter endocrine functions and cause detrimental effects in an organism or its offspring (Colborn, et al, 1996). Most of the endocrine disruptors affect the reproductive system. However, a number of endocrine disruptors are known to affect thyroid functions, for example, ammonium perchlorate.

Thyroid hormones have important roles in the growth and development of various organs and tissues in all classes of vertebrates. Disruption of thyroid function during critical developmental stages can lead to severe morphological and physiological defects in young animals. Thyroid hormones take action by regulating the expression of thyroid-responsive genes in target organs. Therefore, studying the effect of thyroid disruptors on the expression of thyroid-responsive genes can help better understand the mechanisms of thyroid disruption and their effects.

Endocrine disruption

Types of endocrine disruptors and their biological effect. Chemicals that are considered endocrine disruptors are often man-made organic compounds. Examples are dioxins, dichloro-diphenyl-trichloroethane (DDT), Diethylstilbestrol (DES), Bisphenol A (BPA) and polychlorinated biphenyls (PCBs), etc. Many of these chemicals are released into the environment in large amount in industrial waste (e.g., PCBs), pesticides (e.g., DDT) or household materials (e.g., BPA in plastics). These compounds are often not readily decomposed naturally, thus they are persistent in nature after their release and are a long term threat to human and wildlife populations. Many of these compounds are lipophilic and consequently are bioaccumulated up the food chain.

Endocrine disruptors affect endocrine function through different mechanisms. Some of these chemicals are structurally similar to certain hormones and can act as agonists or antagonists to endogenous hormones, thereby altering the hormonal balance within the system. For example, Dichlorodiphenyldichloroethylene (DDE) is a product of natural degradation of DDT which has lower toxicity but is even more persistent in nature than DDT. Both DDT and DDE are known to bind to estrogen receptors and have estrogenic effect. Exposure to DDT and DDE in young animals may cause abnormal development in their reproductive system. For example, it was demonstrated that DDT exposure stimulates uterine growth in immature rats (Robison and Stancel, 1982).

DES is another estrogen agonist. It was a pharmaceutical estrogen once widely prescribed to pregnant women between the 1930s and 1970s to prevent miscarriage. However, exposure to this estrogen appeared to affect the health of the offspring. The use of DES is found to increase the risk of clear-cell carcinoma of the vagina and cervix in female offspring. In addition, a possible association between higher occurrences of breast cancer in DES-exposed female offspring has been reported (Herbst *et al.*, 1971; Palmer *et al.*, 2002). Males exposed to DES have a higher incidence of decreased sperm counts and abnormal genital development as a result of its estrogenic effect (Gill *et al.*, 1979). DES is an example of clear evidence of endocrine disruption in humans.

In addition to DDT, DDE and DES, BPA also has agonist effects on estrogen receptors. BPA exposure leads to estrogen like effect in target cells and may be toxic to developing neurons (Le *et al.*, 2008).

Vinclozolin, a fungicide, and DDE are examples hormone antagonists. Both chemicals are demonstrated to bind to androgen receptors and inhibit the expression of androgen receptor-dependent

genes (Kelce *et al.*, 1995; Kelce *et al.*, 1997). The anti-androgen effects of DDE speculated to have caused decreased plasma testosterone concentrations and abnormal development of external genitalia in male alligators in contaminated areas in Florida (Guillette Jr *et al.*, 1996).

Other chemicals may affect the endocrine system by interfering with hormone synthesis. For example, It has been demonstrated that 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) decreases the activity of 17 α -hydroxylase/17, 20-lyase cytochrome P450 (P450c17), a key enzyme in estradiol synthesis, which leads to decreased estradiol production in human luteinized granulosa cells (Moran *et al.*, 2003).

Endocrine disruptors also can affect hormone metabolism and excretion. Studies in mammals showed PCBs induce uridine diphosphate glucuronosyltransferase (UDP-GT), a phase II biotransformation enzyme in liver that functions to enhance the excretion of PCBs. UDP-GT also facilitates 3, 5, 3', 5'-tetraiodothyronine (T₄) excretion in bile by glucuronidation of the hormone (Barter and Klaassen, 1992; Barter and Klaassen, 1994). Therefore, induction of UDP-GT by PCBs also increases the excretion of T₄, which results in decreased circulating T₄ concentration.

The complexity of the mechanisms of endocrine disruption has led the researchers to incorporate techniques from endocrinology, cell biology, biochemistry and molecular biology to better understand the effect of endocrine disruptors.

Endocrine disruption of thyroid function. Thyroid function is known to be affected by several endocrine disruptors including perchlorate and PCBs. Exposure to these chemicals leads to hypothyroidism and developmental defects.

PCBs were widely used as coolants and insulating fluid for transformers and capacitors. Their production was banned in the 1970s. However, significant amount of PCBs still remain in the environment (Erickson, 1997). In heavily PCB contaminated areas such as the Great Lakes in the Midwest of the United States, decreased reproductive success and declined colony size were observed in herring gulls. High embryonic mortality and reduced embryonic tissue growth also were seen in a number of wild bird species in the area. This led to the speculation that alterations in thyroid functions could be responsible for these problems. Analysis of the contents of the eggs revealed PCBs and other environmental contaminants were present in the eggs (Gilman *et al.*, 1977). Laboratory studies have demonstrated PCB exposure can cause hypothyroidism in developing birds (Roelens *et al.*, 2005). Sustained exposure to PCBs during development may have led to physiological disorders described above in the birds of PCB contaminated areas (reviews, Fox, 1993; McNabb and Fox, 2003). Studies in mammals demonstrated that PCBs induce uridine diphosphate glucuronosyltransferase (UDP-GT), a phase II biotransformation enzyme in liver that facilitates T₄ glucuronidation and its excretion in bile (Barter and Klaassen, 1994). An earlier study in our laboratory showed similar effect of UDP-GT activation led to decreased plasma T₄ concentration and hypothyroidism in PCB exposed birds (Webb and McNabb, 2008).

Perchlorate is another known thyroid disruptor. Ammonium perchlorate is extensively used as an oxidizer in rocket fuel, explosives and fireworks in the United States (Fig. 2.1). Perchlorate contaminant is found in water systems, soil, sediment and vegetation in a number of states such as California and Texas (Urbansky, 1998; Smith *et al.*, 2001; EPA, 2005a; Kendall and Smith, 2006). In 2005, EPA has established an official reference dose of 0.0007 mg/kg/day of perchlorate as the daily exposure level that was not expected to cause adverse health effects in humans (Committee to Assess the Health Implications of Perchlorate Ingestion, 2005; EPA, 2005b). Perchlorate contaminations mostly occur near locations of chemical plants and military bases where large amount of perchlorate is manufactured and used. Concentrations of perchlorate in the ground water from these areas range from less than 1 μ g/L to as high

as 3700mg/l (EPA, 2005a). Perchlorate also is found to accumulate in vegetation and can reach concentrations as high as several hundred milligrams per kilogram wet weight in heavily contaminated areas (Kendall and Smith, 2006). Wildlife populations such as ground dwelling birds are highly dependent on local water and food resources. Therefore, birds in the contaminated area are facing the full impact of the thyroid disrupting effects of perchlorate exposure throughout their post-hatch life. Perchlorate ion also is found to be deposited into eggs by perchlorate exposed hens, indicating maternal perchlorate exposure also may lead to embryonic exposure to perchlorate (Gentles *et al.*, 2005). Moreover, because thyroid hormones have critical roles in the growth and development of young animals (McNabb, 1992), perchlorate induced-hypothyroidism may result in irreversible defect in developing animals.

Perchlorate ion inhibits iodide uptake by into the thyroid gland by the Na-I symporter, resulting in decreased iodide availability in the thyroid gland for thyroid hormone synthesis and hypothyroidism in exposed animals (Wolff, 1998). Recent studies on perchlorate pharmacokinetic modeling demonstrated perchlorate ion is transported into the thyroid gland and may interfere with other processes of thyroid hormone synthesis (Yu *et al.*, 2002; McLanahan *et al.*, 2008). The Na-I symporter is expressed in several other tissues besides thyroid gland such as salivary glands, lactating mammary glands and gastric mucosa. Although little is known about the function of Na-I symporter in these tissues, it is speculated that iodide transportation in these tissues also was mediated by the Na-I symporter. Na-I symporter in the lactating mammary gland may be important for providing the infant with iodide through milk, and Na-I in the gastric mucosa may be involved in the dietary iodide uptake (Dohan *et al.*, 2003). Therefore, perchlorate exposure through drinking water and food may also interfere with iodide uptake in the digestive system and lead to iodide deficiency in exposed animals. Previous studies in our laboratory demonstrated that iodide concentration in Japanese quail eggs was decreased when hens were fed a low iodide diet (McNabb *et al.*, 1985a; McNabb *et al.*, 1985b). In addition, it was demonstrated that in birds, iodide is deposited in the eggs by active transport (Newcomer *et al.*, 1984). It is possible that Na-I symporter also is involved in this iodide transport. Hence, perchlorate exposure of female birds can potentially lead to decreased maternal iodide deposition in their eggs. There is little evidence of direct effect of perchlorate on other tissues than the thyroid gland (Kendall and Smith, 2006).

There is some evidence that birds may adapt to perchlorate exposure. A previous study in our laboratory in bobwhite quail demonstrated that young birds exposed to perchlorate were able to recover from hypothyroidism after sustained exposure. The ability to recover from the initial effect of perchlorate exposure decreased as the dosage of perchlorate increased. No sign of adaptation was seen in birds exposed to perchlorate concentrations higher than 500 mg/l (McNabb *et al.*, 2004a).

Assessment of thyroid disruption. Thyroid status in animals exposed to thyroid disruptors is evaluated by measuring plasma thyroid hormone concentrations (for organismal thyroid status), thyroid gland weight (for evidence of hypothalamus-pituitary-thyroid gland axis activation) and thyroidal hormone contents. Development of hypothyroidism is determined by overall analysis of measurements of all thyroid variables. Previous studies in our laboratory demonstrated that thyroidal T_4 is the most sensitive indicator of decreased thyroid function, while thyroid gland weight and plasma thyroid hormone concentrations were relatively less sensitive indicators. In contrast, body weight and specific tissue growth were very insensitive to changes in thyroid status (McNabb *et al.*, 2004b). In the early stages of hypothyroidism, the first response to minimize the decrease in circulating thyroid hormone concentrations is the release of stored hormones in the thyroid gland. Depletion of stored hormones in the gland leads decreased plasma hormone concentrations. Decreased plasma hormone concentrations activate the

hypothalamus-pituitary-thyroid axis resulting in thyroid gland tissue growth (thyroid gland hypertrophy) to increase thyroid hormone production. Decreased circulating thyroid hormone concentrations usually indicate profound hypothyroidism when the release of stored hormones and increased thyroid hormone production failed to compensate the loss of thyroid hormones in circulation caused by exposure to thyroid disruptors. Significant effects of hypothyroidism on growth may not be observed unless the exposed animal have experienced profound and sustained decrease in circulating thyroid hormone concentrations.

Thyroid function

The thyroid gland and regulation of its function. The thyroid gland is made up of spherical functional units called follicles, which consist of a single layer of follicular epithelial cells surrounding a lumen. There are two types of thyroid hormones: thyroxine (3, 5, 3', 5'- tetraiodothyronine, T₄) and triiodothyronine (3, 5, 3'-triiodothyronine, T₃; Fig.2.2). In the follicle cells, a precursor protein thyroglobulin is produced and thyroid hormones are formed from iodinated tyrosine residues in the thyroglobulin “backbone” and stored in thyroglobulin in colloid in the lumen of the follicles. For hormone release colloid is taken up by the luminal membrane of the follicle cells by endocytosis. The engulfed colloid vesicles containing thyroglobulin then fuse with lysosomes and thyroglobulin molecules are degraded by lysosomal enzymes to produce T₄ or T₃, which are then released into circulation. T₄ is the major product of the thyroid gland but T₃ is the physiologically active form.

Thyroid gland functions are regulated through the hypothalamic-pituitary-thyroid axis (Fig. 2.3). Thyrotropin-releasing hormone from the hypothalamus stimulates the release of thyrotropin which in turn stimulates all aspects of thyroid gland function such as iodide uptake, production and release of thyroid hormone and thyroid gland growth. The concentration of circulating thyroid hormones is controlled through a negative feedback system. Increased circulating thyroid hormone concentrations inhibit the release of thyrotropin from the pituitary (short loop negative feedback) and inhibit the secretion of the thyrotropin-releasing hormone from the hypothalamus (long loop feedback), and thus decrease thyroid hormone release from the thyroid gland. On the other hand, a decrease in circulating thyroid hormone stimulates the secretion of thyrotropin-releasing hormone and thyrotropin resulting in increased thyroid hormone release from the thyroid gland (McNabb, 1992).

Thyroid hormone deiodination. Circulating T₃ is produced mostly from T₄ by extra thyroidal deiodination, primarily in liver and kidney. The conversion is catalyzed by 5'-deiodinases (McNabb, 1992). There are two kinds of deiodinase reactions: 5' (outer ring) deiodination which produces T₃ from T₄, and 5 (inner ring) deiodination which deactivates both T₄ (to inactive reverse T₃) and T₃ (to inactive T₂). The deiodination reactions are catalyzed by three types of deiodinases, type 1 (D1), type 2 (D2) and type 3 (D3) deiodinases. D1 catalyzes both 5' and 5 deiodination activities. D2 only catalyzes 5' deiodination and D3 only catalyzes 5 deiodination. Both D1 and D3 activities are positively correlated with circulating T₄ concentrations. The activity of D2, however, is inversely regulated by both T₄ and T₃ through different mechanisms. High T₄ concentrations facilitate D2 protein degradation by proteasomes thereby decrease D2 activity (Steinsapir *et al.*, 1998). High T₃ concentrations, on the other hand, suppress D2 mRNA levels which ultimately decrease D2 activity (Burmeister *et al.*, 1997). In mammals, D1 is the major deiodinase responsible for the production of circulating T₃ in liver and kidney. D2 is absent in mammalian liver but is responsible for most of the deiodination in the central nervous system (Köhrle, 2000; Bianco *et al.*, 2002). However, in birds, both D2 mRNA and D2 activity have been detected in the liver in addition to in the brain (Hughes and McNabb, 1986; Gereben *et al.*, 1999).

Thyroid hormone regulation in the brain. Thyroid hormones are important for brain

development and function. Thyroid hormones regulate the expression of various thyroid-responsive genes in the brain, most of which are involved in the normal development of the brain, within specific time periods (i.e., critical “windows”) during development. Many of these genes become less responsive or non-responsive to thyroid hormones when those time windows are past. Hypothyroidism during these time windows can affect the expression of these genes and consequently the development of the brain. Such effects may be irreversible if treatment for hypothyroidism does not occur within the critical time windows (Bernal, 2002).

The brain is protected from temporary hypothyroidism by three major mechanisms in both mammals and birds: (a) increasing thyroid hormone uptake into the brain across the blood-brain-barrier, (b) changes in the deiodinase activities to increase T_3 production in the brain and (c) decreasing T_3 degradation and loss from the brain (Rudas *et al.*, 2005). T_3 in the brain is mostly produced locally from T_4 transported into the brain and the uptake of T_4 is significantly increased during hypothyroidism. D2 is the responsible for most of the T_3 production in the brain (Bianco *et al.*, 2002). The expression of D2 is strictly regulated by the thyroid hormone concentrations in the brain. The mRNA level is increased by low T_3 concentrations and decreased by high T_3 concentrations in the brain (Kim *et al.*, 1998; Gereben *et al.*, 2002). T_4 concentrations also play a role in regulating D2 activity by facilitating the degradation of D2. Thus D2 protein has a longer half life under low T_4 concentrations than under high T_4 concentrations in the brain (Burmeister *et al.*, 1997). Overall, decreased thyroid hormone concentrations in the brain, resulting from hypothyroidism, may increase D2 mRNA level and activity. The deactivation of T_3 by D3 and the transport of T_3 out of the blood-brain-barrier are both decreased in the brain during hypothyroidism to help maintain the T_3 concentration in the brain (Rudas *et al.*, 2005). In mammals, with these mechanisms the brain can remain euthyroid condition for up to two weeks after the animal becomes hypothyroid (Bernal, 2002). The time scale for these effects has not been investigated in birds.

Thyroid hormone actions. Thyroid hormones regulate various physiological activities. This section will focus on the role of thyroid hormones in regulating metabolism and development. Thyroid hormones stimulate metabolic activity in most tissues and increase heat production in homeotherms (birds and mammals). Hypothyroidism causes decreased basal metabolic rate, and hyperthyroidism results in increased basal metabolic rate in homeotherms, indicating that thyroid hormones are important for maintaining thermal homeostasis. The development of thermoregulation is different in animals with different developmental patterns. In precocial birds such as Japanese quail, thermoregulation is initiated at hatching. However, in altricial birds such as ring doves, most energy is invested in growth during early post-hatch life and thermoregulation does not fully develop until one or two weeks after hatch (McNabb, 2006).

Thyroid hormones also initiate tissue differentiation during development and are involved in growth and maturation of many organs and tissues (McNabb, 1992). Both thyroid hormones of maternal origin and those from the embryonic thyroid gland are involved in the regulation of embryonic development. In mammals, it was shown that maternal hypothyroidism decreased the thyroid hormone concentrations in fetal organs (Escobar del Rey *et al.*, 1987) and affected fetal organ growth (Morreale de Escobar *et al.*, 1985). Thyroid hormones of the maternal origin also were found to regulate thyroid-responsive genes in the fetal brain in rats (Dowling and Zoeller, 2000). In birds, maternal deposition of thyroid hormones in eggs was found to be dependent on the hen’s thyroid status (Wilson and McNabb, 1997). A number of studies also demonstrated changes in the maternal thyroid hormone deposition in eggs affect development of embryonic organ and tissues such as cartilage (Wilson and McNabb, 1997) and muscle (Maruyama *et al.*, 1995). Disruption of embryonic thyroid function also has detrimental

effects on the embryos development. Embryos from PCB injected eggs developed hypothyroidism, and consequently, they showed delayed hatching and high mortality during hatching (Roelens *et al.*, 2005). Therefore, both maternal and embryonic hypothyroidism affects the development of the embryos.

Thyroid hormones are critical in initiating differentiation and maturation in many tissues including muscle (Maruyama *et al.*, 1995), the skeleton (Wilson and McNabb, 1997) and the central nervous system (Flamant and Samarut, 1998) and are required, at least permissively or indirectly, for growth (McNabb, 1992). Thyroid hormones stimulate muscle cell proliferation, differentiation and muscle fiber hypertrophy. Chickens with altered thyroid function were found to exhibit muscular dystrophy. Thyroid hormones also stimulate matrix production and ossification in cartilage tissues to initiate differentiation and maturation of these tissues. The development of pelvic cartilages in chicken and bobwhite quail was found to be affected by thyroid status (McNabb and King, 1993; Wilson and McNabb, 1997). Thyroid hormones also are involved in the regulation of cell migration, differentiation and maturation of brain cells in mammals (Bernal, 2002). In birds, it was demonstrated that overall growth was decreased after thyroidectomy or goitrogen treatment (McNabb and King, 1993).

Thyroid hormones act by binding to nuclear thyroid receptors the hormone-receptor complex then alters the expression of thyroid-responsive genes which leads to further physiological effects in target organs. Though both thyroid hormones are known to bind thyroid receptors, thyroid receptors exhibit a much stronger affinity (5-10 folds) for T_3 than T_4 ; indicating T_3 is most responsible for thyroid hormone actions. Two thyroid receptor genes have been identified as $TR\alpha$ and $TR\beta$, each can give rise to multiple isoforms of TRs such as $TR\alpha-1$, $TR\alpha-2$, $TR\beta-1$ and $TR\beta-2$ (Munoz and Bernal, 1997). Studies in chicken demonstrated that $TR\alpha$ isoforms were universally expressed in many tissues, whereas the distribution of $TR\beta$ isoforms was restricted to a few tissues including brain, lung and kidney. Developmentally, the expression of $TR\alpha$ isoforms began early in embryonic development before the onset of the embryonic thyroid functions in both mammals and birds. $TR\beta$ isoforms, in contrast, were non-detectable or expressed at very low level during early embryonic development. However, the expression of $TR\beta$ isoforms increased sharply in brain and lung during late embryonic stages in chicken (Forrest *et al.*, 1990). A similar pattern of $TR\beta$ expression also was observed in the brain altricial animals such as rats, where $TR\beta$ first was expressed at very low level in some fetal brain areas then the expression increased and expanded to more brain areas during the postnatal period (Munoz and Bernal, 1997; Bernal, 2002). Therefore, it appears that $TR\alpha$ and $TR\beta$ regulates different physiological processes and $TR\beta$ may have important roles in the late embryonic development in tissues such as brain.

There are many genes in mammalian liver and brain whose expression are regulated by thyroid hormones. Most of the genes were identified by screening the genome for thyroid-responsive elements or microarray studies (Feng *et al.*, 2000; Bernal, 2002; Flores-Morales *et al.*, 2002). The biological and physiology functions were only characterized in a fraction of the identified thyroid-responsive genes. In the liver, many of these thyroid responsive genes are involved regulation of the lipogenesis pathway and mitochondria functions (Feng *et al.*, 2000). In the brain, thyroid responsive genes are believed to have critical roles in normal brain development (Bernal, 2002).

Thyroid hormones and brain development. Development of the vertebrate brain begins as the anterior portion of the neural tube bulges and differentiates into distinct vesicles which give rise to different structures of the brain. The neural tube is composed of a single layer of cells called germinal neuroepithelium, which gives rise to other brain cells (neurons and glia). A neuroepithelium stem cell divides vertically during mitosis, and one of the two daughter cells remains connected to the neural tube and remains as a stem cell while the other daughter cell migrates away from the neural tube. This process

continues and the “later-divided” cells migrate over the “earlier-divided” cells to form the outer layers of the cortex. As a result, the younger cells are found in the more superficial regions of the cortex and the older cells are usually found in the interior regions. The daughter cells that migrate away from the germinal neuroepithelium will not divide again, so the time of their division is called their birthday. Cells having earlier birthdays migrate shorter distances than those having later birthdays. Different types of neurons and glia have different birthdays and migrate different distances to form layers of the brain, each of which is in charge of different functions. Interference of the cell migration to appropriate layers may result in developmental defects in the brain (Gilbert, 2000).

Thyroid hormones play a key role in vertebrate brain development. Evidences of the involvement of thyroid hormones in brain development are mainly collected from studies on hypothyroidism in laboratory mammals (Zoeller and Crofton, 2000; Bernal, 2002). In rats, thyroid hormones of maternal origins are found in the embryonic and fetal tissues before the onset of fetal thyroid gland function (Morreale de Escobar *et al.*, 1985; Escobar del Rey *et al.*, 1987). TRs also can be detected in rat brain several days before the onset of thyroid gland function in the fetus, indicating that maternal thyroid hormones are involved in early brain development (Oppenheimer and Schwartz, 1997). Rats born to hypothyroid dams and rats having congenital hypothyroidism exhibit symptoms like hypomyelination as well as altered cell migration and differentiation. The degree of the defects is closely related to the timing of the onset and severity of the hypothyroidism. There are reports that adult onset hypothyroidism also causes brain defects such as altered dendritic spine number (Ruiz-Marcos *et al.*, 1988; Gould *et al.*, 1990). These defects during adult life, after brain architecture has been established, can be reversed by treatment with exogenous thyroid hormones. Hypothyroidism in the fetus and neonates, however, usually leads to irreversible damage in the brain if thyroid hormone treatment is not conducted within the time window when the genes are under thyroid control (Zoeller and Crofton, 2000; Bernal, 2002).

Studies in rats have identified a number of thyroid responsive genes in the brain. Some of these genes are direct targets of the T₃-thyroid receptor complex whereas some other genes are regulated by T₃ through a secondary medium. For example, T₃ may control the expression of a gene whose product has a regulatory effect on the terminal target, with the end result being the modification of the expression of a second gene. Many details of the mechanisms of thyroid hormones’ regulation of genes in the brain still remain unclear. Most of the thyroid-regulated genes identified in developing brains so far are only sensitive to T₃ during a specific time window. Some of the genes only respond to T₃ transiently, others have a relatively longer period of time during which they are sensitive to T₃. The expression of different genes may be either elevated or suppressed within the time window of responsiveness. The changes in level of expression of the genes during those specific time windows are critical for important events in brain development such as myelination, cell differentiation and migration. In hypothyroid rats, modified gene expressions are found to be associated with flawed brain development. Surprisingly, all these genes become normally expressed in hypothyroid rats 30 days after birth even without thyroid hormone replacement treatments. The majority of these thyroid-responsive genes stop responding to T₃ after their specific time window, except for genes of nerve growth factor (NGF), tyrosine kinase receptor A (TrKA) and RC3/neurogranin, which remain sensitive in adult rats. However, recovery of the gene expression after the critical time window cannot reverse the defects in the brain caused by earlier hypothyroidism (reviewed by Bernal, 2002).

Thyroid disruption and development

Exposure to thyroid disruptors such as perchlorate and PCBs is known to affect thyroid function

by decreasing circulating thyroid hormones and inducing hypothyroidism. Postnatal rats that were developmentally exposed to PCBs suffer sensory deficits (e.g. in hearing development; Goldey *et al.*, 1995). Asymmetric brain development has been observed in wild and laboratory birds exposed to mixture polyhalogenated aromatic hydrocarbons (Henshel *et al.*, 1997; Henshel, 1998). Perchlorate exposure was demonstrated to affect growth of bobwhite quail chicks (McNabb *et al.*, 2004b). Since organ and tissue development is dependent on thyroid hormones, the above mentioned effects can be caused by hypothyroidism induced by chemical exposure.

Alteration of gene expression by thyroid disruption can be divided into two categories according to the nature and function of the genes affected: a) thyroid hormone responsive genes involved in tissue specific thyroid hormone regulation and b) thyroid hormone responsive genes involved in target organ development or function. Examples of the former group are the deiodinase genes (D1, D2 and D3) and examples of the latter include RC3 in the brain and Spot 14 in the liver.

Effects of thyroid disruption on deiodinase expressions in brain and liver. The activities of all three deiodinases are affected by changes in thyroid hormone concentrations. D1 and D3 activities are positively regulated by thyroid hormones whereas D2 activity is inversely regulated by thyroid hormones. D2 is the major, and in several species the only, 5' deiodinase in the brain (Bianco *et al.*, 2002). During temporary hypothyroidism, the brain is capable of maintaining a stable euthyroid T₃ concentration by increasing T₄ uptake and D2 activity as well as by decreasing T₃ deactivation and excretion (Rudas *et al.*, 2005). D2 activity is regulated by both T₄ and T₃ through different mechanisms; T₄ facilitates D2 protein degradation while T₃ affects D2 mRNA level. The effect of T₄ on D2 activity is fast, whereas that of T₃ is usually slow (Burmeister *et al.*, 1997). Because of the increased T₄ uptake when circulating T₄ is low, the brain can maintain a stable T₄ supply of substrate for D2 for an extended period of time. Therefore, changes in D2 activity may not be observed immediately after the onset of hypothyroidism. In addition, changes in T₃ concentrations will be further delayed by the protective mechanisms of the brain against hypothyroidism. Because of the slow response of D2 mRNA level to changes in T₃ concentration, the changes in D2 mRNA level in the brain in response to hypothyroidism may not be observed until after a sustained period of hypothyroidism.

D1 is the major deiodinase in the liver and is believed to be responsible for the production of most of the circulating T₃. Unlike brain, liver is not protected from organismal hypothyroidism, as the thyroid hormone concentrations in the liver co-vary with the plasma thyroid hormone concentrations (Reyns *et al.*, 2002). Therefore, the effect of hypothyroidism on the hepatic deiodinase activity is expected to be seen soon after the onset of hypothyroidism. Decreased circulating T₄ concentrations during hypothyroidism will reduce D1 activity in the liver, which leads to decreased T₃ production and release from the liver into circulation. In avian species, D2 also is expressed in the liver (Hughes and McNabb, 1986; McNabb *et al.*, 1986; Gereben *et al.*, 1999). D2 activity and mRNA level in the liver are expected to be more sensitive to hypothyroidism than that in the brain.

