Effects of induced hypothyroidism on the glucocorticoid stress response in Japanese quail (*Coturnix japonica*)

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Thesis

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Abstract

Many aspects of biological function are affected by hormones, from physiology to behavior, and the synthesis and release of hormones in vertebrates are regulated by the endocrine axes of control. A growing body of research shows that the mechanisms underlying the endocrine axes of control are complex and interconnected, with many hormones having multiple effects, and with many interactions between axes. In this study, I examined the effects of decreased thyroid function on the glucocorticoid stress response in Japanese quail, a potential interaction between the hypothalamic-pituitary thyroid (HPT) and hypothalamic-pituitary adrenal (HPA) axes of control. I used the thyroid inhibitor ammonium perchlorate (AP) for 2 weeks and 5 weeks to induce two states of decreased thyroid function: a thyroid challenged state, in which birds have depleted thyroidal T_4 content, but still maintain euthyroid (normal) concentrations of plasma T_4, and a hypothyroid state, in which birds have depleted thyroidal T_4 content and decreased concentrations of plasma T_4. Thyroid function was assessed by measuring plasma T_4 concentrations, thyroidal T_4 content, and thyroid gland mass. I took blood samples from birds both immediately prior to and

immediately following a 30 minute confinement and agitation stressor to evaluate the effects of decreases in thyroid function on basal and stress-induced plasma corticosterone and plasma T_4 concentrations. I found two key results: First, although baseline levels of plasma corticosterone were unchanged, the corticosterone stress response was significantly blunted in both the thyroid challenged and hypothyroid birds as compared to controls. This finding suggests that the HPT and HPA axes are functionally connected in birds, and other evidence suggests this connection is likely at the pituitary or hypothalamic level. Second, in hypothyroid birds, plasma T_4 concentrations were elevated (into the euthyroid range) in response to the experimental stressor, although no change in plasma T_4 was observed in thyroid challenged or control birds. This finding suggests that plasma T_4 may have a permissive role in mounting a stress response.

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CONTENTS

CHAPTER 1

Literature Review

Introduction

Endocrine control axes are often viewed individually, as separate pathways of communication, but interactions between different endocrine axes appear to play a significant role in normal endocrine function. Some interactions between axes have been well studied. For example, stress, involving hypothalamic-pituitary-adrenal (HPA) axis activation, affects reproductive function, mediated by the hypothalamic-pituitary gonadal (HPG) axis (Tillbrook et al., 2000, review; Moore and Jessop, 2003). Stress and HPA axis activation also affects thyroid function in amphibians (Denver, 1997) and in fetal mammals (Crespi and Denver, 2005). However, to my knowledge, the effects of altered thyroid status on HPA axis function (and the glucocorticoid stress response in particular) have only been studied in mammals (humans: Tsigos et al., 2002; rodents: Nolan et al., 2000) and have only focused on overt hypothyroidism.

Research in wild bird populations exposed to thyroid disrupting chemical contaminants has shown that birds can have decreased thyroid function as indicated by decreased thyroid gland stores of thyroid hormones (THs) and increased thyroid gland mass, even if they are not overtly hypothyroid. In Great Lakes herring gulls (*Larus argentatus*), embryonic and pre-fledgling birds exposed to environmental PCBs were found to have euthyroid (normal) levels of circulating THs, but significantly

depleted thyroidal TH stores (McNabb and Fox, 2003). Similarly, laboratory experiments with bobwhite quail (*Colinus virginianus*) chicks have shown that exposure to ammonium perchlorate (AP) at lower doses and shorter time intervals results in depleted thyroidal hormone stores, while plasma T_4 remains at euthyroid levels (McNabb et al., 2003). McNabb and Fox (2003) suggest that having depleted stores of thyroidal THs may be deleterious for birds facing environmental stressors, when there may be increased demand for thyroid hormone. In addition, states of intermediate decreased thyroid function may also affect the HPA axis and glucocorticoid release during environmental stressors, impacting the physiological and behavioral stress responses mediated by glucocorticoids.