Effects of thyroid disruption on the expression of thyroid hormone target genes in brain and liver. Thyroid hormone regulates the expression of a large number of genes in brain and liver during development. Hypothyroidism resulting from exposure to thyroid disruptors can affect the expression of these genes and thereby interfere with the development and physiological functions of these organs.

RC-3/Neurogranin gene was selected to be the thyroid-responsive gene to investigate in the brain. In mammals, RC3 is a 78 amino acid, neuron-specific protein found in the dendrites, dendritic spines and cell bodies of neurons. This protein binds specifically to calmodulin (CaM) and prevents the CaM from binding calcium ion (Ca²⁺). The affinity of RC3 for CaM decreases as free Ca²⁺ concentration increases.

RC3 is closely related to GAP-43, which is mostly found in axons where RC3 is absent. The two proteins, together with some other proteins are referred to as calpacitins in that they regulate the CaM availability in response to changing Ca^{2+} concentrations. Both proteins bind tightly with CaM at low Ca^{2+} concentration but release CaM in response to a sharp increase in Ca^{2+} concentration to form CaM/ Ca^{2+} thus reducing Ca^{2+} concentration. The rate of CaM release is dependent on the size and rapidity of Ca^{2+} flux (Gerendasy *et al.*, 1994). Serving as capacitor of Ca^{2+} in the dendritic spines, RC3 is considered to be involved in dendritic spine development and remodeling and long-term potentiation. Thus, RC3 may be important for brain functions such as learning and memory (reviewed by (Gerendasy and Sutcliffe, 1997).

In adult rats, RC3 is abundant in forebrain but completely absent from the cerebellum. The protein is not expressed significantly in rat forebrain until after birth and reaches a maximum about 10-15 days after birth then declines to adult levels. The timing of the increase at 10-15 day postnatal in RC3 expression coincides with the onset of synaptogenesis (Gerendasy and Sutcliffe, 1997; Bernal, 2002). Studies in precocial goats, whose synaptogenesis occurs prenatally, shows the increase of RC3 expression begins prenatally and peaks at 1 day after birth thus also indicates a correlation between increasing RC3 concentration and synaptogenesis (Piosik *et al.*, 1995).

T_3 regulates the RC3 gene directly at the transcription level. Dowling and Zoeller (2000) have demonstrated that RC3 expression in fetal rat brain is regulated by thyroid hormone of maternal origin. The RC3 level in fetuses from hypothyroid dams is significantly lower than those from euthyroid dams (Zoeller *et al.*, 2000). RC3 is one of the few genes that respond to T_3 both during development and in adult brain; hypothyroidism reduces RC3 mRNA concentration in both neonatal and adult rat brain and the change in RC3 concentration appears to correlate with changes in numbers and distributions of dendritic spines observed in hypothyroid rats. Thus, the decrease of RC3 mRNA in adult rats can be reversed by thyroid hormone treatment. Because the RC3 gene is sensitive to thyroid hormone over a long period of time and undergoes an increase in expression which appears to be important for brain development, it appears to be the most favorable candidate for my study.

Effects of exposure to thyroid disruptors such as PCB and bisphenol-A on RC3 expression in the brain have been investigated in mammals. PCB exposure decreases circulating T_4 concentration in a dose-dependent manner in these rats. Bisphenol-A exposure increases plasma T_4 concentration in developing rat pups as a result of its antagonist effect on β -TR. However, both studies demonstrated increased RC3 mRNA level in some brain regions in these pups despite changes in plasma T_4 concentrations towards opposite directions in each study compared with the controls (Zoeller *et al.*, 2000; Zoeller *et al.*, 2005). The results indicate the effect of thyroid disruption on RC3 expression in the brain may be complicated.

Similar confounding results also were found in other thyroid sensitive genes. For example: myelin basic protein (MBP) is another gene that is under thyroid hormone control during brain development. MBP expression also has been found to be elevated in the brain following PCB exposure. Nonetheless, changes in mRNA concentration of both RC3 and MBP only occurred in regions that are thyroid hormone sensitive, suggesting that PCBs, as predicted, are in fact interfering with the thyroid functions in the brain but did not induce the reduction of the expression of the two genes as expected (Zoeller *et al.*, 2000). The relationship between the expression of these two genes and PCB-induced hypothyroidism turns out to be more complicated. Thus, although RC3 appeared to be a good candidate for studying thyroid hormone effect in brain, results from above mentioned studies raised the possibility that unpredictable results may be expected. The same subject has not been investigated in any avian

species.

Expressions of thyroid-responsive genes in the liver are more readily affected than those in the brain by thyroid disruption since the T_4 concentration in the liver co-varies with the circulating T_4 . Spot 14 is a protein expressed exclusively in lipogenic tissues such as liver and mammary gland (Jump and Oppenheimer, 1985). Spot 14 regulates the enzymes involved in the lipogenesis in the liver in mammals (Jump *et al.*, 1984). A study in Spot 14 knockout mice demonstrated it also is required for the lipogenesis in the mammary gland. The study also showed that Spot 14 does not affect the expression of the lipogenic enzymes, and the regulatory effects of Spot 14 like occur in the cytoplasm (LaFave *et al.*, 2006). The function of Spot 14 in the liver appears to be more complicated. Although Spot 14 expression is associated with lipogenesis in the liver, deletion of Spot 14 gene has much less effect on the lipogenesis in the liver than it does in the mammary gland. A paralog of Spot 14 gene is found to be expressed at higher level in the liver than in the mammary gland (Zhu *et al.*, 2001; LaFave *et al.*, 2006), suggesting there is alternative mechanisms for lipogenesis regulation in the liver besides Spot 14. In birds, Spot 14 is mostly expressed in liver, fat and ovary. Spot 14 expression is found to be associated with body weight and abdominal fat trait in chicken (Wang *et al.*, 2004; Cao *et al.*, 2007). Mammal studies have demonstrated that Spot 14 expression is regulated by thyroid hormone directly at transcription level (Jump *et al.*, 1997). Elevated thyroid hormone concentrations are shown to increase spot 14 mRNA level in the liver (Jump and Oppenheimer, 1985; Brown *et al.*, 1997). Spot 14 expression also is regulated by other factors such as carbohydrate, fatty acid and other hormones like insulin (Brown *et al.* 1997; Jump, *et al.* 1993; (LaFave *et al.*, 2006).

Traditionally, information on thyroid action in target organs and effect of thyroid disruption were mainly obtained through endocrinology, physiology and histology studies. End effects were commonly observed at tissue and systemic level. Recent development in molecular biology and the identification of tissue specific thyroid-responsive genes provide additional endpoints for the study of thyroid disruption. Many thyroid-responsive genes were identified by microarray studies (Feng *et al.*, 2000; Flores-Morales *et al.*, 2002; Liu and Brent, 2005) and functions of the majority of the identified genes have not been fully investigated. By combining the study of thyroid-responsive gene expression with endocrinology studies, researchers can gain knowledge on the molecular bases of thyroid function as well as detailed mechanism of thyroid disruption by environmental contaminants.

Model organism and methods

In my dissertation I designed two experiments to study the effect of exposure to thyroid disruptor ammonium perchlorate in developing Japanese quail.

Japanese quail were chosen as the organism to study for several reasons. Japanese quail are extensively used in animal studies by many researchers and its development and thyroid function are well understood. Their small size and short lifecycle make them easy to maintain and handle. Japanese quail also serve as a representative of ground-dwelling galliform birds that face the full impact of exposure to environmental pollutants in water and food (Ratnamohan, 1985). A number of studies in our laboratory on thyroid function were performed using Japanese quail and similar organisms (bobwhite quail) and a well established model for assessing thyroid disrupting effect in this species was developed (Wilson and McNabb, 1997; McNabb *et al.*, 2004b; Webb and McNabb, 2008).

Quail chicks were exposed to perchlorate in drinking water. Since it was demonstrated that perchlorate can be transferred from exposed female birds to their eggs (Gentles *et al.*, 2005), embryos were exposed to perchlorate through maternal perchlorate treatment in drinking water. Dosages were

chosen based on previous studies in our laboratory (McNabb *et al.*, 2004a; McNabb *et al.*, 2004b) and the results from several of my pilot experiments on perchlorate exposure of quail chicks. It was determined that 2000 mg/l ammonium perchlorate would cause mild hypothyroidism after two weeks of exposure. Development of overt hypothyroidism may require longer exposure time than two weeks at the dosage of 2000 mg/l ammonium perchlorate. A concentration of 4000 mg/l ammonium perchlorate also was used for the exposure of hens to induce hypothyroidism in shorter exposure time.

Thyroid status in exposed birds was evaluated by measuring plasma thyroid hormone concentrations (for organismal thyroid status), thyroid gland weight (for evidence of hypothalamus - pituitary-thyroid gland axis activation) and thyroidal hormone contents. Development of hypothyroidism is determined by analysis of all measurements. Previous studies in our laboratory demonstrated that thyroidal T₄ is the most sensitive indicator of decreased thyroid function while thyroid gland weight and plasma thyroid hormone concentrations were relatively less sensitive indicators (McNabb *et al.*, 2004b). In my study, all three of these thyroid function indicators were used to provide a more complete picture of thyroid function and they were analyzed together as interdependent variables.

The mRNA levels of D2 were measured in both brain and liver total RNA in exposed embryos and chicks to evaluate the responsiveness of thyroid hormone regulation in these two tissues. D2 is known to be expressed in both brain and liver in chicken and increase in mRNA level was observed in both tissues in thyroidectomized chicken (Gereben *et al.*, 1999).

RC3 mRNA level was measured in the brain of AP exposed birds and was compared to that of the controls. RC3 was chosen because of extensive mammalian studies showing its expression can be altered by hypothyroidism in developing animals (Dowling and Zoeller, 2000; Zoeller *et al.*, 2000; Zoeller *et al.*, 2005).

Spot 14 mRNA level was measured in the liver of AP exposed birds. Spot 14 was chosen because it is rapidly up-regulated by thyroid hormones and was extensively studied in both mammals and birds (Brown *et al.*, 1997; Zhu *et al.*, 2001; Wang *et al.*, 2004; Cao *et al.*, 2007; Ishihara *et al.*, 2007).

Endocrine disruption in avian species has been extensively investigated. However, not many studies have addressed the subject of thyroid disruption in birds using both endocrinology and molecular biology approaches. Even less is known about the effect of thyroid disruption on the thyroid-responsive gene expressions in the brain. The current study will focus on the effect of perchlorate exposure on thyroid function and the expression of thyroid-responsive genes in the brain and the liver in developing Japanese quail. The study also can provide insight on the application of molecular biology endpoint for assessing the thyroid disrupting effects of environmental contaminants.

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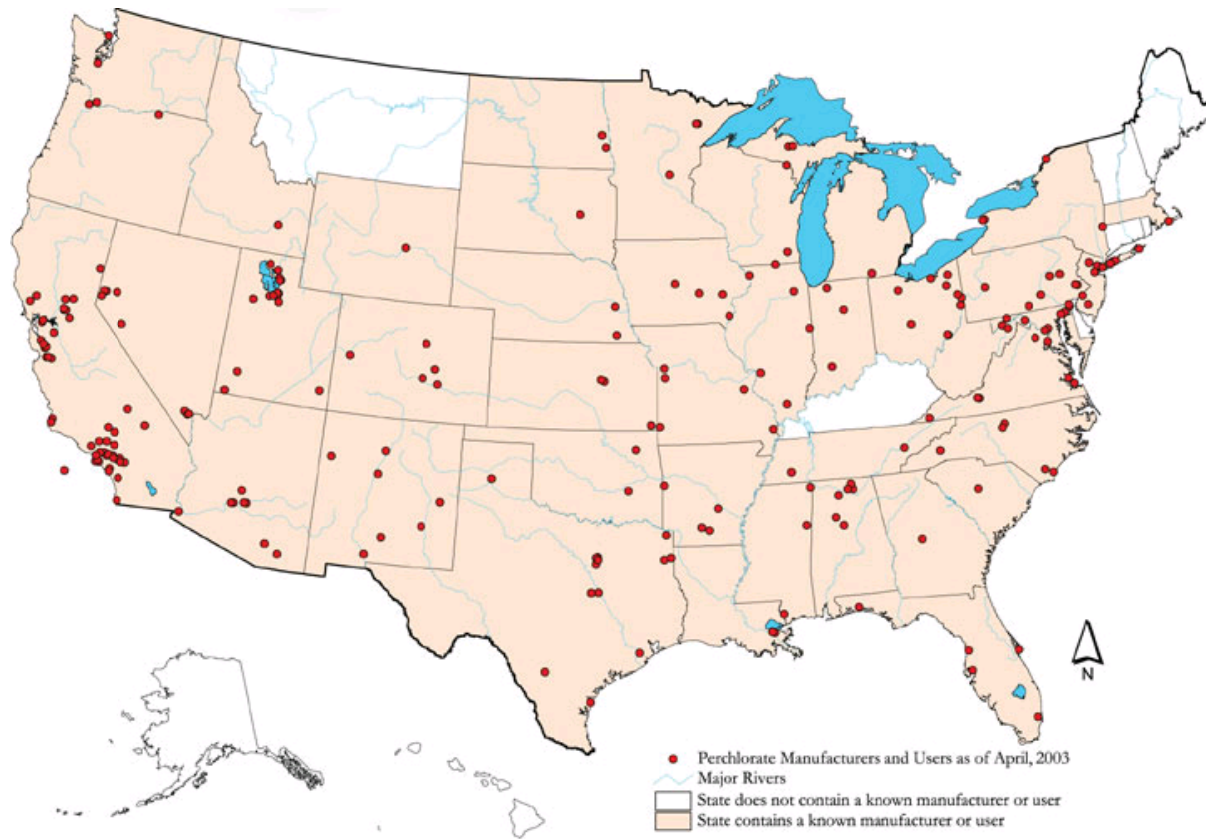


Fig. 2.1 Known perchlorate manufacturers and users in the United States (EPA, 2003). (http://www.epa.gov/swerffrr/images/perchlorate_manuf_users_map.jpg)

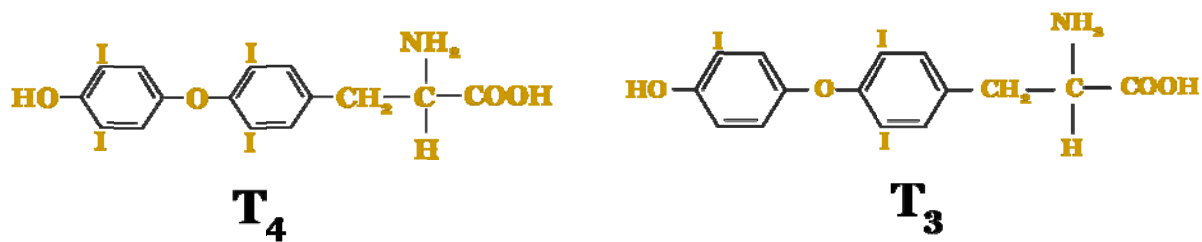


Fig. 2.2 Chemical structure of thyroid hormones.

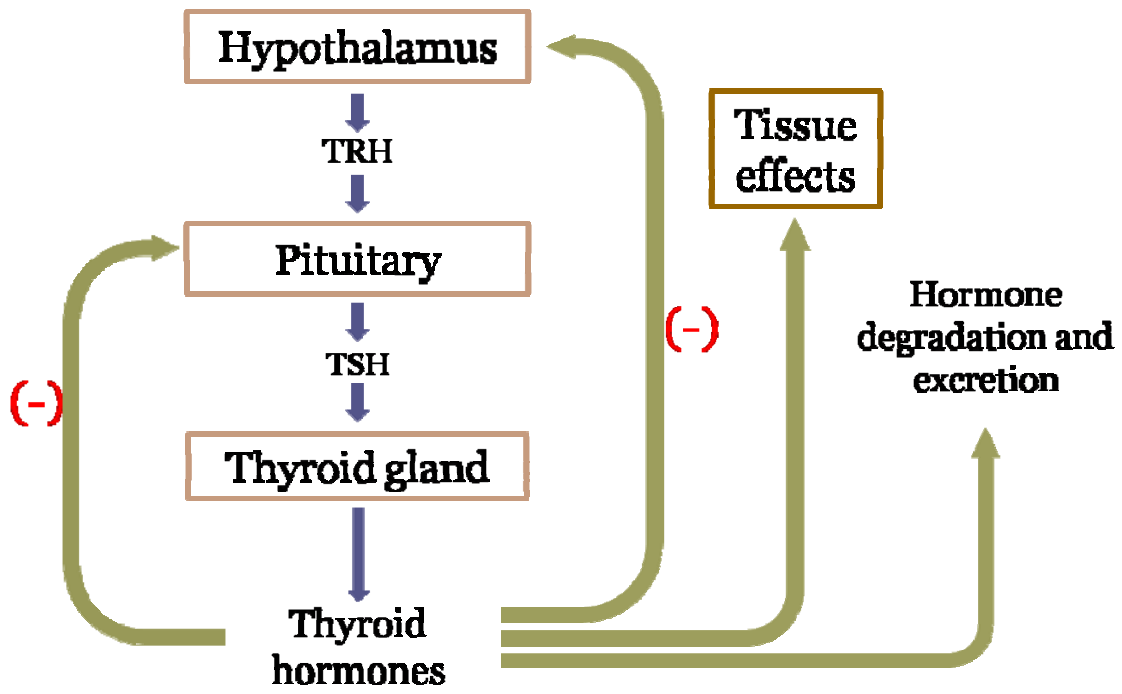


Fig. 2.3 Schematic illustration of thyroid function regulation by the hypothalamus-pituitary-thyroid axis.

Chapter 3

Cloning and Sequencing the Japanese quail homologs of Type 2 deiodinase, RC3/Neurogranin, Spot 14, Nerve growth factor, 18S ribosomal RNA and β -actin

Abstract

Many genes that respond to thyroid hormones have been identified and extensively studied in mammals and chicken, a common avian model species. However, very few genes were studied in Japanese quail, which is a less popular model organism but valuable for the study of avian physiology. In the current study, the cDNA of thyroid responsive genes Type 2 deiodinase, RC3/Neurogranin, Spot 14, and Nerve growth factor expressed in Japanese quail brain a liver were cloned by reverse transcription and polymerase chain reaction (RT-PCR) using primer pairs designed according to available sequences in other avian species such as chicken and canary. Japanese quail 18S ribosomal RNA and β -actin were also cloned as housekeeping for the upcoming studies on the expression of thyroid responsive genes under altered thyroid functions. The cloned cDNAs were sequenced and comparison of their sequence to homologous sequences in other species showed over 90% homology of nucleotide and protein sequence in most of the sequences. The availability of the sequence information of these thyroid responsive genes helps better understand the physiological effect of thyroid hormones in brain and liver of Japanese quail. It also offers new end point for thyroid function studies in Japanese quail.

Introduction

Thyroid hormones initiate various physiological processes in vertebrates by regulating the expression of thyroid responsive genes in target tissues (McNabb 1992). Identification and characterization of these genes in different organisms can provide detailed information on the mechanisms of thyroid functions. Moreover, comparison of the functions of homologous thyroid responsive genes from different species can help understand the evolutionary history thyroid function.

Hundreds of thyroid responsive genes were identified in the liver and brain of mammals (Bernal 2002; Feng, *et al.* 2000; Flores-Morales, *et al.* 2002). Sequences of most of the genes are available for rats and mice. Several of the identified thyroid responsive genes in mammals were extensively studied because of their important physiological functions, e.g. Spot 14 in the liver and RC3/neurogranin in the brain.

Much less information is available for the thyroid responsive genes in the avian species except chicken, in which a number of thyroid responsive genes were identified in the liver (Wang, *et al.* 2007). However, very little is known about the thyroid responsive genes in avian brain. Our laboratory studies the effect of thyroid disruption in Japanese quail and we are interested in the effect of hypothyroidism on the expression of thyroid responsive genes in brain and liver. Japanese quail was chose by our laboratory as the model organism because detailed background research was done on their physiology, development as well as thyroid functions. Its small size and short life cycle also made them suitable for the maintenance and handling in the facilities available to us. We are particularly interested in several genes including type 2 deiodinase (D2), RC3/neurogranin (RC3) and Spot 14 (SP14). None of the sequences were available for Japanese quail but all are available for chicken or rats. The current study describes the cloning and sequencing of Japanese quail thyroid responsive genes D2, RC3, Spot 14, Nerve growth factor (NGF) and two housekeeping genes 18s ribosomal RNA and β -actin.

Materials and methods

Total RNA isolation. To minimize RNA degradation, all equipment was pretreated to eliminate RNase. All glassware used in RNA isolation was baked at least three hours at 200°C. All plastic containers and centrifuge tubes were autoclaved and rinsed with RNaseZap solution (Ambion, Inc., Austin TX) before use. Bench surface, tube racks were sprayed with RNaseZap solution and wiped dry. All pipet tips used were filter tips and were autoclaved and sterilized by UV irradiation before use. Total RNA was isolated from whole brain of day 14 Japanese quail embryo as well as brain and liver of adult quail using Tri-Reagent (Sigma-Aldrich, St. Louis MO) following the protocol provided by the manufacturer. For each 50 mg of tissue, 1 ml of the Tri-Reagent was added. Tissues were homogenized in Tri-Reagent with a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Inc., Westbury NY). After homogenization, insoluble material in the homogenate was eliminated by centrifuging at 12000 g for 10 min. The supernatant was mixed with chloroform (0.2 ml for each milliliter of Tri-Reagent) and centrifuged at 12000 g for 15 min for phase separation. The top aqueous phase containing RNA was transferred to a new tube and mixed with isopropanol (0.5 ml for each milliliter of Tri-Reagent) to precipitate the RNA. RNA pellets were washed in 75% ethanol, dried and resuspended in RNase free water. The RNA samples were then stored at -80 °C for further analysis.

Construction of Japanese quail brain and liver cDNA libraries. The cDNA libraries were constructed by reverse transcription of total RNA isolated from brain and liver. For each reaction, 2 µg of total RNA from each tissue was added into 25 µl reaction mixture containing 0.5 mM dNTP mix, 25 units of ribonuclease inhibitor, 5 µl reaction buffer, 200 units of M-MLV reverse transcriptase (Fisher Scientific, Fair Lawn NJ) and 0.5 µg Oligo(dT)₁₅ primer (Promega, Madison WI) and nuclease free water. All reagents were treated with 0.1 DEPC water then autoclaved to be RNase free. Snap cap tubes were autoclaved and rinsed with RNaseZap before use. The reaction mixtures were incubated at 37°C for 60 min. The reaction was stopped by freezing reaction mixtures at -20 °C.

Polymerase chain reaction (PCR). After searching in the NCBI nucleotide database, the mRNA sequence of β-actin was found in quail. The mRNA sequences D2, Spot 14, NGF and 18S rRNA were found in chicken. The mRNA sequence of RC 3 was found in human, rats and canary. After searching in the chicken EST database, a chicken brain EST was found to share high similarity with the canary RC3 mRNA sequence. The names of the sequences and their GenBank accession numbers are listed in Table 3.1. Primers for PCR were designed based on the chicken sequences. Primers for quail RC3 cDNA were designed based on both canary sequence and the matched chicken EST sequences. The region where the primers were chosen was highly homologous between the chicken and canary sequences with no more than two mismatches within each primer. The bases in the chicken sequence were used in the mismatched positions. Choices of primer sites were restricted within the more conserved protein coding regions of the mRNA sequences. Two pairs of primers were designed for RC3 and D2 and three pairs of primers were designed for NGF to increase the chances of success amplification. All primers were purchased from Operon Biotechnologies, Inc. (Huntsville AB). The PCR reactions were carried out in 50 µl reaction mixtures that each contains 5 µl 10x reaction buffer, 200 µM dNTP mix, 2 units of Taq DNA polymerase (Fisher Scientific, Fair Lawn NJ), 10 pmol of each of the forward and reverse primers and 1 µl of cDNA. The PCR reactions were performed as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95 °C, 45 seconds of annealing and 30 seconds of extension at 72°C. Once the cycles were completed, another 3 min of extension at 72 °C was added. The reaction mixture was then frozen at -20 °C until analysis. Sequences of the primers and the annealing temperatures for each gene are listed in Table 3.2.

Cloning of PCR products. The PCR products were cloned using TOPO TA cloning kit (Invitrogen Corporation, Carlsbad CA) following manufacturer's instructions. 4 μ l of fresh PCR product (less than 24 hr after the PCR reaction finished) was mixed with 1 μ l salt solution and 1 μ l TOPO pCR2.1 vector. The mixture was incubated at room temperature for 5 min and was then placed on ice. 2 μ l of the mixture was added into a vial of One Shot Chemically Competent *E. coli* and was gently mixed. The cells were incubated on ice for 5 min and were then heat-shocked for 30 seconds at 42 °C. After heat-shock, 250 μ l S.O.C. medium (pre-warmed to room temperature) was added to the cells. The tube was then incubated at 37 °C in a shaker (200 rpm) for 1 hr. 50 μ l-100 μ l of the transformed cells were spread on a prewarmed (37 °C) selective plate containing ampicillin. The plates were incubated at 37 °C overnight. 2-3 colonies from each plate was selected from the plate and cultured in 10 ml LB medium containing 50 μ g/ml ampicillin overnight.

Cultured cells were collected by centrifugation and plasmid DNA was isolated by Qiagen Miniprep kit (Qiagen, Valencia CA). Cells were resuspended in 250 μ l buffer P1 in snap cap tubes and mixed with 250 μ l buffer P2. Immediately after mixing with buffer P2, 350 μ l Buffer N3 was added and mixed thoroughly by inverting the tubes several times. The tubes were centrifuged at 17900 g for 10 min to precipitate insoluble materials. Supernatant was transferred to QIAprep spin columns and centrifuged for 1 min. The columns were washed with 0.5 ml buffer PB and 0.75 ml Buffer PE. The plasmid was eluted with 50 μ l deionized water.

DNA sequencing. Plasmids containing inserted PCR products were sequenced by the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg VA). Cleaned PCR products also were sequenced. Both forward and reverse strands of the cDNAs were sequenced to assure the accuracy of the results. The cDNA sequences were compared to mRNA sequences of the corresponding genes in other species using the program Quickalign to confirm their identity. Percentage identities of nucleotide and protein sequences were determined using the program nucleotide blast and blastx from the National Center for Biotechnology Information (NCBI).

Results

All PCR reactions yielded DNA fragment at desired molecular weight except primer pair II for NGF and primer pair II for D2 (Fig. 3.1). The PCR reactions with Spot 14 α and β primers and the one with D2 primer pair I had relatively low yield but enough DNA was acquired for cloning and sequencing.

Sequencing of cloned plasmids was not successful. The results did not match any of the known sequences. All sequences were obtained from the sequencing reactions of uncloned PCR products. Their identities were confirmed by comparing their sequences with the chicken or canary sequences of the corresponding genes. The protein sequences translated from the DNA sequences of PCR products were also compared to the protein sequences of corresponding genes. The percentage of identities between the quail sequences and the sequences of other species were listed in Table 3.3. Most of the quail cDNA and protein sequences are over 90% identical to the corresponding sequences in chicken or canary (RC3). The quail RC3 share no significant similarity in its nucleotide sequence with the human and rat RC3 (RC3 nucleotide sequences of quail, human and rat homologs were compared using both the programs blast and bl2seq) but is 90% homologous with the canary RC3 and 99% homologous with the chicken EST. The protein sequences of quail RC3 is 94% homologous to the canary RC3 protein and only 89% homologous to human and rat RC3 proteins. Alignment of nucleotide and amino acid sequences of the quail cDNA and those of other species are shown in Figs 3.2-3.6.

Discussion

The successful isolation and identification of Japanese quail homologs of chicken and canary brain and liver thyroid responsive genes indicate the coding regions of these genes are well conserved within the avian species. Protein sequences showed higher percentage of homology than nucleotides, most likely because many of the mismatches in the nucleotides are at the wobble position (in most of the sequences over half of the mismatched nucleotides are at the wobble position, Table 3.3), indicating higher degree of flexibility of nucleotide sequences than protein sequences. Therefore, when cloning genes from non model species by RT-PCR, primers should be designed based on the homologous sequences of the most closely related model species.