Thyroid Function and the HPT axis

Thyroid function is controlled by the hypothalamic-pituitary thyroid (HPT) axis. The hypothalamus can stimulate thyroid function by releasing thyrotropin releasing hormone (TRH), and can suppress thyroid function by releasing somatostatin. Thyrotrophs in the anterior pituitary respond to TRH by increasing the release of thyrotropin (thyroid stimulating hormone; TSH) and they respond to somatostatin by suppressing the release of TSH. TSH is the most important controller of thyroid gland activity. When TSH is released it binds to receptors in the thyroid gland and stimulates iodine uptake and the synthesis and release of the two primary THs, thyroxine (T_4), and triiodothyronine, (T_3).

Although the thyroid gland synthesizes both THs, the production of T_4 is significantly greater than that of T_3 and circulating concentrations of T_4 also are significantly greater than circulating T_3 concentrations in most vertebrates (McNabb, 2000). Because T_3 has a higher affinity for TH receptors in target tissue it is generally assumed that most TH effects are stimulated via T_3 action, after T_4 has been converted to T_3 in peripheral tissues such as the liver and kidneys by deiodinase enzymes (see Hadley, 2000, for general review). THs serve two main functions in vertebrates: they play a critical role in the regulation of development, and they act as one of the primary regulators of metabolism in homeotherms (Hadley, 2000).

The thyroid gland synthesizes THs from the amino acid tyrosine and elemental iodine. The number and location of iodine atoms in the central thyronine (made from 2 tyrosines) defines the activity of each TH, with T_3 containing three iodine atoms and T_4 containing four. Because iodine is essential to the synthesis of TH, adequate dietary iodine intake is required for normal thyroid function. The normally functioning thyroid gland maintains significant extracellular 'stores' of THs. The capability of the thyroid to maintain stores of hormone is unique among the endocrine glands and this feature allows thyroid function to be sustained for some time even when an organism's ability to synthesize THs has been compromised (McNabb, 2000). It is generally believed that the adaptive function of thyroidal TH stores is to provide a hormone reserve for organisms experiencing

iodine deficiency, when synthesis of new THs has been compromised (Delange and Ermans, 1996).

Stress and the HPA axis

Stressors have been defined as stimuli which challenge homeostasis (McEwen and Wingfield, 2003). In response to stressors, organisms can activate a range of physiological systems; the two primary systems are the hypothalamic-pituitary adrenal (HPA) axis and the sympathoadrenal system. These systems are functionally integrated and act in concert to create what is often called the 'stress response.' Activation of the HPA axis in response to a stressor is characterized by a signal cascade which originates in the hypothalamus with the release of corticotropin-releasing factor (CRF). In the anterior pituitary, CRF stimulates the release of peptides derived from the pro-hormone pro-opiomelanocortin (POMC), including adrenocorticotropic hormone, (ACTH), which regulates the synthesis and release of glucocorticoids (such as cortisol in most mammals and corticosterone in birds) from the adrenal cortex.

When faced with a stressor, organisms respond with significant changes in physiology and behavior (McEwen and Wingfield, 2003). Glucocorticoids induce many of the key changes that characterize this stress response. Metabolically, elevated glucocorticoids work in concert with the hormones from the sympathoadrenal system (such as epinephrine, which increases glycogen to glucose conversion) to mobilize energy stores, so that an organism can take action to minimize or avoid the source

of stress ('fight or flight'). Glucocorticoids mobilize energy stores by increasing the metabolic breakdown of proteins into amino acids and lipids into free fatty acids. These amino acids and free fatty acids are used to maximize carbohydrate synthesis via gluconeogenesis, so that free glucose is available for immediate energy use (Servatius et al., 2000). Behaviorally, glucocorticoids act to inhibit energetically expensive activities that are not immediately critical to the survival of the organism, such as socialization, territoriality, and reproductive behaviors (McEwen and Wingfield, 2003).

Effects of hypothyroidism on the HPA axis

Research in rats and humans indicates that there are significant effects of hypothyroidism on the HPA axis in mammals. There is general agreement that, under hypothyroid conditions, basal circulating glucocorticoid concentrations remain either unchanged or are reduced (humans, Kamilaris et al., 1987; Tsigos et al., 2002; rats, Tohei et al., 1998; Murikami et al., 1984; Nolan et al., 2000; Shi et al., 1994). Evidence in rats also indicates that the stress or CRH-induced increases in circulating corticosterone and the diurnal corticosterone rhythms that are normally observed in euthyroid animals are attenuated or abolished in hypothyroid animals (Nolan et al., 2000, Murikami et al., 1984). These inhibitions in elevated corticosterone secretion occur no matter how hypothyroidism is induced: by thyroidectomy (Murakami et al., 1984), by thiouracil treatment

(which limits T_4 to T_3 conversion; Nolan et al., 2000; Tohei et al., 1998; Tohei 2004; Shi et al., 1994) or by iodine deficiency (iodine deficient diets; Nolan et al., 2000). Return of normal glucocorticoid secretion, both basal and elevated, occurred in all cases after TH replacement treatment, although full recovery of diurnal rhythms and stress activation responses did not occur immediately in all cases (Nolan et al., 2000, Murikami et al., 1984).

The effects of changes in thyroid function on the higher levels of the HPA axis are less understood. The majority of studies of both rats and humans have indicated that hypothyroidism causes an inhibition of the entire HPA axis. In humans, circulating CRH and ACTH are both decreased in athyreotic patients (Kamilaris et al., 1987) and in thyroidectomized patients (Tsigos et al., 2002; Fommei and Iervasi 2002) and levels of CRH and ACTH were partially or completely restored when T_4 and T_3 were administered. In rats, circulating concentrations of ACTH were observed to decrease to trace levels after thyroidectomy (Murikami et al., 1984). Also in rats, thiouracil treatment caused both CRH mRNA transcripts in the hypothalamus and POMC mRNA transcripts in the anterior pituitary to be decreased to the point that they were barely detectable (Shi et al., 1994). In both experiments, T_3 replacement led to a restoration of normal levels of the measured HPA axis hormones. In contrast to these observations of CRH and ACTH suppression, some studies in rats have found increases in CRF, arginine

vasopressin (AVP), and ACTH after thiouracil treatment (Tohei et al., 1998; Tohei, 2004). The explanation for these conflicting results is currently unknown.

Mechanistically, decreased thyroid function could affect the HPA axis through changes in circulating concentrations of THs, TSH, and/or TRH, and these could be acting at the hypothalamic, pituitary and/or adrenal level. Evidence for thyroid axis hormones (or lack thereof) acting directly upon the adrenal glands is contradictory. Adrenalectomized rats treated with thiouracil show marked reductions in CRH and POMC mRNA transcripts (Shi et al., 1994), which does not support an adrenal gland site of action. In contrast, research performed by Tohei et al. (1998) and Tohei (2004), where CRF and ACTH levels were increased in hypothyroid rats, does suggest adrenal suppression, i.e., an adrenal site of action. Interestingly, an *in vitro* study using mouse adrenal tissue showed that THs may have direct effects on the adrenal glands; increased T_4 and T_3 inhibited corticosterone secretion in rat adrenal cells (Lo et al., 1998). However, this result is inconsistent as an explanation for the either of the observed hypothyroid effects on adrenal activity. (In all studies of hypothyroid animals, the absence of THs leads to an observed decrease in glucocorticoid release. This would suggest that the normal presence of THs should stimulate, not inhibit, glucocorticoid release from the adrenals.)

At the pituitary level, experimental results also have been difficult to interpret. Corticotrophs (ACTH-releasing cells) in

the anterior pituitary have shown increased sensitivity to CRH (Tohei et al., 1998), no change in CRH sensitivity (Kamilaris et al., 1997, and Nolan et al., 2000) and decreased sensitivity to CRH (Shi et al., 1994, and Riedel et al., 2002) during hypothyroidism in rats. At this point it is unclear what the direct effects of hypothyroidism are on pituitary CRH sensitivity.