The alignment of avian RC3 sequences with mammalian homologs showed high level of divergence in the sequences between the two vertebrate groups. However, the protein sequences were highly conserved in all species compared between the 20th and 50th amino acid of the N-terminus, which is critical for function of the protein (Gerendasy and Sutcliffe, 1997). This suggests that the role of this thyroid responsive gene in the brain may be similar in mammals and birds.

It is demonstrated by many studies that thyroid functions on development are very similar among birds and mammals as well as other vertebrate classes. The presence of quail homologs of thyroid responsive genes D2, RC3, NGF, Spot 14 α and β suggested that thyroid hormones may function through similar mechanisms in quail as they do in mammal in organs such as brain and liver. With the sequence of these genes available in quail, researchers can have better understanding of their biochemical characteristics and physiological functions in these birds. Comparison of the similarities and differences in the property and functions of quail genes with those of other vertebrate groups can provide useful information on the evolutionary history of thyroid function in vertebrates. For example, D2 was extensively studied in mammals and most of the studies demonstrated that D2 was absent in the liver. Originally, it was believed the situation is similar in avian species. An earlier study in our laboratory detected D2 like deiodinase activity in the liver of Japanese quail (Hughes and McNabb 1986; McNabb, *et al.* 1986). However, the expression of D2 in avian liver was not confirmed definitively until the D2 mRNA was cloned and characterized in chickens and mRNA of D2 was detected in chicken liver (Gereben, *et al.* 1999). The cloning and sequencing of D2 cDNA from Japanese quail liver cDNA library further supported the results of the early studies on quail hepatic thyroid hormone deiodination in Japanese quail and furthered the understanding D2 function in the liver as well as the organism as a whole.

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Table 3.1 Names and GenBank accession numbers of thyroid responsive genes in chicken, canary and mammals

Genes	Canary RC3	Rat RC3	Human RC3	Chicken EST (RC3 homolog)	Chicken NGF (predicted)
Accession #	U56726	NM_024140	NM_006167	CN223065	XM_418016
Genes	Chicken D2	Chicken Spot 14 alpha	Chicken Spot 14 beta	Chicken 18S rRNA	Quail β -actin
Accession #	NM_204114	AY568629	AY568731	AF173612	AF199488

Table 3.2 Sequences of primer pairs of RT-PCR of thyroid responsive genes from Japanese quail brain and liver total RNA

Gene		Primers sequences (5'→3')		Annealing temperature (°C)	Predicted product size (bp)
RC3	I	Forward	ATGGACTGCTGCAACGAGGG	50	221
		Reverse	TAATCGCCGTTGCGGAGGTCT		
	II	Forward	TGCACGAAGCTGGATGAGGACATC	50	199
		Reverse	CCTAATCGCCGTTGCGGAGGTC		
NGF	I	Forward	CCGGTCTTCTCGGGTCC	55	456
		Reverse	TCTCCCCGATTTCCTGC		
	II	Forward	CTGGGAAGGCAGAGGGA	55	1038
		Reverse	GGCTGAGGTAGGGGGAT		
	III	Forward	CACACACCTTCGTCAAAGC	55	100
		Reverse	GATTTCTGCTGAGCACAC		
D2	I	Forward	ATGGGTCTGTAAAGTGC	50	840
		Reverse	AAGGGACACATCTGTTG		
	II	Forward	GCCTACAAGCAGGTCAAAC	50	477
		Reverse	CACTCGCTCAAATGAAACC		
SP14 α	Forward	AGAAGATGGAGCAGGAGGTG	55	307	
	Reverse	GCTTGGTGTTTTTGGTGAG			
SP14 β	Forward	CGGTACTTCTCGGCCACGC	50	378	
	Reverse	GGGGCTGATCCGCTCCAG			
18S RNA	Forward	TGCATGTCTAAGTACACAC	55	1717	
	Reverse	TAGATAGTCAAGTTCGACC			
β-actin	Forward	CGTGCCGTGTTCCCATC	55	1000	
	Reverse	CCTGCTTGCTGATCCAC			

Table 3.3 Percentage homology of Japanese quail thyroid responsive genes compared with those of other avian and mammalian species

Genes	RC3				NGF	D2	Spot 14 α	Spot 14 β	18S rRNA	β -actin
Organism compared to	Canary	Chicken (EST)	Human	Rats	Chicken	Chicken	Chicken	Chicken	Chicken	Quail
Nucleotide (% identity)	90	99	N/A*	N/A*	94	97	94	90	100	100
% of nucleotide mismatches on wobble position	87	87	N/A	N/A	57	84	50	47	N/A	0
Amino acid (% identity)	94	100	89	89	94	98	91	86	N/A	100

*nucleotide sequences of both human and rat RC3 were aligned with quail RC3 using the program blast and bl2seq and no significant similarity was found.

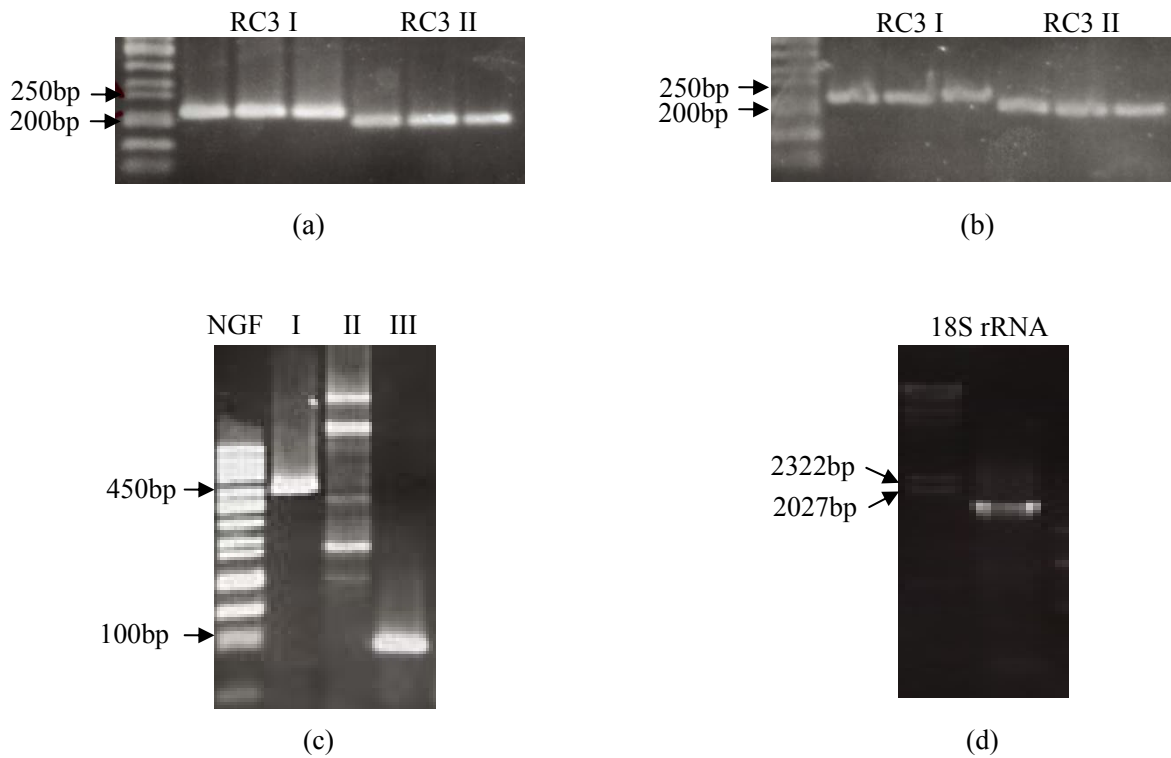


Fig. 3.1 Electrophoresis of products from PCR reactions. The reactions were done using RC3 primer pairs and embryonic brain cDNA (a) and adult brain cDNA (b); NGF primers and embryonic brain cDNA (c) and 18S rRNA and adult liver cDNA (d). Gels of the PCR products with Spot 14 and D2 primers are not included. The low yield from those reactions resulted in very faint band after electrophoresis.

	1	10	20	30	40	50
Quail	?	?	?	?	?	CTAGCTGGAGAGATCCT
Chicken EST	A	T	G	G	A	C
Canary	A	T	G	G	A	C
Rat	A	T	G	G	A	C
Human	A	T	G	G	A	C
Consensus	A	T	G	G	A	C
	51	60	70	80	90	100
Quail	G	G	A	C	A	T
Chicken EST	G	G	A	C	A	T
Canary	G	G	A	C	A	T
Rat	A	G	A	C	A	T
Human	A	G	A	C	A	T
Consensus	G	G	A	C	A	T
	101	110	120	130	140	150
Quail	A	G	G	C	A	G
Chicken EST	A	G	G	C	A	G
Canary	A	G	G	C	A	G
Rat	A	G	G	C	A	G
Human	A	G	G	C	A	G
Consensus	A	G	G	C	A	G
	151	160	170	180	190	200
Quail	A	T	G	G	A	C
Chicken EST	A	T	G	G	A	C
Canary	A	T	G	G	A	C
Rat	T	G	T	G	G	C
Human	C	G	C	G	G	C
Consensus	A	T	G	G	A	C
	201	210	220	230	240	250
Quail	-	C	G	C	G	A
Chicken EST	-	C	G	C	G	A
Canary	-	C	G	C	G	A
Rat	C	C	G	G	G	A
Human	C	C	G	G	G	A
Consensus	C	C	G	G	G	A

(a)

	1	10	20	30	40	50
Quail	?????????L	AGEI	LDI	PLDDP	DANAAAAKI	QASFRGHMTRKKIKCGE
Chicken EST	MDC	NEGACTKLDE	EDI	LDI	PLDDP	DANAAAAKI QASFRGHMTRKKIKCGE
Canary	MDC	NEGACTKLDE	EDI	LDI	PLDDP	DANAAAAKI QASFRGHMTRKKIKCGE
Rat	MDC	CTESACS	KPDDDI	LDI	PLDDP	GANAAAAKI QASFRGHMARKKIKSGE
Human	MDC	CTENACS	KPDDDI	LDI	PLDDP	GANAAAAKI QASFRGHMARKKIKSGE
Consensus	MDC	/E/AC/K	LD/DI	LDI	PLDDP	DANAAAAKI QASFRGHMT
	51	60	70	80	90	100
Quail	MDR	KAKDAECANST	--R-	GGDLRN-	GD*	????????????????
Chicken EST	MDR	KAKDAECANST	R	GGDLRN	GD*	VLLDSPCPSPFPPFPSS
Canary	I	DRKTKDAFCANST	--R-	GGDIRN-	GD*	????????????????
Rat	C	GRKGGPGGP	GGAGGARG	GAGGGPS	GD*	????????????????
Human	R	GRKGGPGGP	GGAGVARG	GAGGGPS	GD*	????????????????
Consensus	/	DRKAKDAECANST	G/	ARGGGDLRN	S	GD*VLLDSPCPSPFPPFPSS
	101	110	119			
Quail	?????????	?????????	?????????			
Chicken EST	PL	PPPPPP	PL	PPPPPP	PPPP	
Canary	????	????	????	????	????	
Rat	????	????	????	????	????	
Human	????	????	????	????	????	
Consensus	PL	PPPPPP	PL	PPPPPP	PPPP	

(b)

Fig. 3.2 Sequence alignment of Japanese quail RC3 and chicken EST, canary, rat and human RC3. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

```

NGF      261          270          280          290          300          310          320          325
Chicken  -----G G G C A G A G T A T G G G A T T T C T C A G C
Consensus G G G T C C T G T T C A G C A C G C A G C C A C C T C C A G T G T C A A G G A A A G G A C A G A G T A C G G G T T T C T C A G C
          326          330          340          350          360          370          380          390
NGF      A G T G C A G C C T C T C T C A A C A G G A C T G C C A G G A C C A A G A G G A C T G C C G A T C C C G T A T T G C A C C G G G G
Chicken  A G T G C A G T C T C T C T C A A C A G G A C T G C C A G G A C C A A G A G G A C T G C C A T C C C C T A T T G C A C C G G G G
Consensus A G T G C A G Y C T C T C T C A A C A G G A C T G C C A G G A C C A A G A G G A C T G C C R C A T C C C C T A Y T G C A C C G G G G
          391          400          410          420          430          440          450          455
NGF      A G A G T T C T C A G T G T G T G A C A G T G T C A C T A T G T G G G T C G G G G A C A A A A C C A C A G C C A C T G A C A T C A
Chicken  A G A G T T C T C A G T G T G T G A C A G T G T C A C C A T G T G G G T C G G G G A C A A A A C C A C C G C C A C C G A C A T C A
Consensus A G A G T T C T C A G T G T G T G A C A G T G T C A C Y A T G T G G G T C G G G G A C A A A A C C A C N G C C A C Y G A C A T C A
          456          460          470          480          490          500          510          520
NGF      A A G G C A A A G A G G T G A C C C T G T T G G G A C A G G T C A A C A T T A A C A A C A A T G T T T T T A A G C A G T A C T T T
Chicken  A A G G C A A A G A G G T G A C C C T G C T G G G A C A G G T C A A C A T T A A C A A C A A C G T T T T T A A G C A G T A C T T T
Consensus A A G G C A A A G A G G T G A C C C T G Y T G G G A C A G G T C A A C A T T A A C A A C A A Y G T T T T T A A G C A G T A C T T T
          521          530          540          550          560          570          580          585
NGF      T T C G A G A C C A A G T G C A G G G A C C C T C G C C C G G T G T C C A G C G G G T G C C G A G G G A T C G A T C G G A A G C A
Chicken  T T C G A G A C C A A G T G C A G G G A C C C T A G C C C G G T G T C C A G C G G G T G C C G A G G G A T C G A T C G G A A G C A
Consensus T T C G A G A C C A A G T G C A G G G A C C C T M G C C C G G T G T C C A G C G G G T G C C G A G G G A T C G A Y C G G A A G C A
          586          590          600          610          620          630          640          550
NGF      T T G G A A C T C A T A C T G C A C C A C A A C A C A C A C C T T C G T C A A A G C A C T G A C C A T C G A G G G C A A C C A A G
Chicken  T T G G A A C T C T A C T G C A C C A C G A C A C A C A C C T T C G T C A A A G C A C T G A C C A T C G A G G G C A A C C A A G
Consensus T T G G A A C I L W A C I G C A C C A C R A C A L A C A C L I I C G I C A A A G C A C I G A C C A I C G A G G C L A A C C A A G
          651          660          670          680          690          700          710          715
NGF      A A G C A T G G A G G T T T A T C C G G A T T G A G A C A G C G T G T G T G T G T G C T C T G C A C G A G A A C C G G G A G A
Chicken  A A G C C T G G A G A T T T A T C C G G A T C G A C A C A G C C T G T G T G T G T G C T C A G C A C G A A A T C G G G G A G A
Consensus M A G C M T G C A G R T T T A T C C G G A T Y C A S A C A G C S T G T C T C T G T G C T C W G C A C C A R A W S C G G G A G A
          716          720          730          740          750          760          770          780
NGF      A C A A G A T G ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Chicken  C C C T G A G G C G G A G C T G A A C C C C A C A A C A A C A T C C T C C T A T C C C C C T A C C T C A G C C T G T A A A T T A T
Consensus M C M W G A K G C G G A G C T G A A C C C C A C A A C A A C A T C C T C C T A T C C C C C T A C C T C A G C C T G T A A A T T A T

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(a)

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NGF      51          60          70          80          90          100
Chicken  A P Q T T H G R F A W M P D G T E D L N I A M D Q N F F K K K R F R S S R V L F S T Q P P P V S R K
Consensus A P Q T T H G R F A W M P D G T E D L N I A M D Q N F F K K K R F R S S R V L F S T Q P P P V S R K
          101          110          120          130          140          150
NGF      G Q S M G F L S S A A S L N R T A R T K R T A H P V L H R G E F S V C D S V S M W V G D K T T A T D
Chicken  G Q S T G F L S S A V S L N R T A R T K R T A H P V L H R G E F S V C D S V S M W V G D K T T A T D
Consensus G Q S / G F L S S A / S L N R T A R T K R T A H P V L H R G E F S V C D S V S M W V G D K T T A T D
          151          160          170          180          190          200
NGF      I K G K E V T V L G E V N I N N N V F K Q Y F F E T K C R D P R P V S S G C R G I D A K H W N S Y C
Chicken  I K G K E V T V L G E V N I N N N V F K Q Y F F E T K C R D P R P V S S G C R G I D A K H W N S Y C
Consensus I K G K E V T V L G E V N I N N N V F K Q Y F F E T K C R D P R P V S S G C R G I D A K H W N S Y C
          201          210          220          230          240          250
NGF      T T T H T F V K A L T M E G K Q E A W R F I R I E T A C V C V L C R R T G R T R & ? ? ? ? ? ? ? ? ? ?
Chicken  T T T H T F V K A L T M E G K Q A A W R F I R I D T A C V C V L S R K S G R P * G G A E P H N N I L
Consensus T T T H T F V K A L T M E G K Q / A W R F I R I / T A C V C V L / R / / G R / & / G A E P H N N I L
          251          260          270          280          290          293
NGF      ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
Chicken  L S P Y L S L * I I L S * K K K K N K D C M V Y L * F I Q Y K E N L I I Y * T L L E T
Consensus L S P Y L S L * I I L S * K K K K N K D C M V Y L * F I Q Y K E N L I I Y * T L L E T

```

(b)

Fig. 3.3 Sequence alignment of Japanese quail NGF and chicken NGF. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

	1	10	20	30	40	50	60	65
Quail	?	?	?	?	?	?	?	?
Chicken	A	T	G	G	G	T	C	T
Consensus	A	T	G	G	G	T	C	T
	66	70	80	90	100	110	120	130
Quail	C	C	T	C	T	T	C	C
Chicken	C	C	T	C	T	T	C	C
Consensus	C	C	T	C	T	T	C	C
	131	140	150	160	170	180	190	195
Quail	C	C	A	A	G	T	C	T
Chicken	C	C	A	A	G	T	C	T
Consensus	C	C	A	A	G	T	C	T
	196	200	210	220	230	240	250	260
Quail	A	G	C	T	T	C	C	T
Chicken	A	G	C	T	T	C	C	T
Consensus	A	G	C	T	T	C	C	T
	261	270	280	290	300	310	320	325
Quail	C	C	A	T	A	G	C	C
Chicken	C	C	A	T	A	G	C	C
Consensus	C	C	A	T	A	G	C	C
	326	330	340	350	360	370	380	390
Quail	C	C	A	A	G	T	G	C
Chicken	C	C	A	A	G	T	G	C
Consensus	C	C	A	A	G	T	G	C
	391	400	410	420	430	440	450	455
Quail	A	C	T	G	A	C	C	A
Chicken	A	C	T	G	A	C	C	A
Consensus	A	C	T	G	A	C	C	A
	456	460	470	480	490	500	510	520
Quail	G	G	C	T	G	A	C	T
Chicken	G	G	C	T	G	A	C	T
Consensus	G	G	C	T	G	A	C	T
	521	530	540	550	560	570	580	585
Quail	T	C	T	C	C	C	C	T
Chicken	T	C	T	C	C	C	C	T
Consensus	T	C	T	C	C	C	C	T
	586	590	600	610	620	630	640	650
Quail	C	A	G	C	T	C	C	T
Chicken	C	A	G	C	T	C	C	T
Consensus	C	A	G	C	T	C	C	T
	651	660	670	680	690	700	710	715
Quail	T	G	C	A	A	T	G	T
Chicken	T	G	C	A	A	T	G	T
Consensus	T	G	C	A	A	T	G	T
	716	720	730	740	750	760	770	780
Quail	A	C	T	G	G	G	A	G
Chicken	A	C	T	G	G	G	A	G
Consensus	A	C	T	G	G	G	A	G
	781	790	800	810	820	830	840	
Quail	T	T	C	A	G	C	A	A
Chicken	T	T	C	A	G	C	A	A
Consensus	T	T	C	A	G	C	A	A

(a)

	1	10	20	30	40	50	60
Quail	? ? ? ? ? ? &	L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L					
Chicken	M G L L S A D L L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L						
Consensus	M G L L S A D L L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L						
	61	70	80	90	100	110	120
Quail	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K D N D G S S S G W K S V G G K C G T K C R L L D F A N S E						
Chicken	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K G N D G S N S S W K S V G G K C G T K C H L L D F A N S E						
Consensus	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K I N D G S I S I W K S V G G K C G T K C I L L D F A N S E						
	121	130	140	150	160	170	180
Quail	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
Chicken	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
Consensus	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
	181	190	200	210	220	230	240
Quail	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
Chicken	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
Consensus	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
	241	250	260	270	280		
Quail	G G K G P F F Y N L Q E V R L W L E Q N & ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?						
Chicken	G G K G P F F Y N L Q E V R L W L E Q N F S K R * N P L S T E D L S T D V S L *						
Consensus	G G K G P F F Y N L Q E V R L W L E Q N F S K R * N P L S T E D L S T D V S L *						

(b)

Fig. 3.4 Sequence alignment of Japanese quail D2 and chicken D2. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

	1	10	20	30	40	50	60	65	
Quail	?	?	?	?	?	?	?	?	
Chicken	A	T	G	G	A	G	C	A	
Consensus	A	T	G	G	A	G	C	A	
	66	70	80	90	100	110	120	130	
Quail	A	G	G	G	T	C	T	T	
Chicken	A	G	G	G	T	C	T	T	
Consensus	A	G	G	G	T	C	T	T	
	131	140	150	160	170	180	190	195	
Quail	A	C	C	A	G	C	T	C	
Chicken	A	C	C	A	G	C	T	C	
Consensus	A	C	C	A	G	C	T	C	
	196	200	210	220	230	240	250	260	
Quail	C	C	C	A	C	A	G	C	
Chicken	C	C	C	A	C	A	G	C	
Consensus	C	C	C	A	C	A	G	C	
	261	270	280	290	300	310	320	325	
Quail	G	G	A	G	A	G	C	G	
Chicken	G	G	A	G	A	G	C	G	
Consensus	G	G	A	G	A	G	C	G	
	326	330	340	350	360	370	380	390	
Quail	C	C	A	A	G	C	A	?	
Chicken	C	C	A	A	G	C	A	?	
Consensus	C	C	A	A	G	C	A	?	
	391	399							
Quail	?	?	?	?	?	?	?	?	
Chicken	A	G	C	T	C	A	T	G	A
Consensus	A	G	C	T	C	A	T	G	A

(a)

	1	10	20	30	40	50	60
Quail	?	?	?	?	?	?	?
Chicken	M	E	Q	Y	F	S	A
Consensus	M	E	Q	Y	F	S	A
	61	70	80	90	100	110	120
Quail	V	T	D	Q	S	P	T
Chicken	V	T	D	Q	S	P	T
Consensus	V	T	D	Q	S	P	T
	121	130	133				
Quail	?	?	?	?	?	?	?
Chicken	E	E	I	N	L	G	E
Consensus	E	E	I	N	L	G	E

(b)

Fig. 3.5 Sequence alignment of Japanese quail Spot 14 α and chicken Spot 14 α . Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

46 50 60 70 80 90
Quail - - - - - CCCAGCCTGCTCAGAGGGGTCTTCCCGCAGGACGGGGC
Chicken GATGTTCCCCAGCCTGCTGCGAGGGGTCTTCCCGCAGGACGGGGC
Consensus GATGTTCCCCAGCCTGCT S M AGGGGTCTTCCCGCAGGACGGGGC

91 100 110 120 130 135
Quail CGACCCGGCTTCAGACGGCTCCGCGGACCTGTACGAGCGCTACCA
Chicken CGACCCAGCCGCGGACGGCCCGCGGACCTCTACGAGCGCTACCA
Consensus CGACCC R G C Y K C M GACGGC Y CCGCGGACCT S TACGAGCGCTACCA

136 140 150 160 170 180
Quail GCTCCTCAAGGCCATCAAGCCCGTGGTGCAAGCGCGGCCTGGCCTC
Chicken GCTCCTCAAGGCCATCAAGCCCGTGGTGGAGCGAGGCCTGGCGTC
Consensus GCTCCTCAAGGCCATCAAGCCCGTGGTG S ACG MGGCCTGGC S TC

181 190 200 210 220 225
Quail CGTCACCGAGCGCAGCTCCGCGAGCGACGACGACGACGACGCCCGC
Chicken CTTCACCGAGCGCAGCTCCGCGGCCACGCGACGCCGACGCCGA
Consensus C K TCACCGAGCGCAGCTCCGC S R GCS ACG MCGACG MCGACGCCG M

226 230 240 250 260 270
Quail AGCC - - - - - GACGGGGCGGACGGCAGCCT
Chicken CGCCGAGGACGCGGCGGCCGCGAGCCGACGGGGCGCCGGCAGCCT
Consensus M G C C G A G G A C G C G G C G G C C G C A G C C G A C G G G G C G G M C G G C A G C C T

271 280 290 300 310 315
Quail GGAGCAGCGGCTGTGCCACCACCTGGCCGGGCTGCAGCAGATCCT
Chicken GGAGCAGCGGCTGTGCCACCACCTGGCCGGGCTGCAGCAGATCCT
Consensus GGAGCAGCGGCTGTGCCACCACCTGGCCGGGCTGCAGCAGATCCT

316 320 330 340 350 360
Quail CAGCCACCTCACCAGGGACACCGCCGCCCTCAGCGCCGCTACAG
Chicken CAGCCACCTGACCAGGGACACCGCCGCCCTGACGCGCCGCTACAG
Consensus CAGCCACCT S ACCAGGGACACCGCCGCCCTSACGCGCCGCTACAG

361 370 380 390 400 405
Quail CCAGATCCTGGAGCGGATCAGCCCC ? ? ? ? ? ? ? ? ? ? ?
Chicken CCAGATCCTGGAGCGGATCAGCCCCGGCGACGCGCAGCCCAGCTG
Consensus CCAGATCCTGGAGCGGATCAGCCCCGGCGACGCGCAGCCCAGCTG

(a)

	1	10	20	30	40
Quail	- - - - -	- - - - -	P S L L R G V F P	Q D G A D P A S	D G S A D L
Chicken	M E R Y F S A T Q K M E Q E V M F P S L L R G V F P	Q D G A D P A A D G P A D L			
Consensus	M E R Y F S A T Q K M E Q E V M F P S L L R G V F P	Q D G A D P A / D G / A D L			
	41	50	60	70	80
Quail	Y E R Y Q L L K A I K P V V Q R G L A S V T E R S S A S D D D D D A A A - - - -				
Chicken	Y E R Y Q L L K A I K P V V E R G L A S F T E R S S A G H A D A D A D A E D A A				
Consensus	Y E R Y Q L L K A I K P V V / R G L A S / T E R S S A / / / D / D A / A E D A A				
	81	90	100	110	120
Quail	- - - D G A D G S L E Q R L C H H L A G L Q Q I L S H L T R D T A A L T R R Y S				
Chicken	A A A D G A A G S L E Q R L C H H L A G L Q Q I L S H L T R D T A A L T R R Y S				
Consensus	A A A D G A / G S L E Q R L C H H L A G L Q Q I L S H L T R D T A A L T R R Y S				
	121	130	135		
Quail	Q I L E R I S P ? ? ? ? ? ? ? ?				
Chicken	Q I L E R I S P G D A Q P S W *				
Consensus	Q I L E R I S P G D A Q P S W *				

(b)

Fig. 3.6 Sequence alignment of Japanese quail Spot 14 β and chicken Spot 14 β . Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

Chapter 4

Effects of maternal exposure to ammonium perchlorate on thyroid function and the expression of thyroid-responsive genes in Japanese quail embryos

Abstract

Perchlorate ion is a known endocrine disruptor which decreases thyroid function resulting in hypothyroidism. Perchlorate ion is deposited in eggs of female birds exposed to ammonium perchlorate (AP), so the embryos from these eggs are subject to the thyroid disrupting effect of perchlorate. Embryonic hypothyroidism resulting from perchlorate exposure may affect the development and function of thyroid-dependent organs. In the present study, laying Japanese quail hens were treated with 2000 mg/l or 4000 mg/l AP in drinking water. Thyroid status and expression of thyroid-responsive genes were examined in the embryos from eggs of exposed hens. Maternal perchlorate exposure was expected to decrease embryonic thyroid function and affect the expression of thyroid-responsive genes in embryonic brain and liver. Perchlorate exposure led to hypothyroidism in hens from both treatment groups but egg production was decreased in the 4000 mg/l group only. Embryos from eggs of perchlorate-exposed hens had hypertrophied thyroid glands and significantly lower thyroidal thyroid hormone storage, indicating hypothyroidism in these embryos. The embryonic hypothyroidism was associated with decreased embryonic growth, delayed hatching and greater mortality during hatching. The mRNA level of type 2 deiodinase (D2) in the liver of embryos from eggs of perchlorate-exposed hens was increased compared to those from eggs of the control hens, a compensatory response that increases the production of metabolically active T₃. The mRNA levels of D2 and RC3 in the brain, however, were not affected by embryonic hypothyroidism resulting from maternal perchlorate exposure. This suggests the embryonic brain is protected from hypothyroidism. This study demonstrated maternal perchlorate exposure led to embryonic hypothyroidism and to changes in the expression of thyroid-responsive genes in embryonic liver. The embryonic effects probably resulted primarily from embryonic perchlorate exposure in the eggs although decreased deposition of maternal thyroid hormones and iodide in the eggs of perchlorate-exposed hens may have played a role.