The best current evidence for a mechanism by which hypothyroidism effects the HPA axis is at the hypothalamic level. Researchers have long suspected that a hypothalamic neuroendocrine factor acting to inhibit corticotropin release may exist, i.e., a hypothetical corticotropin-release inhibiting factor (CRIF). Recent studies have shown that this CRIF exists (McGivern et al., 1997; Redei et al., 1995) and that it may also serve as a mechanistic connector between the HPA and HPT axis. Examination of the hypothalamic prepro-hormone that is modified to produce TRH (prepro-TRH) revealed that a section, prepro-TRH 178-199, inhibited ACTH release *in vitro* (McGivern et al., 1997). Intra-cerebroventricular administration of this protein in rats resulted in decreases in stress-induced ACTH and corticosterone levels, as well as behavioral changes consistent with a less 'stressed' animal (Redei et al., 1995). The injected rats increased novelty-seeking behavior in novel environments, i.e., they decreased their fear reactions and increased their exploratory behavior.

Mechanistically, it has been proposed (Nolan et al., 2000) that this prepro-TRH-derived CRIF may be acting to effect the observed changes in HPA axis hormone profiles that occur during hypothyroidism. When a hypothyroid animal increases its synthesis of TRH in response to decreasing negative feedback from circulating THs, the animal must first increase the synthesis of the TRH precursor protein, prepro-TRH. This increased synthesis of prepro-TRH serves to increase post-translational modifications that increase the production of TRH, as expected, but also leads to an increase in the production of CRIF (prepro-TRH 178-199), another post-translational product of the prepro-TRH protein. This increase in CRIF is likely to be a contributing factor in the inhibition of ACTH (and therefore glucocorticoid) release during hypothyroidism. It should be noted that this CRIF effect is unlikely to be the only mechanistic factor in play, as activational (stress-induced and diurnal) adrenal function does not immediately return to normal after TH replacement (Nolan et al., 2000; Murikami et al., 1984), even when TRH transcripts, previously elevated during hypothyroidism, have returned to normal (Shi et al., 1994).

The observed linking of the HPA and HPT axes, by the CRIF mechanism or otherwise, could be adaptive during stressful events. Mammalian literature indicates that during some stressful events, especially starvation (or other prolonged stressors that indirectly reduce food intake), circulating glucocorticoid levels increase while circulating thyroid hormone levels decline (Engler

et al., 1999). In response to starvation (or perhaps other chronic stressors), it may be adaptive for endotherms to decrease basal metabolic rate and energy demand by lowering circulating thyroid hormone levels, while still increasing glucocorticoid output to mediate processes to alleviate the stressor. This effect could be achieved in an efficient symmetric fashion by decreasing prepro-TRH synthesis (and therefore decreasing CRIF and TRH release.) This effect would work in conjunction with the direct blunting of HPT axis products by glucocorticoids that occurs during fasting (Engler et al., 1999).

Effects of stress and HPA axis activation on thyroid function

Another way in which the HPA and HPT axes are integrated is through thyroid responses to stressors. After a stressor and consequent HPA axis activation, changes in thyroid function (usually quantified by plasma TH levels) have been observed in a variety of experimental settings, but these changes vary by taxa and developmental stage.

During development in birds, amphibians, and mammals, (chicken embryos: De Groef et al., 2003; larval *Xenopus laevis:* Boorse and Denver, 2004; neonatal rats: Meaney et al., 2000), research has shown that increasing hormones from the HPA axis (ACTH, glucocorticoids) work in concert with increasing hormones from the HPT axis (TSH, T_3, T_4) to regulate and increase the rate of tissue differentiation, development, and growth, and that increases in these hormones are usually positively correlated (Kuhn et al., 1998). This concerted relationship between HPA

axis hormones and HPT axis hormones is believed to be adaptive especially during development. Developing vertebrates can respond to environmental stressors by activating their HPA axes and increasing their rate of development (Crespi and Denver, 2005) although trade-offs include decreased body mass and energy reserves (Denver, 1997). In adult and/or juvenile animals, these functional axis connections have been shown to continue beyond early development in some cases; stressors and/or the administration of CRF can also stimulate TH release in all non-mammalian classes of vertebrates (fish: Larsen et al., 1998; amphibians: Denver, 1988; reptiles: Denver et al., 1989; birds: Geris et al., 1996).

It is currently believed that the mechanism above, where TH release is stimulated by stressors, is mediated through the action of CRF. CRF appears to be the primary thyrotropin-releasing factor in amphibians where it is even more effective than TRH in stimulating TH release during metamorphosis , when glucocorticoid and TH levels are tightly linked and are both responsible for regulation of the overall metamorphic rate (Crespi and Denver, 2005, and Boorse and Denver, 2004). Because CRF appears to be thyrotrophic in all non-mammalian classes of vertebrates and in mammals during development, it has been suggested that this functional hypothalamic connection between the HPA and HPT axes is an ancient one. It has even been proposed that CRF may have originally evolved as a thyrotropin-releasing factor and later gained a secondary function as a corticotropin-

releasing factor during the evolutionary history of vertebrates (Crespi and Denver, 2005).

In contrast to these findings, the majority of studies of the interaction between adrenal and thyroid function in adult mammals suggest that thyroid activity is negatively correlated with adrenal activation (Servatius et al., 2000; Engler et al., 1999). Most of these studies show that stressors (or injections of glucocorticoids or CRF) decrease circulating THs. Experimental studies in rats (Servatius et al., 2000), and observational studies in humans (Benker et al., 1990, and Tsigos et al., 2002) support the idea that HPA axis activation decreases thyroid function in adult mammals. It is possible that adult mammals may be functionally different from other vertebrates in this respect.

Mechanistically, current thinking is that this type of inhibitory interaction also is regulated through CRF. However, it is believed that CRF does not inhibit TSH release directly, but instead increases somatostatin release. Research in humans (Riedel et al., 2002) and in rats (Smedh and Uvnas, 1994) indicates that CRF acts directly to stimulate somatostatin release from the hypothalamus, and that this increase in hypothalamic somatostatin results in an inhibition of the synthesis and release of both growth hormone (GH) and TSH from the pituitary.

In conclusion, research in rats indicates that overt hypothyroidism causes blunting of the glucocorticoid stress response. It is believed that this effect is caused, at least

partly, by a CRIF derived from prepro-TRH. Other effects of hypothyroidism at the pituitary and adrenal level appear to be less straightforward. A large body of work indicates that the opposite interaction, of stress effects on thyroid function, is also significant. Stress and HPA axis activation stimulate the HPT axis and thyroid function during development in vertebrates. This axes linkage appears to be maintained into adulthood in all vertebrate classes except mammals, who usually demonstrate the opposite effect, i.e., the suppression of thyroid function during stressors. Although adult mammals appear to be different from other adult vertebrates with respect to the effects of stress on thyroid function, I hypothesize that the blunted glucocorticoid stress responses that have been induced by hypothyroidism in rats will be observable in birds as well. I also hypothesize that this blunting effect will be mediated at the hypothalamic level, by prepro-TRH-derived CRIF.