Introduction

Perchlorate ion is a thyroid disruptor which competitively inhibits the uptake of iodide, a key material for thyroid hormone synthesis, into the thyroid gland (De Groef *et al.*, 2006). Recent evidence also supports the idea that perchlorate itself is transported into the thyroid gland and may have additional effects on gland function (Yu *et al.*, 2002). Ammonium perchlorate (AP), a strong oxidizer in rocket fuel and explosives, is widely manufactured and used in the United States. Perchlorate is found in water system, soil and vegetation in many states with the country (EPA, 2005). Females of ground dwelling birds that are exposed to perchlorate in the contaminated area may develop hypothyroidism, thereby reducing their deposition of thyroid hormones in eggs. Perchlorate ion competes with iodide for the sodium-iodide symporter so this can result in perchlorate deposition in eggs as well as decreased iodide in eggs.

Previous work in our laboratory showed thyroid hormones of maternal origin were found in Japanese quail eggs and their concentrations in eggs co-varied with the circulating thyroid hormone concentrations in hens (Wilson and McNabb, 1997). Thyroid hormones of maternal origin are important for avian embryonic development, especially before the onset of embryonic thyroid function. Several studies have demonstrated that maternal thyroid hormones deposited in avian eggs affect the development

of thyroid-responsive tissues such as pelvic cartilage (Wilson and McNabb, 1997) and muscle tissues (Maruyama *et al.*, 1995). When Bobwhite quail hens are exposed to AP, perchlorate is detected in their eggs (Gentles *et al.*, 2005), indicating the embryonic thyroid glands will be exposed to perchlorate during development. Deficiencies in both maternal hormones in eggs and in embryonic thyroid development due to perchlorate exposure may in turn interfere with thyroid-responsive development of other target tissues.

Thyroid hormones regulate the expression of thyroid-responsive genes in target organs such as brain during specific time windows in development. Irreversible defects may occur in animals that are hypothyroid during these critical times (Bernal, 2002; McNabb, 1992). Two examples of thyroid-responsive genes that are important in development are D2 which regulates brain thyroid hormone concentrations and RC3 which is involved in the structural/functional brain development. T₄ is the main product of the thyroid gland and is converted by type 1 deiodination into physiologically active T₃ mostly in peripheral tissues such as liver and kidney. Most T₃ in the general circulation is from this peripheral deiodination (McNabb, 1992). However, in contrast to the body as a whole, T₃ supply to the central nervous system is tightly controlled, at least partly by Type 2 deiodinase (D2). D2 is the principal 5' deiodinase in the brain and its expression in the brain has been intensively studied (see for example, rats, Bianco *et al.*, 2002; birds, Van der Geyten *et al.*, 2002). D2 activity is strictly regulated by the concentrations of both thyroid hormones, but through different mechanisms. T₃ regulates the transcription of D2 mRNA while T₄ is involved in the control of D2 protein degradation (Burmeister *et al.*, 1997). Overall, D2 activity increases when brain thyroid hormone concentrations are low and decreases when they are elevated. This response of D2 activity to thyroid status is unique among deiodinases and is a key component of the brain's protective mechanisms against fluctuations of circulating thyroid hormone concentrations. Thus, the overall brain T₃ concentration remains close to euthyroid status during conditions such as hypothyroidism (Bianco *et al.*, 2002; Rudas *et al.*, 2005). In addition to brain, the same inverse responses of D2 to changes in thyroid status also have been observed in brown adipose tissue and gonads in mammals (Nguyen *et al.*, 1998; Wagner *et al.*, 2003).

Other thyroid-responsive genes, including RC3/neurogranin, also are critical to brain development. The neural specific RC3 regulates the calcium availability within neurons and is believed to have an important role during synaptogenesis (Gerendasy and Sutcliffe, 1997). In mammals, RC3 expression is up-regulated by thyroid hormones during early development and some hypothyroidism related developmental defects in the brain may be due to the disruption of RC3 expression by decreased thyroid hormone supply to the brain (Bernal, 2002; Piosik *et al.*, 1996; Piosik *et al.*, 1995). Therefore, a number of studies have used RC3 expression as the end point for studying the effect of thyroid disrupting chemicals on the brain in mammals (Xu *et al.*, 2007; Zoeller *et al.*, 2005; Zoeller *et al.*, 2000). RC3 also is present in the brain of avian species (canary, George *et al.*, GenBank accession number 1709258 and Japanese quail, Chen, GenBank accession number EU558133) but it has not been studied in the context of its expression and function during embryonic brain development. There are also a number of thyroid-responsive genes discovered in the liver including Spot 14, a protein that is involved in the regulations of lipogenesis pathway (Jump *et al.*, 1984; Wang *et al.*, 2004).

The current study is focused on the thyroid effects of perchlorate exposure of female birds and their offspring. Japanese quail were chosen as a model for ground dwelling birds that would face full impact of environmental perchlorate contamination. Quail hens were given ammonium perchlorate solutions as drinking water for 6 weeks. Eggs from exposed hens were incubated and embryos were sacrificed on day 14 of the 16.5 day incubation period. Thyroid condition of both hens and embryos was determined. The mRNA levels of D2 and RC3 in the embryonic brain were measured to evaluate the

effect of perchlorate-induced hypothyroidism on the expression of brain thyroid-responsive genes. The mRNA levels of D2 and thyroid-responsive gene Spot 14 (SP14) in the embryonic liver were also measured to study these effects in a peripheral tissue.

Materials and Methods

Animals and perchlorate exposure. Adult Japanese quail hens, ages three to four months, were divided into four groups of ten. Each group of hens was housed in a single cage with two adult males of the same ages. All birds were maintained under 14L:10D photoperiod with game bird feed (Big Spring Mills, Elliston VA) and drinking solutions provided *ad libitum*. Eggs were collected from each group separately and incubated at $39\pm 1^\circ\text{C}$ and $>90\%$ relative humidity in a forced-air incubator (Humidaire Hatchette Incubator; New Madison OH). Two groups of birds were given 2000 mg/l and 4000 mg/l AP (AP powder, molecular weight 117.49, was purchased from Fluka Chemika, Steinheim, Germany) solutions respectively as drinking water. Another group received tap water as drinking water and served as controls. All animal maintenance, handling and sacrifice procedures were approved by the Virginia Tech Animal Care Committee in accordance with federal guidelines.

Sampling procedures. Blood was drawn from the brachial veins of hens at two and four weeks of exposure to determine the plasma T_4 concentrations and assess developing hypothyroidism. Egg collections started a week after the first day of exposure to ensure that the first eggs collected were produced entirely after the treatment began (each egg takes 5-6 days to mature within the hen's reproductive tract, Wilson and McNabb, 1997). Eggs were collected daily in the late afternoon from all three groups, the collections continued for five weeks (week 2 through week 6 of perchlorate exposure) until the egg production from the 4000 mg/l AP decreased to one egg a day from the whole group. Eggs collected within the same week were incubated together. From each week's eggs, ten embryos per group were sacrificed on day 14 of incubation. Embryonic blood was collected from the chorioallantoic artery into heparinized capillary tubes and plasma was stored frozen at -20°C . Embryos were decapitated after blood collection and brains and livers were removed and flash frozen in liquid nitrogen and then stored at -80°C . Thyroid glands of the embryos also were removed, weighed and stored in snap cap tubes at -20°C . Extra eggs were returned to the incubator and hatched. Hens were sacrificed after six weeks of exposure and hatched chicks were sacrificed 1-2 days after hatching.

Hens and chicks were sacrificed by decapitation, trunk blood was collected in heparinized capillary tubes and plasma was frozen at -20°C until analysis. Thyroid glands of the birds were removed, weighed and stored in snap cap tubes at -20°C .

Thyroid assays. Plasma thyroid hormone concentrations were measured using a double antibody radioimmunoassay (RIA) on duplicate aliquots for each sample ($12.5\mu\text{l}$ for T_4 and $25\mu\text{l}$ for T_3) following the method described by Wilson and McNabb (1997). All samples within the same data set (e.g. plasma T_4/T_3 of hens, thyroidal T_4/T_3 of hens, thyroidal T_4/T_3 of embryos of week 2/week 3/week 4/week 5/week 6, etc.) were measured in a single assay. For each assay, a standard curve was constructed using a series of standards prepared in charcoal-stripped chicken plasma with known hormone concentrations. Three replicate aliquots of the standard for each hormone concentration were measured for better accuracy. Primary antibodies were purchased from Fitzgerald Industries International, Inc., (Concord MA). ^{125}I -labeled hormones (high specific activity; $1200\ \mu\text{Ci}/\mu\text{g}$) were purchased from Perkin-Elmer Life Sci (Boston MA,). Three levels of Randox Immunoassay Control serum (Randox Laboratories, San Diego, CA,) were included in each assay to evaluate assay performance. The intra-assay precision of RIA, 95% CI, was 3.1% of the mean for T_4 ($n=6$) and 2.6% of the mean for T_3 ($n=6$; McNabb *et al.*, 2004). For

inter-assay precision, 95% CI was 7.36% of the mean concentration for T₄ (n=5) and 8.65% of the mean for T₃ (n=5). The minimum sensitivity of RIA is 1.25 ng/ml for T₄ and 0.125 ng/ml for T₃. Because the high lipid content in the plasma of the hens interfered with antibody binding of the hormone, an additional step of thyroid hormone extraction from plasma with cold ethanol was included before the measurement of plasma T₄ in the extracts.

The activation of hypothalamic-pituitary-thyroid axis was evaluated by weighing the thyroid glands to the nearest 0.01mg. Thyroid hormone storage in the thyroid gland was determined following the method described by McNabb and Cheng (1985). Each embryonic thyroid gland pair (10 mg or less) was digested in 350 µl of digestion medium containing 25 mg of Pronase (Sigma-Aldrich, St. Louis MO) at 37°C overnight. Thyroid glands from hens were each digested separately due to their larger size. After stopping the digestion by mixing the medium with 1.0 ml ice cold absolute ethanol, the tubes were stored at -20°C for 24 h. The tubes were then centrifuged at 13,500g for five minutes and the supernatant, containing the extracted thyroid hormones, was collected and stored at -20°C until analysis. Dilutions of the supernatant in 75% ethanol were analyzed for T₄ and T₃ by RIA as described above except that the standards for these assays were prepared in 75% ethanol and 25 µl of hormone extract was used for each aliquot for both T₄ and T₃ assays.

Total RNA isolation. Results from thyroid assay suggest the effect of maternal perchlorate exposure time was non-significant. Therefore, mRNA levels of thyroid-responsive genes in embryos from eggs of hens within the same exposure group but incubated and sacrificed at different weeks were not expected to be different. Total RNA was isolated from embryonic brain and liver tissues from eggs collected during week 2, 3 and 4 of hen exposure to perchlorate. The total RNA isolation was performed using Tri-Reagent (Sigma-Aldrich, St. Louis MO) following the manufacturer's suggested protocol. Tissues were mixed with Tri-Reagent at a weight to volume ratio of 50 mg: 1 ml and homogenized with a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Inc, Westbury NY). The homogenate was centrifuged at 12,000 g for ten min to eliminate insoluble material. The supernatant was mixed with chloroform (0.2 ml for each milliliter of Tri-Reagent) and centrifuged at 12,000 g for 15 minutes for phase separation. The top aqueous phase containing RNA was transferred to a new tube and mixed with isopropanol (0.5 ml for each milliliter of Tri-Reagent) then centrifuged at 12,000 g for ten min to precipitate the RNA. RNA pellets were washed in 75% ethanol, dried and resuspended in RNase-free water. The RNA samples were then frozen and stored at -80 °C until further analysis. Altogether brains and livers from 5 different embryos in each group were used for RNA isolation. Total RNA from each brain was isolated separately. RNA from 5 brains in the same group was then pooled together before Northern blotting. Livers from the same treatment group were pooled and homogenized together due to their small size. Liver total RNA were isolated from the pooled homogenate.

Reverse transcription-polymerase chain reaction (RT-PCR). The cDNA libraries of Japanese quail brain and liver were constructed and D2, RC3 and SP14 cDNA were isolated from by RT-PCR for the analysis of thyroid-responsive genes in embryos (see chapter 3). Quail β-actin cDNA was isolated to be used as the housekeeping gene. For cDNA library synthesis, 2µg of total RNA from each tissue was added into 25 µl reaction mixture containing 5µl reaction buffer, 0.5 mM dNTP mix, 25 units of ribonuclease inhibitor, 200 units of M-MLV reverse transcriptase (Fisher Scientific, Fair Lawn NJ) and 0.5 µg Oligo(dT)₁₅ primer (Promega, Madison WI) and nuclease free water. The reaction mixtures were incubated at 37°C for 60 min. The PCR reaction was carried out in 50 µl reaction mixture containing 5 µl 10x reaction buffer, 200 µM dNTP mix, 2 units of Taq DNA polymerase (Fisher Scientific, Fair Lawn NJ), 10 pmol of each of the forward and reverse primers (Operon Biotechnologies, Inc., Huntsville AB) and 1

µl of cDNA. Sequences of the primers for each gene are listed in Table 4.1. The mRNA sequences of D2, RC3 and SP 14 were not available for Japanese quail. Primers for D2 and SP14 were designed based on chicken sequences (Gereben *et al.*, 1999; Wang *et al.*, 2004). The mRNA sequence of RC3 was only available for canary among all avian species. After comparing the RC3 mRNA sequence to the sequences in the chicken brain express sequencing tag (EST) database, several matches were found. Primers for quail RC3 cDNA were designed based on both canary sequence and the matched chicken EST sequences. The PCR reactions were performed as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95 °C, 45 seconds of annealing (annealing temperature for each gene is specified in Table 4.1) and 30 seconds of extension at 72°C. Once the cycles were completed, another 3 min of extension at 72 °C was added. The reaction mixture was then frozen at -20 °C until analysis.

DNA sequencing. After electrophoresis, PCR products were purified using Qiagen gel extraction kits. They were then sequenced by the Virginia Bioinformatics Institute Core Laboratory Facility (Virginia Tech, Blacksburg VA). Both forward and reverse strands of the cDNAs were sequenced to assure the accuracy of the results. The cDNA sequences were compared to mRNA sequences of the corresponding genes in other species to confirm their identity.

Northern analysis. The mRNA levels of D2, RC3 and Spot 14 in brain and liver total RNA were analyzed using Northern blotting as described by Sible *et al* (1997). 20 µg of total RNA was loaded on a 1% denaturing agarose gel containing formaldehyde. The RNA was transferred to a nylon membrane and cross-linked by UV irradiation after electrophoresis. ³²P-labelled DNA probes were synthesized using Random Primed DNA labeling kits (Roche Applied Science, Indianapolis IN) and ³²P-labelled dCTP (Amersham Life Sci, Pittsburg PA). Japanese quail RC3, D2 and β-actin cDNA, acquired from young Japanese quail brain and liver total RNA by RT-PCR, were used as templates for probe synthesis. Membranes were hybridized with 20x10⁶ TCA-perceptible cpm/ml denatured probes in QuikHyb solution at 65°C (Stratagene, La Jolla CA) and then washed at the same temperature twice with 2xSSC containing 0.1% SDS for 15 min and once with 0.2xSSC, 0.1% SDS for 30 minutes. Blots were exposed to Kodak X-ray film overnight.

RC3 mRNA signal was confirmed by comparing Northern blotting results of brain total RNA to that of liver total RNA from one week old euthyroid chicks using RC3 probe. The pattern of RC3 expression during embryonic development was determined for brain tissues collected from euthyroid quail embryos of days 11, 14, 15, 16, 17 of incubation and one week old chicks.

For RNA isolated from embryonic tissues, total brain RNA samples from embryos within the same treatment group were pooled before analysis by Northern blotting. Brain total RNA samples were hybridized with RC3 and D2 probes. Liver total RNA samples were hybridized with D2 and Spot 14 probes. All RNA samples were also hybridized with β-actin probe to test the quality and the consistency of the RNA.

Statistical analysis. One-way ANOVA was used to analyze the plasma T₄ concentrations of the quail hens at 2 weeks and 4 weeks of perchlorate exposure. Multivariate analysis of variance (MANOVA) was used to analyze the thyroid variables measured in hens when they were sacrificed at 6 weeks of perchlorate exposure. Plasma T₄ and T₃ concentrations, thyroid gland weight, thyroidal T₄ and T₃ storage were included as responses. Univariate analysis of variance (ANOVA) was used as post-hoc analysis to investigate the data for each of the individual responses. For the variables that were significantly affected by perchlorate exposure according to MANOVA, one-way ANOVA followed by Tukey's HSD post hoc test was used to compare means between treatment groups (Fig. 4.2). Two-way ANOVA was used to compare the results of embryos from eggs of the 2000 mg/l AP group with the control group for the effect

of maternal perchlorate exposure over the experimental period (week 2 through week 6; Fig. 4.3). Because very few or no embryos were acquired from the eggs of the 4000 mg/l AP group after week 4, data from this group were not included in the two-way ANOVA analysis. A separate week by week comparison of the 2000 mg/l AP and the 4000 mg/l AP group was made using the Wilcoxon test. MANOVA, one-way ANOVA and Tukey's HSD test was performed using Minitab 15 (Minitab, Inc., State College PA). Two-way ANOVA was performed using SAS (SAS Institute Inc., Cary NC) and Wilcoxon test was done using JMP (SAS Institute Inc., Cary NC). Statistically significant differences were defined as probabilities of $p \leq 0.05$.

Results

Egg production by perchlorate-exposed hens. Eggs were collected from each cage every day and daily egg production was calculated by dividing the number of eggs by the number of hens in each cage. Perchlorate exposure with 2000 mg/l AP in the drinking water did not appear to affect egg productivity. The mean number of eggs laid per hen per day in the 2000 mg/l treatment group did not seem to differ from the control group and the number of eggs collected from these groups each week remained consistent during the entire experimental period (Fig. 4.1). Perchlorate exposure with 4000 mg/l AP in the drinking water, however, noticeably reduced egg production. From the beginning of week 3 of perchlorate exposure, the mean number of eggs laid per hen per day declined. After week 4, no more than one egg was collected per day from the entire 4000 mg/l AP group (Fig. 4.1). When the hens were sacrificed, all hens in the control and 2000 mg/l perchlorate exposure groups had ovaries containing a typical sequence of developing eggs while only four out of nine hens in the 4000 mg/l perchlorate exposure group had developing eggs.

Thyroid status of perchlorate-exposed hens. Thyroid status was evaluated based on all the thyroid variables that were measured. Hypothyroidism was determined by decreased thyroidal thyroid hormone storage, thyroid gland hypertrophy and decreased plasma thyroid hormone concentration. Decreased plasma thyroid hormone concentrations indicate profound hypothyroidism and are not as readily observed as the other two indicators under mild hypothyroid conditions. Perchlorate exposure significantly affected thyroid functions in the hens (MANOVA: $F_{10, 42}=12.242$, $p<0.001$). In general, when all thyroid measurements were considered, the degree of hypothyroidism increased with greater perchlorate exposure.

Plasma thyroid hormone concentrations were used to evaluate the progression of developing organismal hypothyroidism. Plasma T_4 concentrations did not differ significantly between the three treatment groups at week 2 (ANOVA: $F_{2, 34}=2.29$, $p=0.117$) and week 4 (ANOVA: $F_{2, 30}=0.54$, $p=0.591$) of perchlorate exposure (Fig. 4.2). After week 6, the mean plasma T_4 concentrations of both treated groups were lower than that of the control group, but only the 4000 mg/l AP group differed significantly from the control group (ANOVA: Fig. 4.3a, $F_{2, 25}=4.58$, $p=0.020$). Plasma T_3 concentrations were only measured at week 6 and no significant difference was observed between the three treatment groups (ANOVA: Fig. 4.3b, $F_{2, 25}=0.37$, $p=0.693$).

Thyroid gland weights, which indicate HPT axis activation, were increased by perchlorate exposure in a dose dependent manner (ANOVA: $F_{2, 25}=17.89$, $p<0.001$; Fig. 4.3c). Significant differences were observed both between the control and 2000 mg/l AP groups and between the 2000 mg/l and the 4000 mg/l AP groups according to results of Tukey's HSD test.

Thyroidal T_4 storage was decreased significantly in both perchlorate-exposed groups (ANOVA: $F_{2, 25}=84.07$; $p<0.001$ Fig. 3.3d). An 11-fold decrease in the mean thyroidal T_4 storage of the 2000 mg/l

AP group and a 32-fold decrease in the mean thyroidal T₄ storage in the 4000 mg/l AP group were observed compared to the control group. However, the two perchlorate-exposed groups did not differ significantly. The thyroidal T₃ content was comparable between the control and the 2000 mg/l AP group but was significantly decreased in the 4000 mg/l AP group compared to the other two groups (ANOVA: $F_{2,25}=4.64$, $p=0.019$; Fig. 4.3e). The mean thyroidal T₃ storage in the 4000 mg/l AP group was 4.4- and 5.7-fold lower than the control and 2000 mg/l AP group, respectively.

Thyroid status of embryos from eggs of perchlorate-exposed hens. Embryonic data from eggs of the control and 2000 mg/l AP hens was analyzed using two-way ANOVA to evaluate the effect of both maternal perchlorate exposure (controls vs. 2000 mg/l AP) and time of exposure (week 2 through week 6) on embryonic thyroid function. Embryonic data from eggs of the hens of the 4000 mg/l AP group was compared with that from eggs of the hens of the 2000 mg/l AP group only between the embryos from the same week.

Body weight in embryos from the eggs of the 2000 mg/l AP hens was significantly lower than in the embryos from the control hens ($F_{1,18}=18.12$, $p=0.005$). Length of perchlorate exposure time of hens was a significant factor in embryonic body weight ($F_{4,67}=2.87$, $p=0.0294$) but with a non-significant interaction term (Fig. 4.4a, $F_{4,67}=1.32$, $p=0.2698$). Thus the mean body weights between the two groups of embryos differed by a constant factor over the time of maternal perchlorate exposure. The mean body weight in embryos from eggs of the 4000 mg/l AP hens was lower than that from eggs of the control group in all weeks. The body weight of embryos from the eggs of 4000 mg/l AP hens was significantly different from those from the eggs of the 2000 mg/l AP hens only in week 4 of the perchlorate exposure ($Z=-2.478$, $p=0.0132$). Only one embryo was obtained from eggs of the 4000 mg/l AP hens on week 5, and it had the second lowest body weight of all embryos sampled during the experiment.

Thyroid gland weight was significantly higher in embryos from the eggs of the 2000 mg/l AP hens than in embryos from the eggs of the control hens ($F_{1,18}=179.17$, $p<0.001$). Both time of exposure ($F_{4,67}=1.02$, $p=0.4045$) and interaction ($F_{4,67}=0.99$, $p=0.4211$) were non-significant (Fig. 4.4b). Therefore the time profiles of thyroid gland weight of the two groups did not differ and the difference in gland weight between the two groups was not constant over time. Thyroid gland weight in the embryos from 4000 mg/l AP hens was significantly higher than in those from the 2000 mg/l AP hens in week 2 ($Z=2.193$, $p=0.0283$) and week 3 ($Z=-2.595$, $p=0.0095$) but not in week 4 ($Z=1.061$, $p=0.2888$) and week 5 (Fig. 4.4b).

Thyroidal T₄ storage in embryos from the eggs of both 2000 mg/l and 4000 mg/l AP hens was significantly lower than that of the control group ($F_{1,18}=237.84$, $p<0.0001$ for control vs. 2000 mg/l AP group). Thyroidal T₄ in embryos from eggs of the 2000 mg/l AP hens was 46-fold less by week 3 and 127-fold less by week 5 than in embryos from eggs of the control group (Fig. 4c). Neither exposure time ($F_{4,67}=0.90$, $p=0.4665$) nor interaction ($F_{4,67}=0.88$, $p=0.4804$) were significant for the thyroidal T₄ storage of the embryos between the control and the 2000 mg/l AP group. So the thyroidal T₄ storage of the embryos in these two groups shared the same time profile but the difference in their mean thyroidal T₄ was not a constant factor over time. There were no significant differences in thyroidal T₄ between the embryos from the eggs of the 2000 mg/l and 4000 mg/l AP hens (Fig. 4.4c).

For the thyroidal T₃ storage in embryos from the eggs of the control and 2000 mg/l AP hens, perchlorate exposure ($F_{1,18}=101.62$, $p<0.0001$), exposure time ($F_{4,63}=4.13$, $p=0.0049$) and interaction ($F_{4,63}=3.66$, $p=0.0096$) were all significant. This shows that the time profiles of the embryonic T₃ storage in these two groups were different (there were stepwise increases in the thyroidal T₃ storage in embryos from the eggs of the 2000 mg/l AP hens in the last three weeks; Fig. 4.4d) and the mean thyroidal T₃

storage in the two groups do not differ by a constant factor over time. There were no significant differences in thyroidal T3 in embryos from the eggs of 2000 mg/l and 4000 mg/l AP hens (Fig. 4.4d).

Among the extra eggs that were returned to the incubator after sampling, several from both control and 2000 mg/l AP group hatched. Eggs of the 2000 mg/l AP hens all hatched one or two days later than the control group (Fig. 4.5). Although some of the eggs in the 4000 mg/l AP group had embryos that pipped the shell, none hatched successfully.

Of all the thyroid variables measured in the hatched chicks, only the thyroid gland weight in those from the eggs of the 2000 mg/l AP hens was consistently different than those of the control hens (Fig. 4.6a-e). Statistical analysis was not performed on these results due to the small and variable numbers of chicks obtained each week.

RT-PCR and DNA sequencing. All PCR reactions yielded single DNA bands of expected sizes. Alignment of sequence of the cDNA fragments amplified using RC3, D2 and SP14 primers with chicken (canary and chicken EST for RC3) sequences confirmed that they are indeed partial sequences of Japanese quail RC3 (189 bp, Fig. 4.7), D2 (758 bp, Fig. 4.8) and SP14 (287 bp, Fig. 4.9). All three sequences were submitted to the GenBank and were assigned GenBank accession numbers EU558133 (RC3), EU558134 (D2) and EU558135 (SP14). Sequence of cDNA fragments amplified using β -actin primers was by comparing the cDNA sequence with the sequences in the NCBI nucleotide database using the program BLAST and Japanese quail β -actin mRNA sequence was found to be the best match.

The mRNA levels of thyroid-responsive genes in the brain and liver in embryos from eggs of perchlorate-exposed hens. No differences were observed in the mRNA D2 levels in the brain of embryos from eggs of either of the two perchlorate exposure groups compared to the control group. In the liver, however, the D2 mRNA levels in embryos from both perchlorate-exposed groups were higher than those from the control group, in which D2 mRNA was not detectable by Northern blotting. Liver D2 mRNA level was higher in embryos from eggs of the 2000 mg/l AP hens than from eggs of the 4000 mg/l AP hens (Fig. 4.10). The mRNA level of SP14, the other thyroid-responsive gene studied in the liver, is not detectable by Northern blotting in the liver total RNA of embryos from eggs of control as well as perchlorate-exposed hens.