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CHAPTER 2

Effects of induced hypothyroidism on the glucocorticoid stress response in Japanese quail (*Coturnix japonica*)

Abstract

Decreased thyroid function can have significant impacts on organismal health, but many of the effects of decreased thyroid function are not yet well understood, including potential interactions with other endocrine axes of control. The objective of this study was to investigate the effect of decreased thyroid function on the glucocorticoid stress response, a potential interaction between two endocrine axes of control, the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-adrenal (HPA) axis. I used the thyroid inhibitor ammonium perchlorate (AP) for 5 weeks and 2 weeks to induce two states of decreased thyroid function: a hypothyroid state, in which birds have depleted thyroidal T_4 content and decreased concentrations of plasma T_4, and a thyroid challenged state, in which birds have depleted thyroidal T_4 content, but still maintain euthyroid (normal) concentrations of plasma T_4. Thyroid function was assessed by measuring plasma T_4 concentrations, thyroidal T_4 content, and thyroid gland mass. I took blood samples from birds both immediately prior to and immediately following a 30 minute confinement and agitation stressor to evaluate the effects of decreases in thyroid function on basal and stress-induced plasma corticosterone and plasma T_4

concentrations. I found two key results: First, although baseline levels of plasma corticosterone were unchanged, the corticosterone stress response was significantly blunted in both the hypothyroid birds and the thyroid challenged birds, compared to controls. This finding suggests that the HPT and HPA axes are functionally connected in birds, and other evidence suggests this connection is likely at the pituitary or hypothalamic level. Second, in hypothyroid birds, plasma T_4 concentrations were increased (into the euthyroid range). This increase in plasma T_4 with stress was not observed in thyroid challenged or control birds. This finding suggests that plasma T_4 may have a permissive role in mounting a stress response.

Introduction

Changes in circulating hormones can have significant impacts on all aspects of organismal function, from physiology to behavior. In addition, changing the circulating levels of one hormone can induce further changes in the levels of other hormone(s), leading to even more secondary effects. This study addresses an effect of altered endocrine function that has not been studied in depth, namely, the effect of decreased thyroid function on the glucocorticoid stress response. Because both overt and intermediate states of decreased thyroid function are found in wild animal populations and in human beings in the clinical setting, the potential ecological and medical consequences of alterations to the glucocorticoid stress response during decreased thyroid function may be of significant concern.

Research into the effects of environmental thyroid disruptors, such as polychlorinated biphenyls (PCBs) and ammonium perchlorate (AP), has shown that birds can maintain euthyroid levels of circulating thyroid hormones when subjected to some exposure levels of thyroid disruptors. This response is possible because of the extra-cellular stores of thyroid hormones that are maintained by the thyroid glands (a feature unique among the endocrine glands). In Great Lakes herring gulls (*Larus argentatus*), embryonic and pre-fledgling birds exposed to environmental PCBs were found to have euthyroid levels of circulating T_4, but significantly depleted thyroidal T_4 stores (McNabb and Fox, 2003). Similarly, laboratory experiments with

bobwhite quail chicks (*Colinus virginianus*) have shown that exposure to AP at lower doses and shorter time intervals results in depleted thyroidal T_4, while plasma T_4 remains at euthyroid levels (McNabb et al., 2004). McNabb and Fox (2003) suggest that having depleted stores of thyroidal T_4 may be deleterious for birds facing environmental stressors, when there may be increased demand for thyroid hormone. In addition, intermediate states of decreased thyroid function may also affect the HPA axis and glucocorticoid release during environmental stressors, impacting the physiological and behavioral stress responses mediated by glucocorticoids.

Endocrine axes of control are often viewed individually as separate pathways of communication, but interactions between different endocrine axes appear to play a significant role in normal endocrine function. Some interactions between axes have been well studied. For example, stress, involving HPA axis activation, affects reproductive function, mediated by the hypothalamic-pituitary gonadal (HPG) axis (Tillbrook et al., 2000; Moore and Jessop, 2003). Stress and HPA axis activation also can affect thyroid function as documented in amphibians (Denver, 1997) and in fetal mammals (Crespi and Denver, 2005). However, to my knowledge, the effects of altered thyroid status on HPA axis function (and the glucocorticoid stress response in particular) have only been studied in mammals (humans: Tsigos et al., 2002; rodents: Nolan et al., 2000), and have only focused on overt hypothyroidism.