Northern blotting using RC3 cDNA probe detected two bands at lower molecular weight in brain total RNA but not in liver total RNA of one week old chicks (Fig. 4.11a). The same two bands also were detected in the brain total RNA of embryos at day 11 and days 14-17 of incubation and in one week old chicks. There was a gradual increase in the intensity of both bands in the brain total RNA from day 11 to day 16 embryos. The intensity of the bands was similar in day16 and day17 embryos and was slightly higher in one week old chicks (Fig. 4.11b). Embryos from hens of both treatment groups showed no difference in their RC3 mRNA levels in brain total RNA compared to those from the control hens (Fig. 4.12).

Northern blotting analysis was repeated on the total RNA isolated from tissues of embryos from week 2, 3 and 4. Same results described above were observed in all three replicates. The pictures presented in the figures were from the Northern blotting on total RNA isolated from tissues of embryos from week 3.

Discussion

This study demonstrates that maternal exposure to perchlorate can lead to disruption of embryonic thyroid function in eggs laid by exposed Japanese quail hens. Maternal exposure to perchlorate can result in perchlorate deposition in eggs and decreased maternal thyroid hormone

deposition in eggs if the hens become hypothyroid from the perchlorate exposure (Gentles *et al.*, 2005; Wilson and McNabb, 1997). Moreover, competitive inhibition of iodide transport into eggs by perchlorate may lead to iodide deficiency in eggs laid by perchlorate-exposed hens. All three factors may have contributed either directly or indirectly to the hypothyroidism observed in the embryos in eggs from perchlorate-exposed hens in this study. Hypothyroidism in these embryos was found to affect their growth and interfere with important developmental events such as hatching.

Egg production by Japanese quail hens exposed to perchlorate was sustained under mild hypothyroid conditions, but was drastically reduced or stopped as hypothyroidism becomes more severe. In the present study, egg production was not affected in hens exposed to 2000 mg/l AP throughout the six week exposure. Egg production was markedly decreased in the hens exposed to 4000 mg/l AP by the 4th week of exposure and thereafter. These results are in agreement with the findings of a previous study in our laboratory in which Japanese quail hens were orally dosed with 4 mg/day methimazole, a thyroid hormone synthesis inhibitor, for one month then 8 mg/day in the following month. Soon after the higher dosage treatment started, egg production ceased completely (Wilson and McNabb, 1997). In the present study, plasma T₄ concentrations, which indicate organismal thyroid hormone exposure in the birds, were monitored to assess the development of hypothyroidism in the hens (Fig. 4.2). On this basis, hens exposed to 2000 mg/l AP showed the first trends toward decreases in plasma T₄ at four weeks of exposure. The decrease remained non-significant at six weeks of exposure although other indicators (decreased thyroidal thyroid hormone storage and thyroid gland hypertrophy) indicated significant loss of thyroid function by this time (Fig. 4.3). It appeared that hens in this treatment group were releasing stored thyroid hormones to maintain circulating thyroid hormones for as long as possible during the perchlorate exposure. Hens exposed to 4000 mg/l AP exhibited overt hypothyroidism by six weeks of treatment as indicated by significant changes, compared to the controls, for each of the thyroid variables measured.

Embryos from eggs laid by perchlorate-exposed hens in both treatment groups exhibited deficiencies in thyroid functions indicating hypothyroidism. It is presumed that perchlorate was deposited in the eggs in proportion to hen exposures so that perchlorate would affect embryonic thyroid gland development and thyroid hormone production. Likewise, any deficiency in iodide deposition in eggs resulting from perchlorate exposure in the hens should further limit thyroid hormone production. Embryos, at day 14, showed evidence of HPT axis activation (thyroid gland weight was significantly higher than in controls) and significantly less thyroid hormone storage in both treatment groups than in the controls. A similar thyroid condition was created in a previous study by our laboratory in which laying hens were given low iodide diets, causing low iodide content in their eggs and resulting in thyroid gland hypertrophy in the embryos (McNabb *et al.*, 1985a; McNabb *et al.*, 1985b). Therefore, the increased gland weight and low thyroid hormone storage (both indicative of embryonic hypothyroidism) in the embryos from perchlorate-exposed hens in the present study likely resulted from iodide deficiency in the embryonic thyroid glands caused by perchlorate exposure. Deficient maternal iodide deposition in the eggs may have added to this effect. However, without parallel studies of perchlorate exposure and iodide deficiency, it is difficult to determine how important a role any decrease in egg iodide played in the reduced embryonic thyroid function observed in the embryos from eggs of perchlorate-exposed hens in the present study.

The lower body weights of the embryos from perchlorate-exposed hens compared to the controls indicated embryonic hypothyroidism had an effect on body growth. Although embryonic plasma thyroid hormones could not be measured because of lipid interference with the assay, both thyroid gland hypertrophy and very low thyroidal thyroid hormone storages in the embryos from eggs of perchlorate-

exposed hens indicated hypothyroid conditions in these embryos. Thus, it appears that thyroid deficiencies are the primary cause of the decreased growth in these embryos compared to the controls. In the present study, the possibility that reduced maternal hormones deposited in the eggs of perchlorate-exposed hens played a role in this decreased growth needs to be considered. Maternal plasma T_4 decreased stepwise with increasing perchlorate exposure in the present study and an earlier study in our laboratory demonstrated that the amount of maternal thyroid hormone deposited in Japanese quail eggs was correlated with the thyroid status of the hens. In that same study, changes in maternal thyroid hormone concentrations in the egg affected growth of a specific thyroid-responsive tissue (pelvic cartilage) in the embryos but had no significant effect on overall embryonic growth (Wilson and McNabb, 1997). Surprisingly, decreases in embryonic growth have not been observed in all studies where embryonic hypothyroidism has been produced. For example, when eggs from euthyroid chickens were injected with PCB 77, although plasma thyroid hormone concentrations were significantly decreased during late incubation, mean body weight of the PCB-exposed embryos did not differ from that in the controls (Roelens *et al.*, 2005). It is difficult to evaluate the lack of growth effect in the study by Roelens (2005) because the timing of the development of hypothyroidism in PCB-exposed embryos was not clear therefore the length of time the embryos were exposed to hypothyroidism could not be determined.

The embryonic hypothyroidism induced by maternal perchlorate exposure appeared to have caused delayed or unsuccessful hatching of embryos from perchlorate-exposed hens in the present study. Deficiencies in embryonic thyroid function are known to decrease hatching success in birds (Freeman, 1964). In the present study, no embryos successfully hatched in the 4000 mg/l group and the hatching of embryos in the 2000 mg/l AP group was delayed by one to three days compared to the control group. Likewise, when chicken embryos were exposed to PCB 77, they had decreased plasma thyroid hormone concentrations at the end of incubation, delayed hatching and increased deaths during the hatching process (Roelens *et al.*, 2005).

In the present study, some chicks hatched from eggs laid by 2000 mg/l AP hens. These chicks were not exposed to perchlorate post-hatch and thyroid function appeared to increase rapidly after hatching. In these chicks, all thyroid variables measured except the thyroid gland weight were equivalent to those of the controls within two days post-hatch. Perchlorate ion, which is water soluble, can be excreted rapidly so it does not accumulate in the body (Yu *et al.*, 2002). Therefore, recovery from perchlorate-induced hypothyroidism may start soon after exposure is discontinued. Thyroid gland hypertrophy, which reflects earlier thyroid tissue growth (i.e. goiter), presumably required a longer time to be fully reversed than the other thyroid variables.

Under hypothyroid conditions, the expression of brain D2 is usually elevated to increase thyroid hormone activation of T_4 to T_3 (Bianco *et al.*, 2002; Rudas *et al.*, 2005). Increased D2 mRNA levels were seen in the brain of thyroidectomized adult chickens (Gereben *et al.*, 1999). Thus, in the present study, hypothyroidism observed in embryos from eggs of perchlorate-exposed hens was expected to increase the D2 mRNA level in brain and liver. However, no discernible difference was observed in the D2 mRNA level in the brain of d14 embryos from either of the perchlorate-exposed groups compared to that of the control group. The D2 expression is inversely regulated by both thyroid hormones through different mechanisms: T_3 regulates D2 mRNA levels at the transcriptional level and T_4 regulates the degradation of the D2 protein (Burmeister *et al.*, 1997). Therefore, mRNA level (as measured in the present study) may not accurately reflect the full effect of hypothyroidism on the D2 activity. When chicken eggs were injected with PCB 77, plasma and brain thyroid hormone concentrations were reduced and D2 activity in the brain was increased (Beck *et al.*, 2006). Therefore, although no difference was observed in the D2

mRNA level in the brains of perchlorate-exposed quail embryos, brain D2 activity might still have been altered by the AP-induced hypothyroidism in these embryos.

D2 activity has been reported in liver in birds (Japanese quail, Hughes and McNabb, 1986; chicken, Van Der Geyten *et al.*, 2002). Although it is not present in mammalian liver, a recent study in our laboratory demonstrated that D2 also was expressed in the liver of young Japanese quail chicks and the level of D2 mRNA in the liver of these quail chicks was increased after two weeks of exposure to 2000 mg/l AP (chicks were three weeks old when sacrificed; Chen, chapter 5 of this dissertation). The present study shows that D2 mRNA was not detectable in the liver of control embryos but was present in the liver of embryos from hens in both perchlorate-exposed groups. Thus, hepatic D2 mRNA level appears to be inversely influenced by embryonic hypothyroidism. The increase in liver D2 mRNA level of quail embryos from eggs of perchlorate-exposed hens and of chicks exposed to perchlorate post-hatch indicate that D2 may play a role in regulating thyroid hormone concentrations in hypothyroid embryos.

The effect of maternal perchlorate exposure on the expression of thyroid responsive genes that affect brain functional development was assessed by measuring the mRNA level RC3 in the embryonic brain. The gene is up-regulated by thyroid hormones in mammals. In precocial mammals such as goat, RC3 expression increases significantly during late embryonic development and peaks at birth before it drops to the adult level (Gerendasy and Sutcliffe, 1997). Pattern of RC3 expression in precocial quail embryos resembled that in goats as the quail RC3 mRNA level increased gradually during the late incubation period and remained relatively stable between the end of incubation and one week after hatching. In goats, hypothyroidism decreases RC3 mRNA level in specific brain regions (Piosik *et al.*, 1996; Piosik *et al.*, 1995). The RC3 mRNA levels in the brains of embryos from eggs of perchlorate-exposed hens, however, are not different from that in the brains of embryos from eggs of control hens. The RC3 mRNA levels in my experiment were measured in the whole brain total RNA using Northern blotting, so I did not address region-specific changes in mRNA levels within the brain. Thus it is possible that RC3 expression was affected in some areas in the brain of perchlorate-exposed embryos.

In conclusion, maternal perchlorate exposure altered egg conditions and disrupted the thyroid function of Japanese quail embryos, resulting in embryonic hypothyroidism which in turn affected embryonic growth. Hypothyroidism in the embryos altered the D2 mRNA level in the liver in a compensatory direction. Level of spot 14 mRNA was not detectable by Northern blotting in D14 embryos, possibly due to the low activity of hepatic functions before hatching. In the brain no changes in mRNA levels of D2 and RC3 could be detected by Northern blotting in embryos from eggs of perchlorate-exposed hens in comparison to controls. Changes in mRNA levels of thyroid-responsive genes in the brain may be subtle and were likely restricted to specific brain regions. Techniques capable of providing information on spacial distribution of mRNA in the brain such as in situ hybridization can offer more details about the expression of brain thyroid-responsive genes in quail embryos from eggs of perchlorate-exposed hens. The current study demonstrated perchlorate exposure can affect the health of the offspring of the exposed birds. For wild birds in perchlorate contaminated areas, such effect may cause a decrease in the survival rate of the offspring of the exposed birds and consequently lead to decline in wild bird population in these areas.

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Table 4.1 Primer sequences and annealing temperatures for the PCR of cDNA of selected Japanese quail genes

Gene	Primers sequences (5'→3')		Annealing temperature (°C)	Predicted product size (bp)
RC3	Forward	ATGGACTGCTGCAACGAGGG	50	221
	Reverse	TAATCGCCGTTGCGGAGGTCT		
D2	Forward	ATGGGTCTGTTAAGTGC	50	840
	Reverse	AAGGGACACATCTGTTG		
SP14 α	Forward	AGAAGATGGAGCAGGAGGTG	55	307
	Reverse	GCTTGGTGTTTTTGGTGAG		
β -actin	Forward	CGTGCCGTGTTCCCATC	55	1000
	Reverse	CCTGCTTGCTGATCCAC		

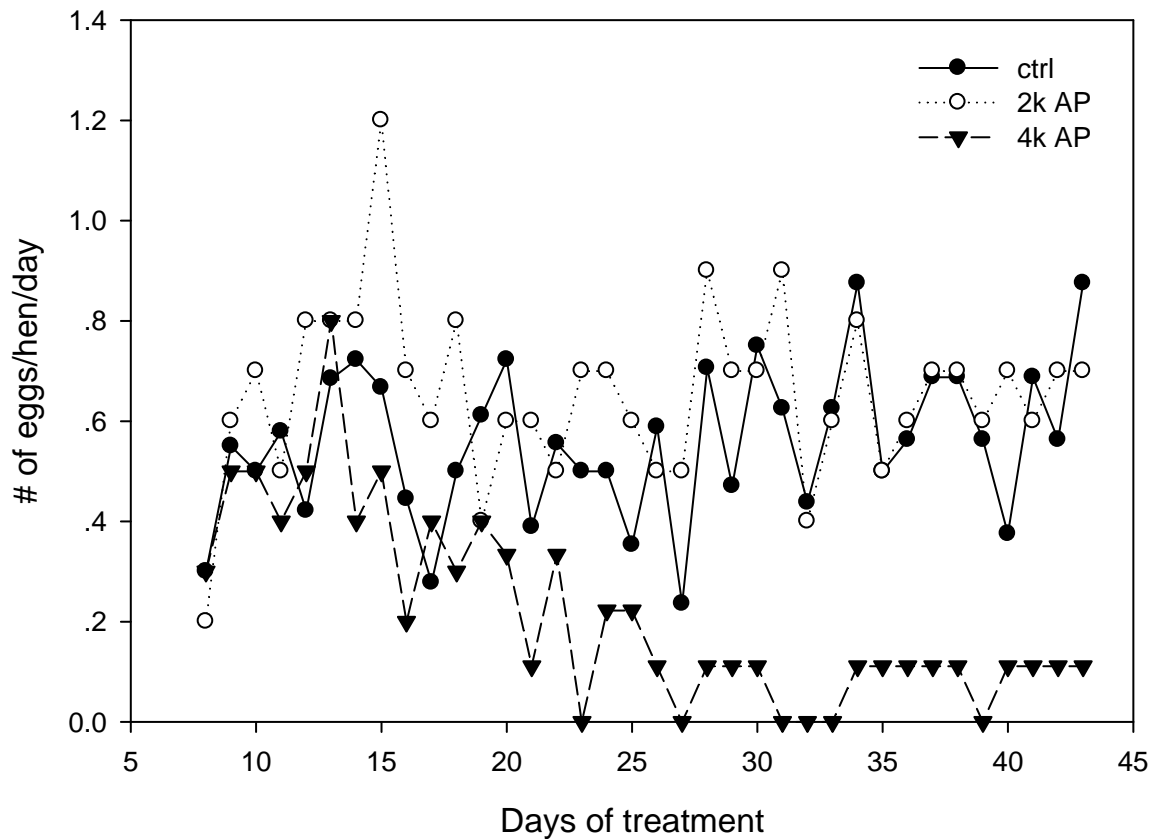


Fig. 4.1 Mean numbers of eggs laid per hen per day from the three treatment groups over the period of perchlorate exposure.

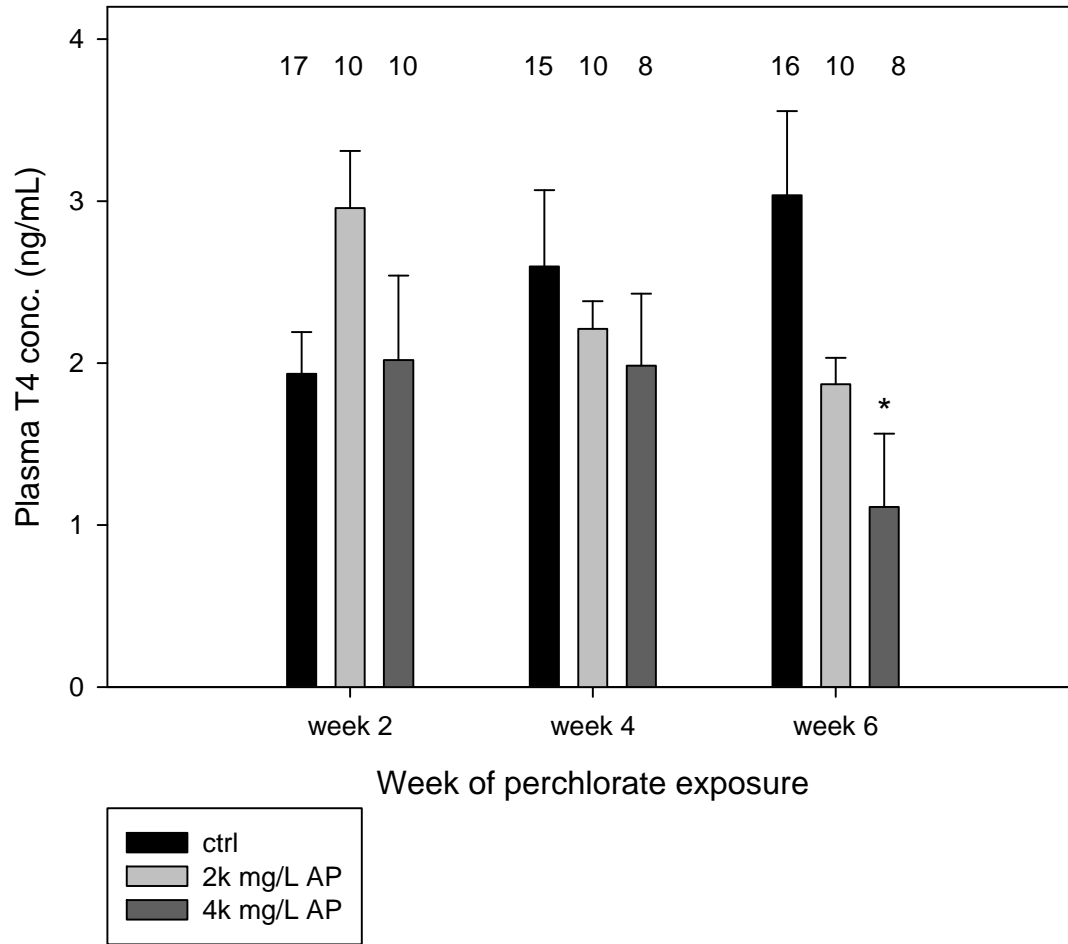


Fig. 4.2 Plasma T₄ concentration of hens exposed to ammonium perchlorate. T₄ concentration was measured in the ethanol extract of plasma from blood drawn from the brachial veins of hens at week 2 and week 4 of perchlorate exposure. Hens were sacrificed at week 6 of perchlorate exposure and T₄ concentration was measured in the ethanol extract of plasma from trunk blood of the hens. Values are means ± SE. Significant differences (p<0.05) are indicated by *. N values are above each bar. During the experiment, four hens in the control group and one hen in the 4000 mg/l perchlorate exposure group died of natural causes.

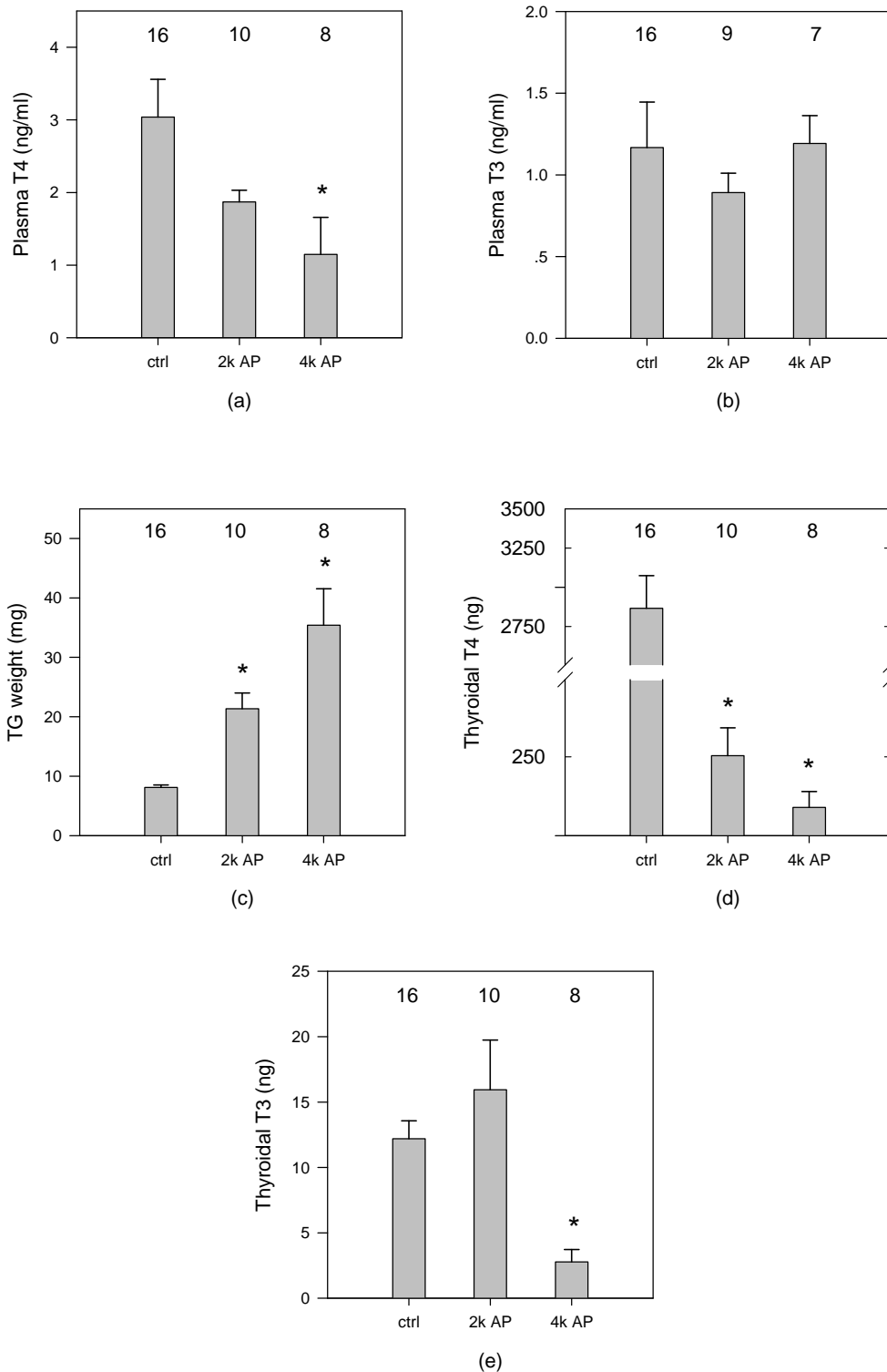


Fig. 4.3 Thyroid status of hens exposed to ammonium perchlorate. Hens were treated with 2000 mg/l and 4000 mg/l AP solution as drinking water for six weeks before sacrificed and plasma T₄ concentration (a), plasma T₃ concentration (b), thyroid gland weight (c), thyroidal T₄ storage (d) and thyroidal T₃ (e) storage were measured. Values are means ± SE. Significant differences (p<0.05) between control and treated groups are indicated by *. N values are above each bar

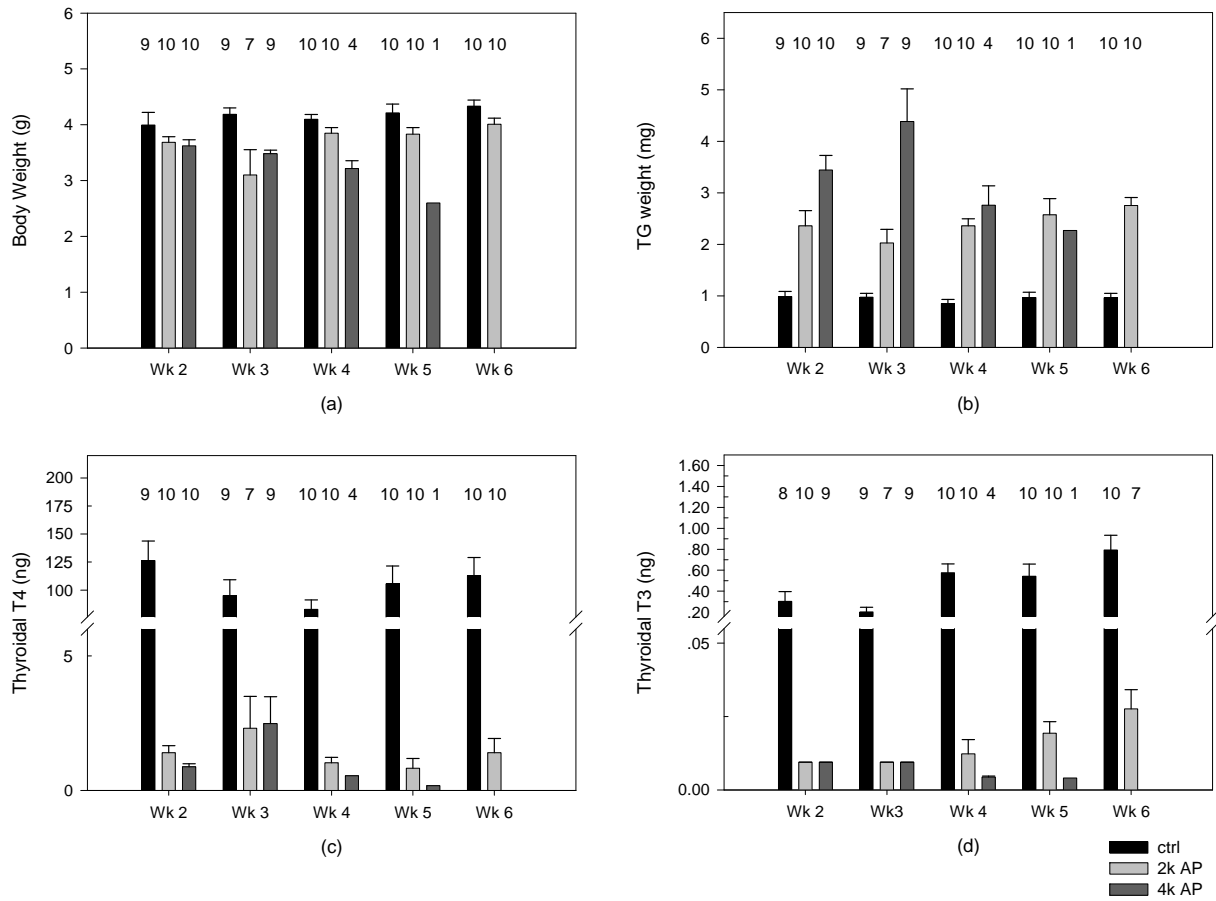


Fig. 4.4 Effects of hens' exposure to ammonium perchlorate on the growth and thyroid status of the embryos. All embryos were sacrificed at d14 of incubation and body weight (a), thyroid gland weight (b), thyroidal T₄ storage (c) and thyroidal T₃ storage (d) were measured. All values shown are means \pm SE. N values are above each bar.

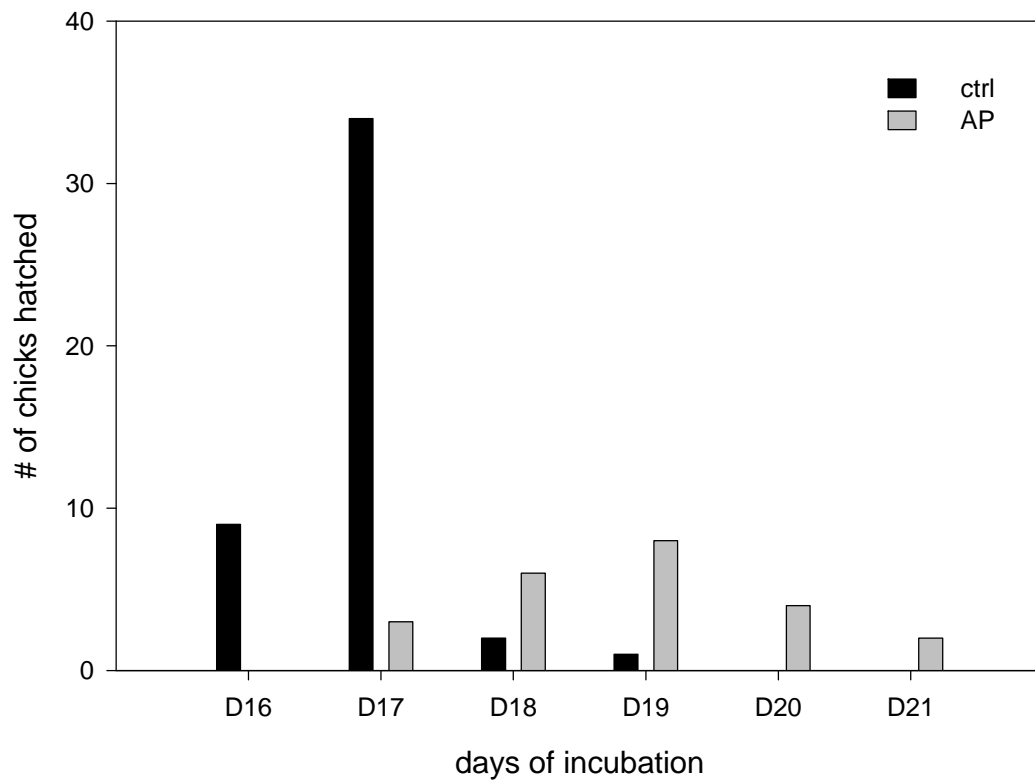


Fig. 4.5 Number of chicks hatched from eggs collected from control and ammonium perchlorate-exposed hens each day between d16 and d21 of incubation. Japanese quail commonly hatch between day 16 and day 17 of incubation.

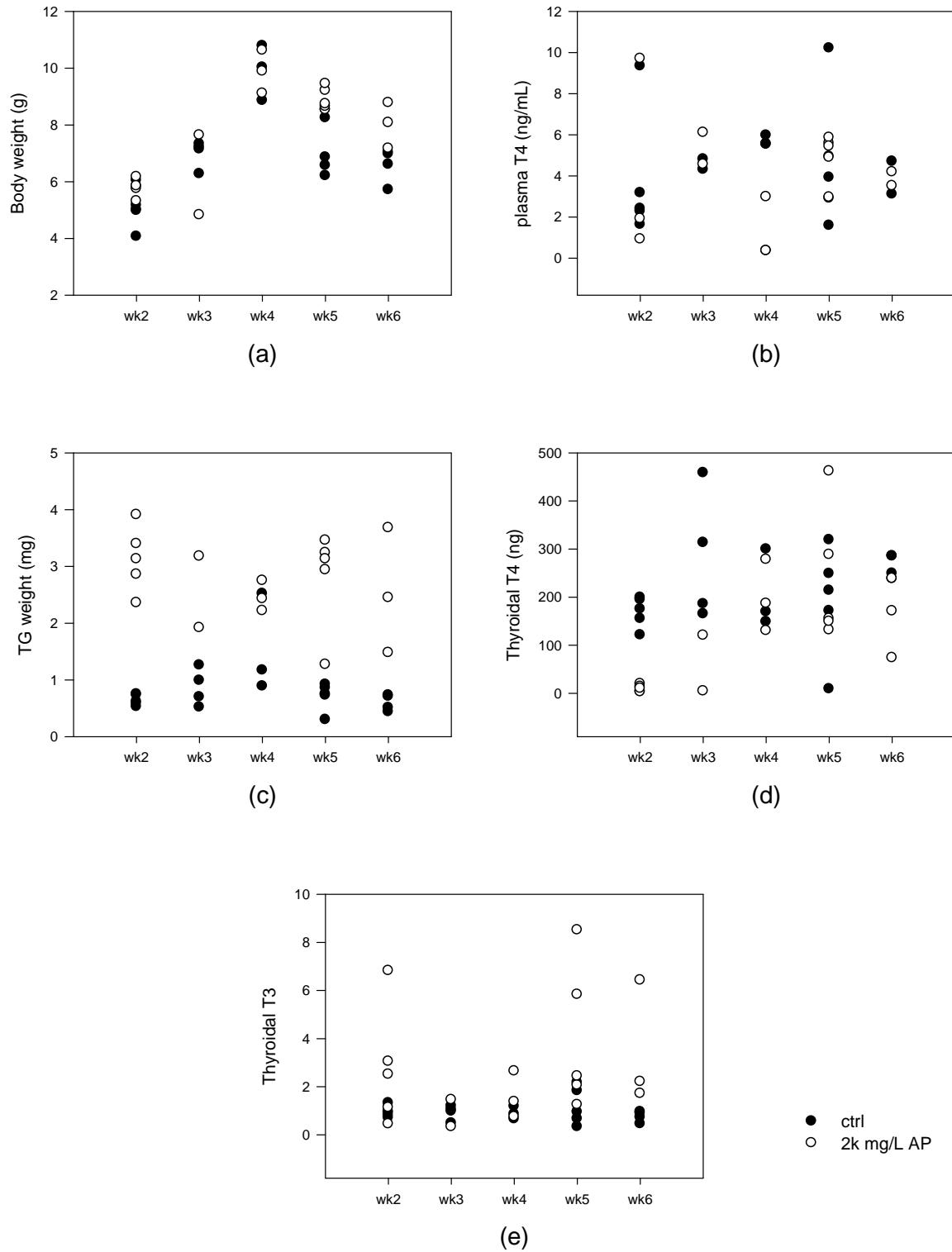


Fig. 4.6 Thyroid status of chicks hatched from eggs collected from control and ammonium perchlorate-exposed hens. Chicks were sacrificed within two days after hatching; body weight (a), plasma T₄ (b), thyroid gland weight (c), thyroidal T₄ storage (d) and thyroidal T₃ storage (e) were measured. Solid circles represent chicks from the control group and open circles represent chicks from the 2000 mg/l perchlorate exposure group.

	1	10	20	30	40	50	60	65
Quail	?	?	?	?	?	?	?	?
Chicken EST	?	?	?	?	?	?	?	?
Canary	?	?	?	?	?	?	?	?
Consensus	?	?	?	?	?	?	?	?

	66	70	80	90	100	110	120	130
Quail	C	G	A	C	C	C	C	G
Chicken EST	C	G	A	C	C	C	C	G
Canary	C	G	A	C	C	C	C	G
Consensus	C	G	A	C	C	C	C	G

	131	140	150	160	170	180	190	195
Quail	A	G	A	G	A	T	C	A
Chicken EST	A	G	A	G	A	T	C	A
Canary	A	G	A	G	A	T	C	A
Consensus	A	G	A	G	A	T	C	A

	196	200	210	220	230	240	250	260
Quail	G	G	C	G	A	G	A	C
Chicken EST	G	G	C	G	A	G	A	C
Canary	G	G	C	G	A	G	A	C
Consensus	G	G	C	G	A	G	A	C

	261	270	280	290	300	310	320	325
Quail	?	?	?	?	?	?	?	?
Chicken EST	?	?	?	?	?	?	?	?
Canary	?	?	?	?	?	?	?	?
Consensus	?	?	?	?	?	?	?	?

	326	330	340	343
Quail	?	?	?	?
Chicken EST	?	?	?	?
Canary	?	?	?	?
Consensus	?	?	?	?

(a)

	1	10	20	30	40	50	55
Quail	L	A	G	E	I	L	D
Chicken EST	L	A	G	E	I	L	D
Canary	L	A	G	E	I	L	D
Consensus	L	A	G	E	I	L	D

	56	60	70	80	90	100	110
Quail	K	D	A	E	C	A	N
Chicken EST	K	D	A	E	C	A	N
Canary	K	D	A	E	C	A	N
Consensus	K	D	A	E	C	A	N

	111	114
Quail	P	P
Chicken EST	P	P
Canary	P	P
Consensus	P	P

(b)

Fig. 4.7 Sequence alignment of Japanese quail RC3, chicken EST and Canary RC3. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment.

	1	10	20	30	40	50	60	65
Quail	?	?	?	?	?	?	?	?
Chicken	A	T	G	G	G	T	C	T
Consensus	A	T	G	G	G	T	C	T
	66	70	80	90	100	110	120	130
Quail	C	C	T	C	T	T	C	C
Chicken	C	C	T	C	T	T	C	C
Consensus	C	C	T	C	T	T	C	C
	131	140	150	160	170	180	190	195
Quail	C	C	A	A	G	T	C	T
Chicken	C	C	A	A	G	T	C	T
Consensus	C	C	A	A	G	T	C	T
	196	200	210	220	230	240	250	260
Quail	A	G	C	T	T	C	C	T
Chicken	A	G	C	T	T	C	C	T
Consensus	A	G	C	T	T	C	C	T
	261	270	280	290	300	310	320	325
Quail	C	C	A	T	A	G	C	C
Chicken	C	C	A	T	A	G	C	C
Consensus	C	C	A	T	A	G	C	C
	326	330	340	350	360	370	380	390
Quail	C	C	A	A	G	T	C	C
Chicken	C	C	A	A	G	T	C	C
Consensus	C	C	A	A	G	T	C	C
	391	400	410	420	430	440	450	455
Quail	A	C	T	G	A	C	C	A
Chicken	A	C	T	G	A	C	C	A
Consensus	A	C	T	G	A	C	C	A
	456	460	470	480	490	500	510	520
Quail	G	G	C	T	G	A	C	T
Chicken	G	G	C	T	G	A	C	T
Consensus	G	G	C	T	G	A	C	T
	521	530	540	550	560	570	580	585
Quail	T	C	T	C	C	C	C	T
Chicken	T	C	T	C	C	C	C	T
Consensus	T	C	T	C	C	C	C	T
	586	590	600	610	620	630	640	650
Quail	C	A	G	C	T	C	C	T
Chicken	C	A	G	C	T	C	C	T
Consensus	C	A	G	C	T	C	C	T
	651	660	670	680	690	700	710	715
Quail	T	G	C	A	A	T	G	T
Chicken	T	G	C	A	A	T	G	T
Consensus	T	G	C	A	A	T	G	T
	716	720	730	740	750	760	770	780
Quail	A	C	T	G	G	G	A	G
Chicken	A	C	T	G	G	G	A	G
Consensus	A	C	T	G	G	G	A	G
	781	790	800	810	820	830	840	
Quail	T	T	C	A	G	C	A	A
Chicken	T	T	C	A	G	C	A	A
Consensus	T	T	C	A	G	C	A	A

(a)

	1	10	20	30	40	50	60
Quail	? ? ? ? ? ?	& L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L					
Chicken	M G L L S A D L L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L						
Consensus	M G L L S A D L L I T L Q I L P V F F S N C L F L A L Y D S V I L L K H M V L F L S R S K S A R G E W R R M L T S E G L						
	61	70	80	90	100	110	120
Quail	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K D N D G S S S G W K S V G G K C G T K C R L L D F A N S E						
Chicken	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K G N D G S N S S W K S V G G K C G T K C H L L D F A N S E						
Consensus	R C V W N S F L L D A Y K Q V K L G G E A P N S S V I H I A K I N D G S I S I W K S V G G K C G T K C I L L D F A N S E						
	121	130	140	150	160	170	180
Quail	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
Chicken	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
Consensus	R P L V V N F G S A T * P P F T S Q L S A F S K L V E E F S G V A D F L L V Y I D E A H P S D G W A A P G I S P S S F E						
	181	190	200	210	220	230	240
Quail	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
Chicken	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
Consensus	V K K H R N Q E D R C A A A H Q L L E R F S L P P Q C Q V V A D C M D N N A N V A Y G V S F E R V C I V Q R Q K I A Y L						
	241	250	260	270	280		
Quail	G G K G P F F Y N L Q E V R L W L E Q N & ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?						
Chicken	G G K G P F F Y N L Q E V R L W L E Q N F S K R * N P L S T E D L S T D V S L *						
Consensus	G G K G P F F Y N L Q E V R L W L E Q N F S K R * N P L S T E D L S T D V S L *						

(b)

Fig. 4.8 Sequence alignment of Japanese quail D2 and Chicken D2. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

	1	10	20	30	40	50	60	65
Quail	? ? ? ? ? ? ? ?	? ? ? ? ? ? ? ?	? ? ? ? ? ? ? ?	? ? ? ? ? ? ? ?	? ? ? ? ? ? ? ?	AGGAGGTGATGTTCCCCAGCCTGCTCAG		
Chicken	ATGGAGCAGTACTTCTCGGCCACGCAGAAGATGGAGCAGGAGGTGATGTTCCCCAGCCTGCTGCG							
Consensus	ATGGAGCAGTACTTCTCGGCCACGCAGAAGATGGAGCAGGAGGTGATGTTCCCCAGCCTGCT						S M G	

	66	70	80	90	100	110	120	130
Quail	AGGGGTCTTCCACAGGATGGGGCTGACCCAGCCACCAGCGGGCTCCGGGACCTCTACGAGCGCT							
Chicken	AGGGGTCTTCCCGCAGGACGGGGCCGACCCAGCCACCAGCGGGCCCGCACCTCTACGAGCACT							
Consensus	AGGGGTCTTCCC	R	CAGGA	Y	GGGGC	Y	GACCCAGCCACC	R

	131	140	150	160	170	180	190	195
Quail	ACCAGCTCCTCAAGTCCATCAAGCCCATGGTGGAGCGAGGCCTGGCCTCCGTCACTGATCAGAGC							
Chicken	ACCAGCTCCTCAAGGCCATCAAGCCCGTGGTGGAGCGAGGCCTGGCCTCCGTCACTCAGAGC							
Consensus	ACCAGCTCCTCAAG	K	CCATCAAGCCC	R	TGGTGGAGCGAGGCCTGGCCTCCGTCACT			Y

	196	200	210	220	230	240	250	260
Quail	CCCACCAGCAATGACGACGCCAACACAGCCCCAGATGATGGCATA-----GATGGGAATCT							
Chicken	CCCACCAGCAATGCCGACGCCGACACGGCCCCATATGATGGCATAAGATGGCATAAGATGGGAATCT							
Consensus	CCCACCAGCAATG	M	CGACGCC	R	ACAC	R	GCCCCA	K

	261	270	280	290	300	310	320	325
Quail	GGAGGAGCGGCTGTCCCACCATATGAATGGCTTGCAGCAGGTTCTGACCGACCTCACCAAAACA							
Chicken	GGAGGAGCGGCTGTCCCACCATATGAATGGCTTGCAGCAGGTTCTGACCGACCTCACCAAAACA							
Consensus	GGAGGAGCGGCTGTCCCACCA	Y	ATGAATGGCTTGCAGCAGGTTCTGACCGACCTCACCAAAACA					

	326	330	340	350	360	370	380	390
Quail	CCAAAGCA? ?							
Chicken	CCAAAGCTCTCACCCGGAGGTACAGCCAGATCCTGGAGGAGATCAACCTCGGTGAAGGTCAGAGC							
Consensus	CCAAAGC	W	CTCACCCGGAGGTACAGCCAGATCCTGGAGGAGATCAACCTCGGTGAAGGTCAGAGC					

	391	399
Quail	? ? ? ? ? ? ? ?	
Chicken	AGCTCATGA	
Consensus	AGCTCATGA	

(a)

	1	10	20	30	40	50	60
Quail	? ? ? ? ? ? ? ?	& E V M F P S L L R G V F P Q D G A D P A T S G S A D L Y E R Y Q L L K S I K P M V E R G L A S					
Chicken	M E Q Y F S A T Q K M E Q E V M F P S L L R G V F P Q D G A D P A T G G P A D L Y E H Y Q L L K A I K P V V E R G L A S						
Consensus	M E Q Y F S A T Q K M E Q E V M F P S L L R G V F P Q D G A D P A T	G	G	A D L Y E	Y	Q L L K	I

	61	70	80	90	100	110	120
Quail	V T D Q S P T S N D D A N T A P D D G I - - - D G N L E E R L S H H M N G L Q Q V L T D L T K N T K A ? ? ? ? ? ? ? ? ? ? ? ? ?						
Chicken	V T D Q S P T S N A D A D T A P Y D G I D G I D G N L E E R L S H H M N G L Q Q V L T D L T K N T K A L T R R Y S Q I L						
Consensus	V T D Q S P T S N	D	A	T	A P	D G I D G I D G N L E E R L S H H M N G L Q Q V L T D L T K N T K A L T R R Y S Q I L	

	121	130	133
Quail	? ? ? ? ? ? ? ? ? ?		
Chicken	E E I N L G E G Q S S S *		
Consensus	E E I N L G E G Q S S S *		

(b)

Fig. 4.9 Sequence alignment of Japanese quail SP14 and Chicken SP14. Both cDNA sequences (a) and amino acid sequences (b) were compared. Consensus sequence was included as the bottom row of the alignment. Position of unmatched nucleotides and amino acids were highlighted in the consensus sequence.

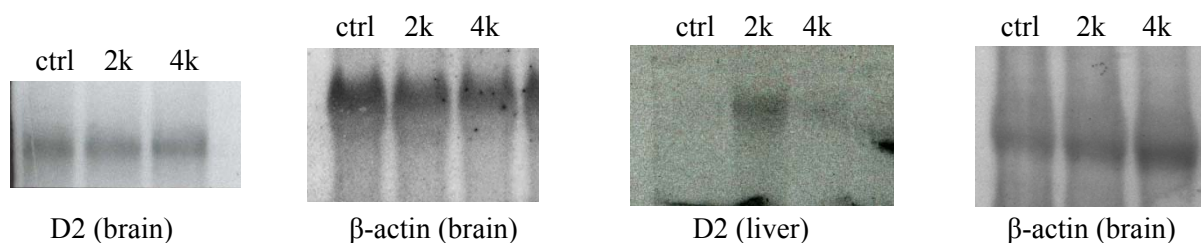


Fig. 4.10 The mRNA levels of D2 in brain and liver total RNA of embryos from hens exposed to AP. Same RNA samples were hybridized with β -actin probe to test the quality and consistency of the RNA.

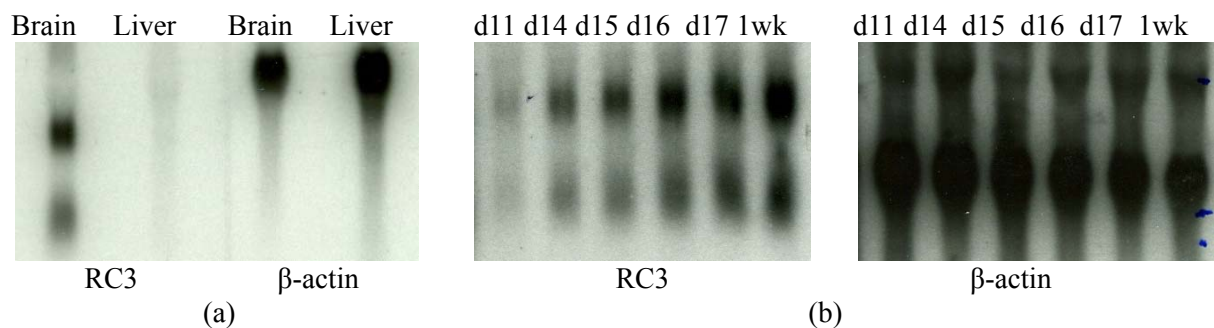


Fig. 4.11 RC3 mRNA level during late embryonic and early post hatching stage. Total RNA were isolated from brains of euthyroid embryos and chicks. Northern blots of RC3 in brain and liver total RNA were compared to confirm the specificity of the probe (a). The pattern of RC3 mRNA level from d11 embryo to one week old chick is shown in (b). β -actin was used as reference gene to test the quality and consistency of the RNA.

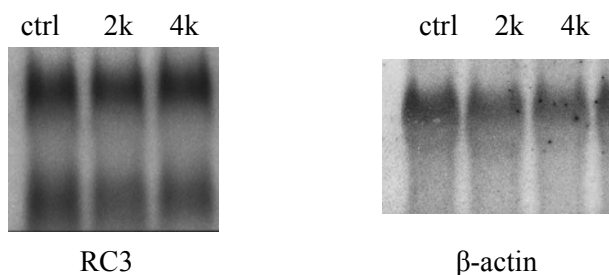


Fig. 4.12 The mRNA levels of RC3 in the brain total RNA of embryos from hens exposed to AP. Same RNA samples were hybridized with β -actin probe to test the quality and consistency of the RNA.

Chapter 5

Ammonium perchlorate exposure induces hypothyroidism in young Japanese quail and affects the expression of Type 2 deiodinase and thyroid-responsive genes in the liver but not in the brain

Abstract

Ground dwelling birds living in perchlorate contaminated areas are exposed to perchlorate ion, a known thyroid disruptor, in drinking water. Developing young may be particularly vulnerable to the developmental effects of hypothyroidism resulting from perchlorate exposure. Perchlorate exposure decreases thyroid function and may affect the expression of thyroid-responsive genes. To investigate the effects of perchlorate-induced hypothyroidism on developing birds, Japanese quail chicks were exposed to 2000 mg/l ammonium perchlorate (AP) in drinking water starting from day 5 post-hatch to 7.5 weeks of age. Quail were sacrificed after 2 and 7.5 weeks of continuous perchlorate exposure to evaluate the effects of short-term and long-term perchlorate exposure. Hypothyroidism resulting from competitive inhibition by perchlorate of iodide transport into the thyroid gland was evident in perchlorate exposed quail chicks after 2 weeks as lower plasma thyroid hormone concentrations and lower thyroidal thyroid hormone content than in the control group. The degree of hypothyroidism was increased with continued perchlorate exposure as indicated by significant thyroid gland hypertrophy, in addition to low plasma thyroid hormones and low thyroidal thyroid hormone content in quail after 7.5 weeks. Perchlorate-induced hypothyroidism increased type 2 deiodinase (D2) mRNA level and decreased spot 14 mRNA level in the liver while D2 mRNA and RC3 mRNA levels in the brain were not affected after 2 weeks of exposure. No change in mRNA levels was observed in the genes studied in either liver or brain after 7.5 weeks of perchlorate exposure despite the increase in the degree of hypothyroidism. These results suggest that the brain, but not the liver, was protected from the effects of perchlorate-induced hypothyroidism. Regulation of thyroid-responsive gene expression by thyroid hormones is complex and the response to changes of thyroid status may not be restricted to mRNA levels.

Introduction

Thyroid hormones play essential roles in the development of vertebrates by regulating thyroid responsive genes in target organs. Thyroid hormones control the expression of thyroid responsive genes during critical developmental windows and are thus required for tissue or organ development. Hypothyroidism during these critical time windows may result in irreversible defects (McNabb, 1992).

Perchlorate ion is a known thyroid disruptor. It inhibits iodine uptake by the thyroid gland and consequently reduces the production of thyroid hormones. The manufacture and use of ammonium perchlorate as an oxidizer in solid rocket fuels has led to the contamination of ground water, rivers and lakes in many states in the United States (De Groef *et al.*, 2006; Stoker *et al.*, 2006). Wildlife species such as ground dwelling birds in perchlorate contaminated areas may develop hypothyroidism at young ages. Perchlorate-induced hypothyroidism may alter the expression of thyroid-responsive genes in these young animals and thereby interfere with their growth and development.

Of the two forms of thyroid hormones, T_4 is the primary product from the thyroid gland, whereas T_3 is the physiologically active, receptor-binding form (McNabb, 1992). T_4 is converted to T_3 by 5'-deiodination, and the conversion is catalyzed by Type 1 and Type 2 deiodinases (D1 and D2). D2 is the major 5' deiodinase in the central nervous system and D2 activity is influenced by thyroid hormone concentrations at both transcriptional (mRNA) and translational (protein) levels (Burmeister *et al.*, 1997). D2 activity is enhanced by low thyroid hormone concentrations but inhibited by high thyroid hormone

concentrations. Such responses prevent T_3 concentration from major fluctuations of T_3 concentration due to changes in circulating thyroid hormone concentrations. D2 activity is also found in several other organs such as brown adipose tissue, skeletal muscle in mammals, and liver in both mammals and bird (Bianco *et al.*, 2002). Studies in chickens have demonstrated that brain T_3 concentration is relatively less affected by changes in plasma T_4 concentration than is the case in other organs, and D2 is one of the key contributors to this stability (Rudas *et al.*, 2005).

In mammals, D2 in the brain maintains a stable supply of T_3 , which in turn regulates the expression of many thyroid responsive genes such as RC3/neurogranin (RC3). RC3 is a neuron specific, calmodulin-binding protein that regulates calcium availability in neurons. The expression of RC3 is under thyroid hormone control in both developing and adult animals (Gerendasy and Sutcliffe, 1997). A significant increase in the RC3 expression during development coincides with the timing of synaptogenesis, which leads to the assumption that RC3 plays an important role during brain development. In mammals, hypothyroidism reduces RC3 mRNA and protein levels in a number of brain regions and such reductions of RC3 expression during brain development are potentially related to some irreversible mental deficits (Bernal, 2002).

Thyroid hormones also are involved in the control of metabolic processes in the liver. The mRNA level of Spot 14 (SP14), a protein involved in the lipogenic pathway in the liver, is directly and rapidly up-regulated by T_3 . The functions of SP14 in relation to thyroid status were well studied in both mammals and birds. Hypothyroid animals typically have lower SP14 mRNA levels than euthyroid animals and their lipogenic function may be disrupted (Brown *et al.*, 1997; Wang *et al.*, 2004).

In this study, I evaluated the effects of perchlorate-induced hypothyroidism on the mRNA level of thyroid responsive genes in brain and liver in young Japanese quail, which were used as a model for ground-dwelling galliform birds in general. Hypothyroidism in perchlorate exposed birds was evaluated by measurements of plasma thyroid hormone concentrations, thyroid gland weight (to assess activation of the hypothalamic-pituitary-thyroid axis) and thyroid hormone storage in the thyroid gland. The D2 mRNA levels were measured in brain and liver, RC3 mRNA levels were measured in the brain and SP14 mRNA levels were measured in the liver to evaluate tissue specific responses of thyroid responsive gene expression.

Materials and methods

Animals. Japanese quail eggs were collected from a breeding colony in the animal care facilities in the Department of Biological Sciences at Virginia Tech. Eggs were incubated and hatched at $39 \pm 1^\circ\text{C}$ and $>90\%$ relative humidity in a forced-air incubator (Humidaire Hatchette Incubator; New Madison OH). Newly hatched chicks were divided into two treatment groups, banded and kept in separate shelves in a commercial brooder. Game bird feed (Big Spring Mills, Ellison VA) and drinking solutions were provided ad libitum. Birds were moved to taller cages at 4 weeks of age until the end of the experiment. All maintenance, handling and sacrifice procedures of the animals were approved by the Virginia Tech Animal Care Committee in accordance with federal guidelines (IACUC).

Exposure of juvenile quail to perchlorate. Chicks, 4-5 days old, were divided into two groups and housed in separate cages. One group of 17 chicks was given 2000 mg/l AP (powder, Fluka Chimka, Steinheim, Germany) solution in tap water as drinking water. Previous work in our laboratory on Bobwhite quail chicks showed that this concentration of AP was sufficient to cause decreased thyroid function after a 2 week exposure period (McNabb *et al.*, 2004b). The other group of 13 chicks was given tap water as controls. After 2 weeks, 5 chicks randomly selected from each group were sacrificed to

evaluate the short-term effects of perchlorate exposure. The rest of the chicks were kept on the same treatment and blood samples were drawn from brachial vein at 4 weeks and 6 weeks of exposure to determine their plasma T₄ concentrations. These chicks were sacrificed at 7.5 weeks of exposure after it was shown that at 6 weeks the mean plasma T₄ concentration was significantly lower in the AP group than the control group.