Research in mammals indicates that decreases in thyroid function can have profound impacts on HPA axis function and glucocorticoid release. Evidence indicates that stress or corticotropin-releasing hormone (CRH)-induced increases in plasma corticosterone are attenuated or abolished during hypothyroidism (Murikami et al., 1984, Nolan et al., 2000, Shi et al., 1994, Tohei et al., 1998). However, there is general agreement that, under hypothyroid conditions, basal plasma glucocorticoid concentrations remain either unchanged or are slightly reduced (humans: Kamilaris et al., 1987, Tsigos et al., 2002; rats: Murikami et al., 1984, Nolan et al., 2000, Shi et al., 1994, Tohei et al., 1998). Studies exploring the opposite interaction (effect of stress on thyroid function) have shown that adult mammals may be functionally different from other vertebrates. Stressors (and/or CRH release) can induce thyroid hormone release during development in amphibians, birds, and mammals (larval *Xenopus laevis*: Boorse and Denver, 2004; chicken embryos: De Groef et al., 2003; neonatal rats: Meaney et al., 2000), and in adults or juveniles of all non-mammalian vertebrate classes (fish: Larsen et al., 1998; amphibians: Denver, 1988; reptiles: Denver et al., 1989; juvenile chickens: Geris et al., 1996). In contrast, in adult mammals, stressors either have no effect on thyroid function or they have an inhibitory effect (Servatius et al., 2000, for review).

The objective of this study was to examine the effects of different degrees of decreased thyroid function on the stress

response as indicated by basal and stress-induced plasma corticosterone. To this end, I created an AP dosing regime that induced two levels of decreased thyroid function in two experimental groups: thyroid challenged status in one group, (defined as euthyroid plasma T_4 with depleted gland T_4), and hypothyroid status in another group (defined as lowered plasma T_4 with depleted gland T_4). All birds were then subjected to a confinement and agitation stressor, and plasma corticosterone concentrations before (basal) and post-stressor were assessed and compared along with measures of thyroid function. I predicted that Japanese quail would respond to both thyroid challenged status and hypothyroid status with blunted corticosterone stress responses as compared to controls.

Materials and Methods

Animals

Japanese quail (*Coturnix japonica*) were reared in the breeding colony maintained in the Department of Biological Sciences animal care facilities at Virginia Tech (Blacksburg, VA, USA). Adult quail (4 to 6 months old) were maintained in group housing in 98 x 132 x 66 cm cages, with separate cages for each treatment group. Drinking solutions and chick starter feed (Big Spring Mills, Ellison, VA) were available *ad libitum.* Birds were placed into experimental groups with equal numbers of males and females per group (when both genders were used in the dose/response experiment). Birds were weighed and individually banded immediately before beginning treatment so that individuals could be identified and changes in body mass could be recorded. Procedures for animal maintenance, handling and sacrifice were approved by Virginia Tech's Animal Care Committee in accordance with federal guidelines.

Experimental design

Different concentrations of ammonium perchlorate (AP) were dissolved in distilled water and administered to the birds in three experiments. These solutions were the only source of drinking water, however, individual drinking rates were not measured.

Dose/Response (Exp. 1). Male and female birds were treated with concentrations of: 0 (control), 50, 100, 250, 500, 1000, or 2000

mg/L AP in drinking water for two weeks to determine the AP dose required to induce the two levels of decreased thyroid function of interest (thyroid challenged and hypothyroidism). The control and 2,000 mg/L AP contained eight birds each, and the five remaining treatment groups contained six birds each. Thyroid status was assessed by measuring plasma T_4 and T_3, content of T_4 and T_3 in the thyroid glands (TH stores) and thyroid gland mass (an indirect measure of HPT axis activation).