Tissue collection. All chicks were sacrificed by decapitation, and trunk blood was collected in heparinized capillary tubes and plasma was stored at -20°C until analysis. Brains and livers were dissected immediately after sacrifice, flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. Thyroid glands were removed, weighed, frozen and stored at -20°C in snap cap tubes.

Thyroid assays. Plasma thyroid hormone concentrations were determined by a double antibody radioimmunoassay (RIA) described by Wilson and McNabb (1997). Hormone standards were prepared in charcoal-stripped chicken plasma. Duplicate aliquots for each plasma sample were used and sample volumes were 12.5 µl for T₄ and 25 µl for T₃. Primary antibodies were purchased from Fitzgerald (Fitzgerald Industries International, Inc, Concord MA). ¹²⁵I-labeled hormones (high specific activity; 1200 µCi/µg) were purchased from Perkin-Elmer Life Sci (Boston MA). Three levels of Randox Immunoassay Control serum (Randox Laboratories, San Diego CA) were included in each assay to evaluate assay performance. The intra-assay precision of RIA, ±2SE, was 3.1% of the mean for T₄ (n=6) and 2.6% of the mean for T₃ (n=6; McNabb *et al.*, 2004a). The plasma of quail at 6 weeks contains high lipid content that interferes with the antibody binding during RIA. The thyroid hormones in the plasma of these birds were extracted by mixing equal volumes of plasma with 100% ethanol. The mixture was centrifuged at 12000 g to remove insoluble material. The relative hormone concentrations in the supernatant after centrifugation were measured to estimate plasma hormone concentrations.

Activation of the hypothalamic-pituitary-thyroid axis was evaluated by comparing mean thyroid gland weights from the control and AP exposed groups. The pair of thyroid glands from each bird was weighed to the nearest 0.01 mg. Weights of the thyroid glands from the treated group were compared to those from the control group.

Thyroidal hormone content of the glands was measured using the method described by McNabb and Cheng (1985). Thyroid gland tissue (10 mg or less) was digested in 350 µl of digestion medium containing 25 mg of Pronase (Sigma-Aldrich, St. Louis, MO) at 37 °C for 24 h. When the combined weight of the gland pair exceeded 10 mg, each gland was digested separately. The digestion was stopped by the addition of 1.0 ml of absolute ethanol and the tubes were stored at -20 °C for 24 h to extract the thyroid hormones. The tubes were then centrifuged at 13,500 g for 5 min and the supernatant was collected and stored at -20 °C until analysis. Dilutions of the supernatant in 75% ethanol were analyzed for T₄ and T₃ by RIA as described above except the standards for these assays were prepared in 75% ethanol instead of plasma.

Thyroid hormone concentrations in samples within the same data set (plasma T₄/T₃ at 2 weeks, plasma T₄/T₃ at 6 weeks, thyroidal T₄/T₃ at 2 weeks and thyroidal T₄/T₃ at 7.5 weeks) were measured in one single assay. Therefore, all comparison of measurements between the different treatment groups were made with data acquired from the same assay. There was no inter-assay comparison of data.

Total RNA isolation. Total RNA was isolated from brain and liver tissues using Tri-Reagent (Sigma-Aldrich, St. Louis MO) following the protocol provided by the manufacturer. Total RNA from each brain or liver tissue was isolated individually. Tissues were homogenized in Tri-Reagent with a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Inc., Westbury NY). For each 50 mg of tissue, 1 ml of the Tri-Reagent was added. Insoluble material in the homogenate was eliminated by

centrifuging at 12000 g for 10 min. The supernatant was mixed with chloroform (0.2 ml for each milliliter of Tri-Reagent) and centrifuged at 12000 g for 15 min for phase separation. The top aqueous phase containing RNA was transferred to a new tube and mixed with isopropanol (0.5 ml for each milliliter of Tri-Reagent) to precipitate the RNA. RNA pellets were washed in 75% ethanol, dried and resuspended in RNase free water. The RNA samples were then stored at -80 °C for further analysis.

Northern blotting. The mRNA levels of thyroid responsive genes were determined by northern blot following the protocol described by Sible *et al.* (1997). Before Northern blotting, total RNA samples within the same group were pooled. 20 µg from each pooled total RNA sample was loaded on a 1% denaturing agarose gel containing formaldehyde for electrophoresis. After electrophoresis, the RNA was transferred to a nylon membrane and cross-linked by UV irradiation. ³²P-labelled probes were prepared using Random Primed DNA labeling kit (Roche Applied Science, Indianapolis IN) and ³²P-labeled dCTP (Amersham Life Sci, Pittsburg PA). The cDNA templates of Japanese quail D2, RC3, SP14 and β-actin were obtained from young Japanese quail brain and liver total RNA by RT-PCR. Sequence information of the templates is available in the NCBI nucleotide database (NCBI accession numbers: RC3, EU558133; DII, EU558134 and SP14, EU558135). Membranes were hybridized with 20x10⁶ TCA-perceptible cpm/ml denatured probes in QuikHyb solution (Stratagene, La Jolla CA) and washed following manufacturer's instruction. Brain total RNA was hybridized with D2 and RC3 probes and liver total RNA was hybridized with D2 and SP14 probes. In addition, both brain and liver total RNAs were hybridized with β-actin probe to test the consistency of the RNA.

Statistical analyses. Multivariate analysis of variance (MANOVA) was used to analyze the thyroid variables measured. For chicks sacrificed at 2 weeks, plasma T₄ and T₃ concentration, thyroid gland weight and thyroidal T₄ and T₃ storages were included as responses. For chicks sacrificed at 7.5 weeks, thyroid gland weight and thyroidal T₄ and T₃ storages were included as responses. Univariate analysis of variance (ANOVA) was used as post-hoc analysis to investigate the data for each individual response. Body weight at both 2 and 7.5 weeks and plasma T₄ and T₃ concentrations at 6 weeks were analyzed using student t-test. Statistically significant differences were defined as probabilities of p ≤ 0.05. Statistical analyses were performed using Minitab 15 (Minitab, Inc., State College PA). Birds sacrificed at 2 weeks of exposure were randomly selected and the sex of the birds could not be distinguished by appearance at that age. As a result, all 5 chicks in the control group at 2 weeks turned out to be females and 3 out of the 5 chicks in the perchlorate-exposed group were females. Of the birds sacrificed at 7.5 weeks, only 3 out of 8 birds in the control group were females while 6 out of 12 birds in the exposed group were females. Moreover, it was assumed that the sex differences in Japanese chicks at the ages studied were negligible. Therefore, effects of perchlorate exposure on difference sexes were not statistically analyzed.

Results

Growth and Development. Perchlorate exposure did not affect the overall growth of the birds. Body weight of the control group and perchlorate-exposed group were not significantly different at either 2 weeks (p=0.168, mean_{ctrl}=33.49±3.7, mean_{AP}=27.07±1.0) or 7.5 weeks (p=0.657, mean_{ctrl}=105.25±2.3, mean_{AP}=103.3±3.6) of treatment. In males of the perchlorate-exposed group, the change of their plumage from the dull immature color to a brighter adult-like color was delayed for about one week compared with those of the control group. When sacrificed at 7.5 weeks, 4 of the 6 males in the perchlorate-exposed group were sexually mature and the other two had underdeveloped testes; all control males had testicular development characteristic of sexually mature adults.

Thyroid function. Perchlorate exposure significantly affected overall thyroid function at both 2 weeks (MANOVA: $F_{3,4}=12.258$; $p=0.015$) and 7.5 weeks (MANOVA: $F_{3,16}=81.228$; $p<0.001$). The development of organismal hypothyroidism was assessed by measuring thyroid hormones in blood samples collected at 2 weeks (sacrificed chicks) and 6 weeks (blood drawn from the brachial vein of the remaining birds) of perchlorate exposure. Plasma T_4 concentrations in the perchlorate-exposed group were significantly lower than those in the control group at 2 weeks of treatment (ANOVA: $F_{1,8}=36.55$; $p<0.001$, Fig. 5.1a). The relative T_4 concentration after ethanol extraction from the quail plasma at 6 weeks of treatment also were significantly lower in the perchlorate-exposed group than those in the control group (t-test: $p<0.0001$, Fig. 5.1b). The proportional difference in T_4 concentration (perchlorate group/control group) between groups was very similar at 2 weeks (~ 0.31) and 6 weeks (~ 0.29) of treatment. Plasma T_3 concentrations were significantly lower in the perchlorate-exposed group than in the control group at 2 weeks (ANOVA: $F_{1,8}=49.31$; $p<0.001$; Fig. 5.1c) but did not differ significantly in the control group and the perchlorate-exposed group at 6 weeks (t-test: $p=0.057$; Fig. 5.1d) due to greater variability at the later time. T_3 assays on plasma samples collected at 6 weeks were carried out without ethanol extraction because the lipid in the plasma had relatively less interference with the binding of T_3 primary antibody to the hormone than it did in T_4 assays. Note that T_3 concentrations in several samples in both groups were not measurable due to the lipid interference in the plasma at 6 weeks. Plasma thyroid hormone concentrations of birds when sacrificed at 7.5 weeks of perchlorate exposure were not measurable. Most of the birds were sexually mature at the time of sacrifice and the lipid content in the plasma increased dramatically between 6 and 7.5 weeks of perchlorate exposure. Ethanol extraction was not effective in eliminating lipid interference with antibody binding in the RIA at this age.

The involvement of the hypothalamic-pituitary-thyroid axis in developing hypothyroidism was assessed by thyroid gland weight. Thyroid gland weight in the perchlorate-exposed group was not significantly different from the control group at 2 weeks of treatment (ANOVA: $F_{1,8}=3.29$; $p=0.107$, Fig. 5.2a). At 7.5 weeks, the thyroid gland weight was significantly higher in the perchlorate-exposed group than in the control group (ANOVA: $F_{1,18}=31.44$; $p<0.001$; Fig. 5.2b).

Thyroid gland storage of T_4 per bird (ng T_4 /pair thyroid glands) was significantly lower in the perchlorate-exposed group at both 2 weeks (ANOVA: $F_{1,8}=11.98$; $p=0.009$, Fig. 5.2c) and 7.5 weeks of treatment (ANOVA: $F_{1,18}=52.50$; $p<0.0001$, Fig. 5.2d). The perchlorate-exposed group showed a 7.5-fold decrease in gland T_4 after 2 weeks compared with the control group and a slightly greater 8.1-fold decrease after 7.5 weeks. Thyroidal T_3 content in perchlorate-exposed group was not significantly different from controls at 2 weeks of treatment (ANOVA: $F_{1,8}=0.57$; $p=0.472$, Fig. 5.2e) but was significantly lower at 7.5 weeks of treatment (ANOVA: $F_{1,18}=175.37$; $p<0.001$, Fig. 5.2f).

Tissue mRNA levels. D2 mRNA level in the brain was not affected by perchlorate exposure at either 2 weeks or 7.5 weeks (Fig. 5.3a). In the liver, D2 mRNA level was higher in the perchlorate-exposed group than the control group at 2 weeks. At 7.5 weeks, however, no difference in D2 mRNA levels could be observed between the control and perchlorate-exposed groups. In addition, D2 mRNA levels in the liver at 7.5 weeks of perchlorate exposure in both control and treated groups appeared to be slightly lower than those at 2 weeks of perchlorate exposure (Fig. 5.3a). The SP14 mRNA level in the liver was lower in the perchlorate-exposed group compared with the control group at 2 weeks but was about the same in both groups at 7.5 weeks. The SP14 mRNA of both the perchlorate-exposed group and the control group at 7.5 weeks, moreover, was expressed at a higher level than the control group at 2 weeks (Fig. 5.3b). There was no difference in the RC3 mRNA levels in the brain between the control and perchlorate-exposed groups at either 2 or 7.5 weeks of perchlorate exposure (Fig. 5.3c).

Discussion

Hypothyroidism, resulting from perchlorate exposure, was evident in quail chicks after 2 weeks of exposure. The treated group, at this time point, showed decreased plasma thyroid hormone concentrations (organismal hypothyroidism) and largely depleted thyroidal thyroid hormone content compare to the control group. The degree of hypothyroidism was increased with continued duration of perchlorate exposure. Plasma T_4 in the perchlorate-exposed birds was significantly lower than that of the control birds at 2 weeks and decreased slightly more by 6 weeks of treatment (perchlorate group/control group; 0.31 vs. 0.29, respectively). Thyroidal T_4 stores also continued to decrease slightly from 2 weeks to 7.5 weeks of treatment (perchlorate group/control group; 0.13 vs. 0.12, respectively).

The thyroid gland is unique among the endocrine glands in that it contains extracellular thyroid hormone storage within the gland. Release of stored thyroidal thyroid hormones is an early response to a decrease in plasma thyroid hormone concentrations (McNabb, 1992). The low perchlorate group/control group ratios of thyroidal thyroid hormone contents observed in this study indicates that a large proportion of the stored thyroid hormones in the perchlorate-exposed birds had been released into the circulation to limit the decline of plasma thyroid hormone concentrations. The release of stored hormones occurs because decreases in plasma thyroid hormones have a negative feedback effect on the HPT axis, which increases the release of thyrotropin from the pituitary. Increased thyrotropin, in addition to stimulating thyroid hormone release, also stimulates thyroid gland growth. This activation of the HPT axis, as indicated by thyroid gland hypertrophy, is much more obvious at 7.5 weeks than at 2 weeks of treatment. The thyroid gland weight in the perchlorate-exposed birds at 2 weeks was not significantly altered by the perchlorate exposure. This resulted from variation in thyroid gland weight data. At 7.5 weeks, mean thyroid gland weight is 3.2 fold higher in the perchlorate-exposed group than the control group with much less variation among individual data, indicating the treatment is long enough that all perchlorate-exposed birds had developed overt hypothyroidism. It is likely that due to individual differences in their response to perchlorate, not all birds had developed hypothyroidism after only 2 weeks of perchlorate exposure.

The mRNA level of D2 was not affected in the brain of Japanese quail chicks after 2 weeks of perchlorate exposure. Profound hypothyroidism was expected to increase D2 mRNA level in the brain because of studies on rats (Bernal, 2002) and young chickens (Gereben *et al.*, 1999; Rudas *et al.*, 2005). However, brain is known to be protected from short-term hypothyroidism by several mechanisms: (1) increases in D2 activity which increases T_3 production in organismal hypothyroidism, (2) increases in the uptake of thyroid hormones into the brain and (3) decreases in the loss of thyroid hormones from the brain. The third of these results from decreased thyroid hormone deactivation by inner ring deiodination Type 3 deiodinase (a 5-deiodinase) and consequent decreased loss of T_3 to the circulation (Rudas *et al.*, 2005). Another possible explanation for the lack of D2 responses is based on the mechanism of D2 gene regulation. Thus if the second and third mechanisms protected brain T_3 in this study, D2 alteration would not occur. Levels of mRNA may not be a direct reflection of the D2 activity in specific tissues. D2 activity is regulated by both T_3 and T_4 through different mechanisms. Studies in mammals have revealed that T_4 alters D2 enzyme activity at a post-translational level, i.e., high T_4 concentration facilitates the degradation of the enzyme (Kim *et al.*, 1998; Steinsapir *et al.*, 1998). In contrast, T_3 regulates D2 mRNA level (a transcriptional effect), which also ultimately changes the activity of the enzyme. High T_3 concentrations decrease and low T_3 concentrations increase D2 mRNA levels. The effect of T_4 is usually fast while that of T_3 is slow (Burmeister *et al.*, 1997). The slow response of D2 mRNA level to changes in thyroid hormone concentrations and the additional protective mechanism the brain has against short-term hypothyroidism might have kept the brain of quail chicks in this study free from the effect of

hypothyroidism. This would be consistent with the lack of significant changes in the D2 mRNA level at 2 weeks of perchlorate exposure.

Prolonged perchlorate exposure (7.5 weeks) depleted thyroid hormone stores in the thyroid glands in addition to decreasing circulating thyroid hormones in treated birds, thus thyroid hormone supply to target organs in these birds was slightly lower than those treated for 2 weeks. Moreover, changes D2 mRNA levels that were not visible in the brain at 2 weeks may become noticeable at 7.5 weeks. Therefore, perchlorate-exposed birds at 7.5 weeks were expected to have higher D2 mRNA levels in the brain than the control birds as a response to sustained decreased thyroid hormone availability. However, D2 mRNA remained unaffected by the longer period of treatment. It is still possible that D2 activity could have been altered in these birds due to the complexity in D2 regulation. Besides the thyroid hormones, several other regulatory mechanisms also exist that can affect D2 synthesis at post-transcriptional level (Gereben *et al.*, 2002) and not all of these mechanisms involve changes in mRNA levels (e.g. alternative splicing of mRNA which leads to the translation of an inactive enzyme). Therefore, northern blot may not detect the effects of all these mechanisms. Moreover, D2 may not be affected by hypothyroidism to the same degree in all brain areas. Verhoelst *et al.* (2004) showed that D2 was actively expressed in epithelial cells of the choroid plexus in the chicken embryo brain. This led to a new hypothesis of thyroid hormone-uptake and regulation in the avian brain. In mammals, T_4 is believed to be transported through the blood-brain-barrier and is then transformed into T_3 by D2 in neural cells such as astrocytes (Bianco *et al.*, 2002; Southwell *et al.*, 1993). In birds, it is likely that T_4 is taken up by the choroid plexus and is deiodinated within the choroid plexus to generate T_3 . T_3 then binds to transthyretin and enters the brain (Verhoelst *et al.*, 2004). According to this new hypothesis, T_3 concentration in the avian brain is regulated solely through the deiodination by the choroid plexus instead of through D2 activity in other brain areas. Another study by Gereben *et al.* (2004) demonstrated D2 mRNA in chicken brain underwent ontogenic redistribution. D2 mRNA was detected in elongated cell clusters throughout the brain in chick embryo but the intensity of the signal decreased in adult chicken. Also in adult chicken, D2 mRNA emerged in some regions of the wall of the third ventricle (Gereben *et al.*, 2004). In the present study, when the quail were sacrificed at 7.5 weeks, they are already young adults, thus D2 mRNA distribution is likely similar to that in adult chicken. D2 mRNA level was measured in total RNA extracted from whole quail brains. Although there might be changes in the mRNA level in areas like the choroid plexus, those changes might have been masked by the D2 mRNA from other brain areas and remained undetected.

D2 mRNA also was detected in quail liver in this study and its level was increased at 2 weeks but not at 7.5 weeks of perchlorate exposure in comparison to controls. In humans and rats, D2 is predominantly expressed in the CNS and brown adipose tissue (BAT) but is absent in the liver (Bates *et al.*, 1999; Bianco *et al.*, 2002). In avian species, however, the presence of hepatic D2 was confirmed by both detection of PTU insensitive 5'-deiodinase activity in Japanese quail liver (Hughes and McNabb, 1986; McNabb *et al.*, 1986) and hepatic D2 mRNA in adult chickens (Gereben *et al.*, 1999). In adult chickens, the level of D2 mRNA in liver is comparable to that in brain but its role in the liver is as yet unclear (Gereben *et al.*, 1999). Liver is one of the tissues responsible for the production of a major fraction of circulating T_3 from T_4 and historically D1 was believed to be the sole source of T_3 from peripheral deiodination in mammals (Chopra, 1991). Nonetheless, some studies in rats indicate that D2 in BAT and perhaps in other tissues may account for as much as 50% of the T_3 production for release to the circulation. The ratio may be even higher in hypothyroid rats where D1 activity is reduced by low circulating T_4 (Nguyen *et al.*, 1998; Silva and Larsen, 1985). Therefore, the hepatic D2 in the liver in

birds also may contribute to circulating T₃ and its activity may be elevated in the liver of hypothyroid birds.

Hypothyroidism increased D2 mRNA level in the liver of Japanese quail chicks after 2 weeks of perchlorate exposure. Unlike the brain, for which hormone entry and exit are regulated, liver is not protected against hypothyroid conditions (i.e. it is exposed to circulating thyroid hormone concentrations). Decreased plasma thyroid hormones may affect the expression of D2 and other thyroid responsive genes in the liver. The elevated D2 mRNA level in the livers of perchlorate-exposed birds indicates the liver initially responded to hypothyroidism by increasing T₃ production by the liver D2 presumably for release into the circulation. In contrast to 2 weeks, at 7.5 weeks, although the perchlorate-exposed birds remained hypothyroid, their liver D2 mRNA level was not significantly different from that of the control group.

The liver also differs from the brain in that D1, instead of D2, is the predominant 5' deiodinase. D2 activity in Japanese quail liver has been estimated to account for less than 10% of the total 5'-monodeiodinase activity throughout their post-hatching development (Hughes and McNabb, 1986). Moreover, there is evidence from other studies showing D2 expression changes during the course of development. In rats, D2/D1 activity ratio decreases substantially from neonatal period to adult in such tissues as thyroid gland and pituitary (Bates *et al.*, 1999). Hence, developmentally, D2 function and regulation may be different in quail chicks from adults. Furthermore, Gereben *et al* (2002) discovered that D2 might be regulated differently in the liver from the brain in adult chicken. While D2 mRNA levels were comparable in both tissues, D2 activity is much lower in the liver than in the brain, likely a result of alternative splicing of D2 mRNA in the liver. Birds sacrificed at 7.5 weeks of treatment were already young adults. Therefore, D2 mRNA level could have been less sensitive to hypothyroidism by 7.5 weeks of treatment and changes in them may not have been detectable by northern blot.

The mRNA level of RC3, a thyroid-responsive gene in the brain, was not affected by perchlorate-induced hypothyroidism in the brain at either 2 or 7.5 weeks of perchlorate exposure. Because the brain D2 mRNA level also was not affected in the perchlorate-exposed group, this argues that T₃ concentrations in the brain did not change appreciably. Many of the previous studies on RC3 were done by in-situ hybridization of RC3 mRNA in brain tissues and have found that RC3 mRNA level does not change the same way in all brain areas (Ishitobi *et al.*, 2007; Lein *et al.*, 2007; Piosik *et al.*, 1996; Piosik *et al.*, 1995; Wilcoxon *et al.*, 2007; Zoeller *et al.*, 2000). My study used northern blot on total brain RNA, which does not provide information on the spatial distribution or regional changes of the mRNA within the tissue studied.

In the liver, hypothyroidism lowered the SP14 mRNA level at 2 weeks of perchlorate exposure. SP14 codes for a protein that regulates enzymes in the lipogenic pathway in the liver and its mRNA level is rapidly up-regulated by the thyroid hormones (Jump *et al.*, 1984; Narayan *et al.*, 1984). This result indicates some liver functions may have been affected by perchlorate-induced hypothyroidism. At 7.5 weeks, no significant difference was seen between the perchlorate-exposed and control groups. With increasing maturity, SP14 mRNA levels were increased considerably at 7.5 weeks compared to those at 2 weeks in both control and treated birds. SP14 is known to be responsive to factors other than plasma thyroid hormone concentrations (e.g., circulating insulin concentrations as well as carbohydrate content of the diet, (Jump *et al.*, 2001; LaFave *et al.*, 2006). At 7.5 weeks of treatment, quail are entering breeding age. We observed plasma lipid concentration increased markedly, indicating that lipogenesis was highly active at the time. In such conditions SP14 expression and lipogenesis may be regulated by multiple mechanisms and the influence of thyroid hormones may not be as significant as at 2 week of perchlorate

exposure.

In summary, perchlorate exposure causes hypothyroidism in young Japanese quail and thyroid function indicators suggest the degree of hypothyroidism increased as the time of exposure lengthened. The effects of perchlorate exposure on the expression of thyroid-responsive genes were more complicated. Genes in different tissues responded differently and developmental changes also may alter the way genes respond to hypothyroidism. Modifications of the methods or a wider selection of thyroid-responsive genes may yield more information on this subject.

The study suggests that wild birds in perchlorate contaminated areas may develop hypothyroidism from chronic perchlorate exposure during their post-hatch life. The effects of hypothyroidism on these may differ with age. Nonetheless, hypothyroidism reduces the overall fitness of both young and adult birds thereby making them vulnerable to adverse conditions in wild habitat. Therefore, perchlorate contamination may potentially affect the wild avian population in contaminated areas.

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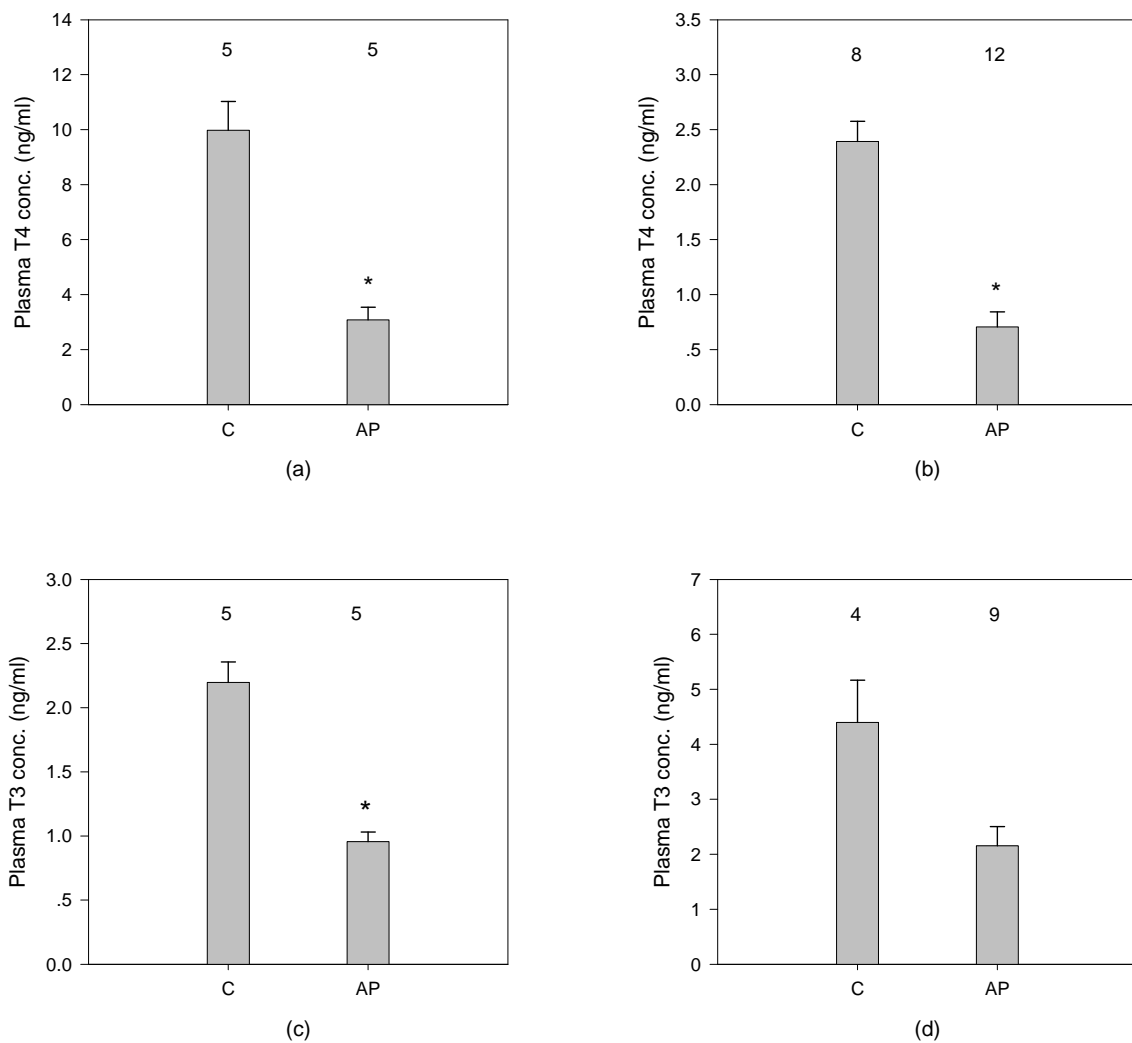


Fig. 5.1 Plasma T₄ concentrations in perchlorate-exposed young Japanese quail: after 2 weeks (a) and 6 weeks (b) of ammonium perchlorate (AP) treatment and plasma T₃ concentrations after 2 weeks (c) and 7.5 weeks (d) of ammonium perchlorate (AP) treatment. AP was administered, beginning at 4-5 days of age, at 2000mg AP/l in drinking water. Values are means ±SE. Asterisks indicate significant differences at p<0.05 between control (C) and perchlorate-exposed groups. N values are above each bar.

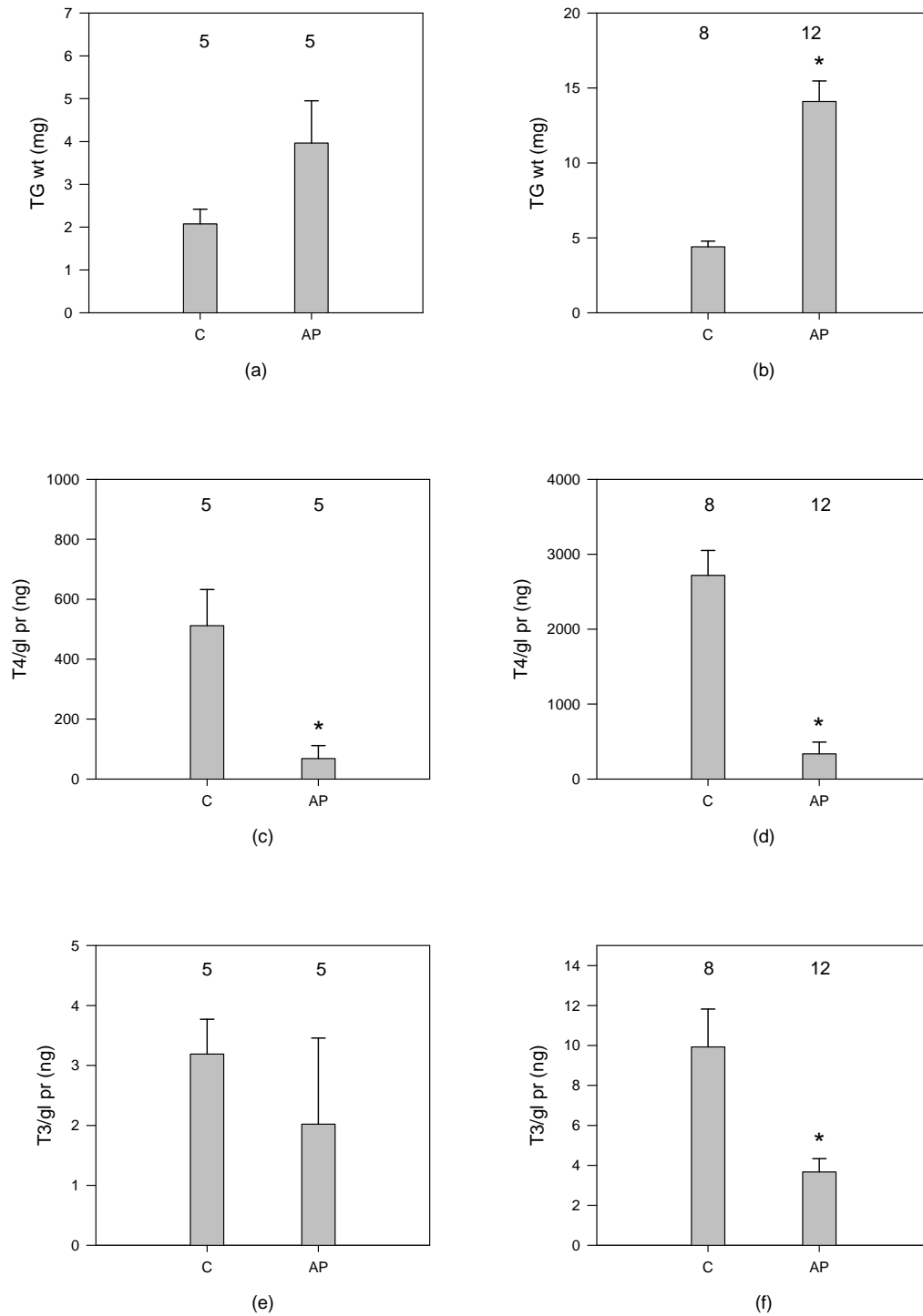
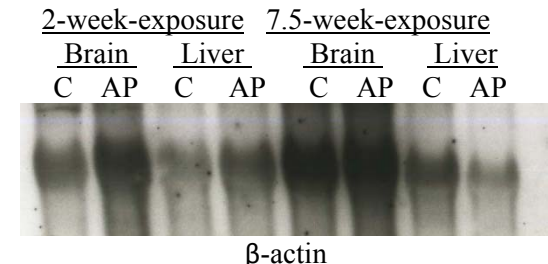
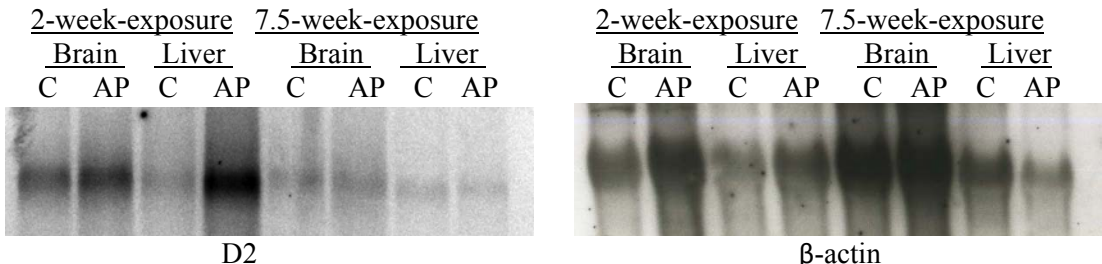
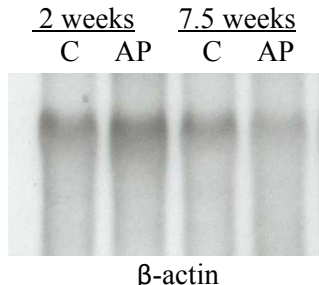
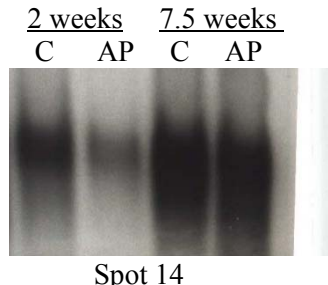


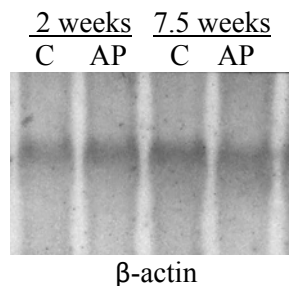
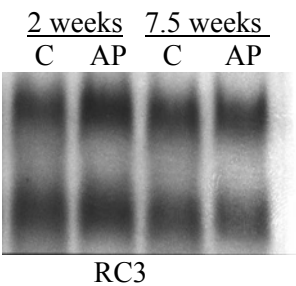
Fig. 5.2 Thyroid gland weight and thyroidal thyroid hormone storage of perchlorate-exposed young Japanese quails: thyroid gland weight after 2 weeks (a) and 7.5 weeks (b) of perchlorate exposure; thyroidal T₄ storage after 2 weeks (c) and 7.5 weeks (d) of perchlorate exposure; thyroidal T₃ storage after 2 weeks and 7.5 weeks of perchlorate exposure. AP was administered, beginning at 4-5 days of age, at 2000mg AP/l in drinking water. Values are means ± SE. Asterisks indicate significant differences at p<0.05 between control (C) and perchlorate-exposed groups. N values are above each bar.



(a)



(b)



(c)

Fig. 5.3 Effect of ammonium perchlorate exposure on the mRNA level of thyroid-responsive genes: D2 (a), RC3 (b) and Spot 14 (c). All RNA samples also were probed with Actin to test the quality and consistency of the RNA.

Chapter 6

Conclusions

The present study demonstrates that maternal perchlorate exposure in Japanese quail hens led to hypothyroidism, assessed by measuring several thyroid functions in embryos from their eggs. Embryonic hypothyroidism is likely to be the main cause of the decreased growth and delayed hatching of the embryos from eggs of perchlorate-exposed hens. This hypothyroidism presumably resulted from the exposure to maternal perchlorate deposition in eggs as has been shown in other studies (Gentles *et al.*, 2005). Hens in both perchlorate-exposed groups developed hypothyroidism and showed decreased plasma T₄ concentrations. This decreased plasma T₄ in hens may have led to decreased maternal T₄ deposition in the eggs as has been previously observed (Wilson and McNabb 1997). Egg T₄ of maternal origin was demonstrated to affect growth and differentiation of various tissues during early embryonic development in birds (Flamant and Samarut 1998; Maruyama, *et al.* 1995; Wilson and McNabb 1997). Therefore, this maternal hypothyroidism may have partly contributed to decreased body growth in the embryos. Moreover, perchlorate may interfere iodide transport in extra thyroidal tissues wherever the Na/I symporter is expressed. If iodide is deposited into ovaries and eggs by the same mechanism as it is transported into the thyroid gland, maternal perchlorate exposure may decrease iodide content in the eggs as well. As demonstrated in a previous study in our laboratory, decreased iodide content in Japanese eggs can affect embryonic thyroid function (McNabb, *et al.* 1985). Therefore, it is possible that the embryonic hypothyroidism observed in this study was partly a result of a potential decrease in the iodide content in the eggs of perchlorate-exposed hens.

Effects of maternal thyroid disruption on embryonic development have been studied primarily in mammals because of the active exchange of some materials between the mother and the fetus and because of evidence of fetal dependence on maternal thyroid hormone during pregnancy (Dowling and Zoeller 2000; Escobar, *et al.* 2004). In studies on avian model organisms, a more frequently used method is injecting the eggs with thyroid disrupting chemicals at early stages of incubation (Beck, *et al.* 2006; Roelens, *et al.* 2005). This method gives the researchers better control of the levels of exposure of the embryos. However, the procedure is invasive and may cause high mortality of the embryos in injected eggs. Moreover, it may not result in egg conditions that reflect multiple maternal influences on egg conditions. The approach we used in this study better simulates the real situation of thyroid disruption in avian embryos in the environment. Several studies have used eggs from euthyroid female birds and demonstrated that thyroid disruptors injected into these eggs affected embryonic development of specific thyroid responsive tissues. However, the effects on overall body growth observed in our study were not found in those egg injection studies. This suggests that embryonic growth is related to both embryonic and maternal thyroid status. Additional understanding of the relative roles of egg perchlorate, maternal hormones and egg iodide content would be possible in a study that measured egg perchlorate, maternal thyroid hormones and iodide concentration in eggs from exposed hens to obtain more precise conditions of the environment in which the quail embryos develop.

Japanese quail chicks exposed to perchlorate after hatching also developed hypothyroidism. Signs of hypothyroidism were observed after two weeks of perchlorate exposure. Longer exposure to perchlorate (7.5 weeks) increased the degree of hypothyroidism. In contrast to the results from the embryos, post-hatch perchlorate exposure did not affect the overall growth of the birds. Therefore, it is likely that the bodyweight gain after hatch in Japanese quail is more dependent on factors other than thyroid hormones (e.g. growth hormone, diet, etc.) than is the case for embryos. Nonetheless, we did

observe delayed development of gonads and adult plumage color in two of the males in the perchlorate-exposed group. This suggests that the hypothyroidism in these males may have affected other endocrine functions such as the gonadal hormone system. Thus, post-hatch perchlorate exposure may have an effect on the reproductive success of the birds through interference with male reproductive system development and reduced fitness of the offspring from perchlorate exposed females. Such effects may result in reduced health and size of wild bird populations in perchlorate contaminated areas.

It is worthwhile to point out that each of the thyroid variables used in the current study (plasma thyroid hormone concentrations, thyroid gland weight and thyroidal hormone contents) to assess hypothyroidism is a component of an integrated system that regulates the overall thyroid functions. Changes in plasma thyroid hormone concentrations trigger the feedback system in the hypothalamic-pituitary-thyroid axis. The feedback system regulates the release of stored hormones in the gland and thyroid gland tissue growth which in turn determine the plasma thyroid hormone concentrations. Each variable is constantly adjusting to or being restricted by the changes in the other two. Therefore, individual variables cannot be used independently to assess thyroid status. Rather, the thyroid status of an organism is reflected by the combination of the conditions of all variables. In the current study, data of thyroid variables were analyzed using MANOVA to include all variables as dependent factors for consideration when assessing the relative thyroid conditions of perchlorate exposed birds comparing to controls.

Increased D2 mRNA levels were observed in the liver of embryos from eggs of perchlorate-exposed hens and of chicks after two weeks of post hatch perchlorate exposure, indicating that D2 is involved in some compensatory regulation of thyroid hormone concentrations in developing quail under hypothyroid conditions. Studies in rats demonstrated that D2, primarily expressed in such tissues as central nervous system and brown fat in mammals, accounted for a significant portion of circulating T_3 production (Nguyen, *et al.* 1998), and the ratio of T_3 production by D2 vs. D1 increase during hypothyroidism (Silva and Larsen 1985). The increase in hepatic D2 mRNA level in embryos from eggs of perchlorate-exposed hens as well as in chicks after short term perchlorate exposure suggests a similar contribution of circulating T_3 by D2 in birds at least at young ages. This is the first finding suggesting that hepatic D2 in birds may have compensatory effects on circulating thyroid hormones in hypothyroid conditions. The lack of a similar response in D2 mRNA level in the young adult birds after 7.5 weeks of perchlorate exposure indicates that this contribution may be less significant in adult birds. The higher sensitivity of hepatic D2 in developing birds could be a mechanism with adaptive value that reduces the impact of hypothyroidism during development.

Spot 14 mRNA level was increased in quail chicks after two weeks of perchlorate exposure, indicating perchlorate-induced hypothyroidism may have affected thyroid regulated functions in the liver. The response of Spot 14 to hypothyroidism was not observed in the liver of the chicks after 7.5 weeks of perchlorate exposure. When sacrificed at 7.5 weeks, the birds were close to entering breeding condition and the blood lipid content increased significantly, indicating high lipogenesis activity in these birds. The many physiological changes in these birds such as the maturation of the reproductive system may have caused the regulation of Spot 14 to be more dependent on factors other than thyroid hormones at this age.

The mRNA levels of D2 and RC3 in the brain were not affected by perchlorate exposure in either the embryo or chick experiments. It seems likely that the protective mechanisms controlling thyroid hormone entry into the brain and thyroid hormone loss out of the brain prevented the development of hypothyroid conditions in the brain in this study. Alternatively, changes in the expression of thyroid-responsive genes in the brain may have been below the detection of the techniques used in this study.

Moreover, earlier studies indicated there was a significant increase in D2 expression in late embryonic stages in chicken and this increase may not be regulated by thyroid hormones (Beck *et al.* 2006; Reyns, *et al.* 2003). Therefore, changes in embryonic thyroid status may not have a significant impact on the D2 mRNA level in Japanese quail embryonic brain. Because the regulation of gene expression may not be restricted at the mRNA level, future studies should include measurements of D2 and RC3 protein levels as well as deiodinase activity assay to gain detailed understanding of effect of thyroid disruption on gene expressions in the Japanese quail brain.

In conclusion, perchlorate exposure, either indirect (via hens) or direct (chicks), disrupt thyroid function of developing Japanese quail. Expression of thyroid-responsive genes in the liver was affected in embryos and young chicks by perchlorate-induced hypothyroidism. The expression of thyroid-responsive genes in the developing quail brain was not affected by perchlorate exposure in the present study.

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Appendix

Experimental procedures:

Exposure of animals to ammonium perchlorate

All animal procedures were approved by the Virginia Tech Animal Care Committee in accordance with federal guidelines (IACUC). Ammonium perchlorate solutions (2000 mg/l and 4000 mg/l) were administered to Japanese quail through drinking water. Solutions in water bottles used to provide drinking water to the birds were discarded every two days. The bottles were then cleaned to eliminate food and bird waste contamination in them and were filled with fresh solutions before returning to the cage.

Sacrifice of the birds and tissue collection

Adult and young Japanese quail were sacrificed by decapitation. Trunk blood was collected in heparinized Micro-Hematocrit capillary tubes (Fisher Scientific, Fair Lawn NJ). For each bird, four to six tubes of blood were collected. The tubes were then sealed at one end using Hemato-seal tube sealing compound (Fisher Scientific, Fair Lawn NJ).

Body weight was measured after the birds stopped bleeding and moving. Immediately after weighing, brain and liver tissues were dissected. Brains were dissected by opening the skull from the back of the head with a pair of sharp scissors. When the skull was removed, the whole brain was taken out using a pair of tweezers and transferred into a 15 ml centrifuge tube. The tube was placed in liquid nitrogen to flash freeze the tissue before being stored at -80°C . For the liver, only the top right lobe was collected, which was sufficient for RNA isolation. The liver also was transferred into a 15 ml centrifuge tube, flash frozen in liquid nitrogen and stored at -80°C .

After brain and liver tissues were collected, the thyroid glands were dissected. Each gland was placed on a small piece of aluminum foil and weighed to the nearest 0.01 mg. The glands were transferred into a snap-cap tube and stored at -20°C .

For embryonic tissue collection, eggs were opened at the sharper end. Embryonic blood was collected from the chorioallantoic artery into heparinized capillary tubes. One or two tubes of blood were usually collected from each embryo. The tubes were sealed at one end with tube sealing compound. After blood collection, the embryos were sacrificed by decapitation and weight to the nearest 0.01 g.

Embryonic brains and livers were dissected immediately after embryos were weighed. The skin of the heads was cut with a pair of small scissors. The skulls of the embryos were soft and could be removed with a pair of tweezers. The brain was taken out with the tweezers and transferred into snap-cap tubes. The tubes were then flash frozen in liquid nitrogen and stored at -80°C .

Embryonic thyroid glands were dissected under a dissecting microscope. The glands were placed on a piece of aluminum foil and weighed to the nearest 0.01 mg before being transferred into snap-cap tubes. The glands were stored at -20°C before analysis.

Plasma isolation, thyroid hormone extraction and RIA

All blood tubes were centrifuged to separate the plasma from blood cells. After centrifugation, the blood in the tubes was separated into two layers, the upper light yellow plasma layer and the dark red bottom layer containing the blood cells. The tubes were cut with a file at the interface of the two layers and the plasma was blown into a snap-cap tube. The plasma was then stored at -20°C before further

analysis.

The plasma of adult quail contains high lipid content that interferes with the antibody binding during RIA. The thyroid hormones in the plasma of these birds were extracted by mixing equal volumes of plasma with 100% ethanol. The mixture was centrifuged at 12,000×g to remove insoluble material. The relative hormone concentrations in the supernatant after centrifugation were measured to estimate plasma hormone concentration in adult quail.

Thyroid hormones in the thyroid glands were extracted by Pronase digestion of the gland tissue followed by ethanol extraction of hormones from the digestion mix. Each embryonic thyroid gland pair (10mg or less) was digested in 350 µl of digestion medium containing 25 mg of Pronase (Sigma-Aldrich, St. Louis, MO,) at 37°C overnight. Thyroid glands from hens were each digested separately due to their larger size. After stopping the digestion by mixing the medium with 1.0ml ice cold absolute ethanol, the tubes were stored at -20°C for 24 h. The tubes were then centrifuged at 13,500×g for five minutes and the supernatant, containing the extracted thyroid hormones, was collected and stored at -20°C until analysis.

Plasma thyroid hormone concentrations were measured using a double antibody RIA on duplicate aliquots for each hormone (12.5µl for T₄ and 25µl for T₃) following the method described by Wilson and McNabb (1997). Primary antibodies were purchased from Fitzgerald (Fitzgerald Industries International, Inc, Concord, MA,). ¹²⁵I-labeled hormones (high specific activity; 1200 µCi/µg) were purchased from Perkin-Elmer Life Sci (Boston, MA,). The standard curve was constructed using a series of standards prepared in charcoal-stripped chicken plasma with known hormone concentrations. Three replicates of the standard for each hormone concentration were measured for better accuracy. Three levels of Randox Immunoassay Control serum (Randox Laboratories, San Diego, CA,) were included in each assay to evaluate assay performance. For ethanol extracted hormone samples, duplicate aliquots of 25 µl solutions were used and the standards were prepared in 75% ethanol instead of charcoal-stripped chicken plasma.

Total RNA isolation

To minimize RNA degradation, all equipment was pretreated to eliminate RNase. All glassware used in RNA isolation was baked at least three hours at 200°C. All plastic containers and centrifuge tubes were autoclaved and rinsed RNaseZap solution (Ambion, Inc., Austin TX) before use. Bench surface, tube racks were sprayed with RNaseZap solution and wiped dry with a KimWipe (Kimberly-Clark, Irving TX). All pipet tips used were filter tips and were autoclaved and sterilized by UV irradiation before use. Total RNA was isolated from whole brain of day 14 Japanese quail embryo as well as brain and liver of adult quail using Tri-Reagent (Sigma-Aldrich, St. Louis, MO,) following the protocol provided by the manufacturer. For each 50 mg of tissue, 1 ml of the Tri-Reagent was added. Tissues were homogenized in Tri-Reagent with a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Inc., Westbury, NY,). After homogenization, insoluble material in the homogenate was eliminated by centrifuging at 12,000×g for 10 min at 4°C. The supernatant was mixed with chloroform (0.2 ml for each milliliter of Tri-Reagent) and centrifuged at 12,000×g at 4°C for 15 min for phase separation. The top aqueous phase containing RNA was transferred to a new tube and mixed with isopropanol (0.5 ml for each milliliter of Tri-Reagent) to precipitate the RNA. RNA pellets were washed in 75% ethanol, dried and resuspended in RNase-free water. The RNA samples were then stored at -80 °C for further analysis.

Construction of Japanese quail brain and liver cDNA libraries

The cDNA libraries were constructed by reverse transcription of total RNA isolated from brain and liver. For each reaction, 2 µg of total RNA from each tissue was added into 25 µl reaction mixture

containing 0.5 mM dNTP mix, 25 units of ribonuclease inhibitor, 5 µl reaction buffer, 200 units of M-MLV reverse transcriptase (Fisher Scientific, Fair Lawn NJ) and 0.5 µg Oligo(dT)₁₅ primer (Promega, Madison WI) and nuclease free water. All reagents were treated with 0.1 DEPC water then autoclaved to be RNase free. Snap cap tubes were autoclaved and rinsed with RNaseZap before use. The reaction mixtures were incubated at 37°C for 60 min. The reaction was stopped by freezing reaction mixtures at -20 °C.

Polymerase chain reaction (PCR)

After searching in the NCBI nucleotide database, the mRNA sequence of β-actin was found for quail. The mRNA sequences D2, Spot 14, NGF and 18S rRNA were found for chicken. The mRNA sequence of RC 3 was found in human, rats and canary. After searching in the chicken EST database, a chicken brain EST was found to share high similarity with the canary RC3 mRNA sequence. The names of the sequences and their GenBank accession numbers are listed in Table 3.1. Primers for PCR were designed based on the chicken sequences. Primers for quail RC3 cDNA were designed based on both canary sequence and the matched chicken EST sequences. The regions where the primers were chosen were highly homologous between the chicken and canary sequences with no more than two mismatches within each primer. The bases in the chicken sequence were used in the mismatched positions. Choices of primer sites were restricted within the more conserved protein coding regions of the mRNA sequences. Two pairs of primers were designed for RC3 and D2 and three pairs of primers were designed for NGF to increase the chances of successful amplification. All primers were purchased from Operon Biotechnologies, Inc. (Huntsville AB). The PCR reactions were carried out in 50 µl reaction mixtures that each contains 5 µl 10x reaction buffer, 200 µM dNTP mix, 2 units of Taq DNA polymerase (Fisher Scientific, Fair Lawn NJ), 10 pmol of each of the forward and reverse primers and 1 µl of cDNA. The PCR reactions were performed as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95 °C, 45 seconds of annealing and 30 seconds of extension at 72°C. Once the cycles were completed, another 3 min of extension at 72 °C was added. The reaction mixture was then frozen at -20 °C until analysis. Sequences of the primers and the annealing temperatures for each gene are listed in Table 3.2.

Cloning of PCR products

The PCR products were cloned using TOPO TA cloning kit (Invitrogen Corporation, Carlsbad CA) following manufacturer's instructions. 4 µl of fresh PCR product (less than 24 hr after the PCR reaction finished) was mixed with 1 µl salt solution and 1 µl TOPO pCR2.1 vector. The mixture was incubated at room temperature for 5 min and was then placed on ice. 2 µl of the mixture was added into a vial of One Shot Chemically Competent *E. coli* and was gently mixed. The cells were incubated on ice for 5 min and were then heat-shocked for 30 seconds at 42 °C. After heat-shock, 250 µl S.O.C. medium (pre-warmed to room temperature) was added to the cells. The tube was then incubated at 37 °C in a shaker (200 rpm) for 1 hr. 50 µl-100 µl of the transformed cells were spread on a prewarmed (37 °C) selective plate containing ampicillin. The plates were incubated at 37 °C overnight. 2-3 colonies from each plate was selected from the plate and cultured in 10 ml LB medium containing 50 µg/ml ampicillin overnight.

Cultured cells were collected by centrifugation and plasmid was isolated by Qiagen Miniprep kit (Qiagen, Valencia CA). Cells were resuspended in 250 µl buffer P1 in snap cap tubes and mixed with 250 µl buffer P2. Immediately after mixing with buffer P2, 350 µl Buffer N3 was added and mixed thoroughly by inverting the tubes several times. The tubes were centrifuged at 17,900×g for 10 min to

precipitate insoluble materials. Supernatant was transferred to QIAprep spin columns and centrifuged for 1 min. The columns were washed with 0.5 ml buffer PB and 0.75 ml Buffer PE. The plasmid was eluted with 50 µl deionized water.

The isolated plasmid was analyzed by gel electrophoresis. 5 µl of plasmid sample were premixed with 6× gel loading buffer and loaded on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide. 1 µl of TOPO pCR2.1 vector was loaded on a separate lane as reference. The gel was run in 1×TBE buffer for 40 min. Plasmid bands were visualized by UV irradiation.

DNA sequencing

Plasmids containing inserted PCR products were sequenced by the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg VA). Cleaned PCR products also were sequenced. Both forward and reverse strands of the cDNAs were sequenced to assure the accuracy of the results. The cDNA sequences were compared to mRNA sequences of the corresponding genes in other species using the program Quickalign to confirm their identity.

Northern blotting

Total RNA (20 µg from each sample) was loaded on a 1% denaturing agarose gel containing formaldehyde. The gel was run in 1x MOPS buffer at 65 V for half an hour then 100 V for three more hours. After electrophoresis, the RNA was transferred to a nylon membrane and cross-linked by UV irradiation. ³²P-labelled probes were prepared using Random Primed DNA labeling kit (Roche Applied Science, Indianapolis IN) and ³²P-labeled dCTP (Amersham Life Sci, Pittsburg, PA.). Membranes were hybridized with TCA-perceptible cpm/ml denatured probes in QuickHyb solution (Stratagene, La Jolla CA) and washed following manufacturer's instruction. For each 10 ml of QuickHyb, 100 µl of sheared salmon sperm DNA (10 mg/ml) was mixed with 20 x 10⁶ cpm of probe and heated to 100°C for 5 min. Membranes were first incubated in QuickHyb solution at 65 °C for 30 min. Denatured DNA and probes were then added to QuickHyb and continued incubation at 65 °C for 3 hrs. After hybridization, the membranes were washed twice in 2 x SSC, 0.1% SDS at 65 °C for 15 min and once in 0.2 x SSC, 0.1% SDS at 65°C for 30 min. The membrane was then air dried and exposed to KODAK X-ray films (usually 24-48 hrs). Exposure time for each membrane was determined individually depending on the strength of the signal. Some blots had high levels of background noise. Those experiments were repeated and the membrane was washed once in 2 x SSC, 0.1 SDS at 65°C for 15 min and twice in 0.2 x SSC, 0.1% SDS at 65 °C for 30 min to reduce background noise.

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