Effect of decreased thyroid function on the corticosterone stress response I (Exp. 2). Male birds were treated with concentrations of: 0 (control), 500, 1000, or 2000 mg/L AP in drinking water for two weeks. Each experimental group contained 12 birds. At the end of the treatment period, birds were sampled. Blood was sampled at two times, within 3 minutes of removal from cages (baseline sample), and after 30 minutes of confinement and agitation in a cloth bag (stressed sample). Treatment groups were sampled over 3 days in varying order: Day 1: control, 2000, 500, 1000; Day 2: 2000, 1000, 500, control; Day 3: control, 500, 1000, 2000. Thyroid status was assessed as in the first experiment. Plasma corticosterone concentrations were measured in the baseline and stressed blood samples to assess the corticosterone stress response.

Effect of decreased thyroid function on the corticosterone stress response II (Exp. 3). Male birds were treated with 2,000 mg/L AP in drinking water for either 2 weeks (thyroid challenged group) or 5 weeks (hypothyroid group), or with normal drinking water

(controls). Each experimental group contained 16 birds. At the end of the treatment period, blood was sampled at two times, within 3 minutes of removal from cages (baseline sample), and after 30 minutes of confinement and agitation in a cloth bag (stressed sample) as in the previous experiment. Treatment groups were sampled over 3 days in varying order: Day 1: control, 2 week group, 5 week group; Day 2: 5 week group, 2 week group; control; Day 3: 2 week group, 5 week group, control. Thyroid status and corticosterone stress response profiles were assessed as in the previous experiment.

Sampling

All birds were sampled between 14:00 and 16:00 in all three experiments. A baseline blood sample was obtained from each bird within 3 minutes of removal from its cage by alar vein puncture with a 21 gauge needle. The post-stress sample was obtained in the same fashion after 30 minutes of confinement and agitation in a cloth bag. Four to six heparinized microcapillary tubes of blood were collected from each bird during both the sampling periods. Plasma was separated by centrifugation and stored at -20°C until analysis. After all blood samples were obtained, birds were sacrificed by decapitation and final body weights were recorded to 0.01 g. The pair of thyroid glands were removed and weighed immediately to 0.01 mg and stored in snap-cap vials at -20°C until analysis. During dissection, the sex of each bird was verified by inspection of the gonads.

Hormone analysis

Thyroid hormones were measured using a double antibody radioimmunoassay (RIA) (McNabb and Hughes, 1983) adapted for small sample volumes (McNabb et al., 2004). Sample volumes were 12.5 µl for T_4 and 25 µl for T_3. RIAs on plasma samples used hormone standards prepared in charcoal-stripped chicken plasma. Primary antibodies were obtained through Sigma-Aldrich Chemical (St. Louis, MO, USA). High specific activity ^{125}I-labelled hormones (1200 µCi/µg) were obtained from Perkin-Elmer Life Sciences (Boston, MA, USA). Three levels of control serum from Randox Laboratories Ltd. (Crumlin, UK) were included with each assay to evaluate assay performance.

Thyroid gland hormone content was measured by the method described by McNabb and Cheng (1985). In brief, thyroid tissue (approx. 5 mg) was digested in 350 µl of digestion medium containing 25 mg of Pronase (Sigma-Aldrich Chemical, St. Louis, MO, USA) at 37°C in a water bath for 24 h. After digestion, 1.0 ml of ice-cold absolute ethanol was added to the tubes, and the tubes were vortexed. Hormones were extracted into the ethanol for 24 h at -20°C, and extracts were centrifuged at 13,500 g for 5 min. An aliquot of the supernatant was removed and stored at -20°C until analysis. Dilutions of the supernatants were prepared using 75% ethanol and analyzed for T_4 and T_3 by RIA as described

above, except that the standards used were prepared in 75% ethanol.

Plasma corticosterone levels were determined following the methods of Moore et al., (2002). Briefly, 5-25 µl plasma samples were allowed to equilibrate overnight with 2,000 cpm of tritiated corticosterone for determination of individual extraction efficiency. Each sample was extracted with 4.0 ml of dichloromethane, dried under nitrogen, and resuspended in phosphate-buffered saline. Samples were assayed in duplicate, and assay values were corrected for plasma volume and individual recoveries after extraction (extraction efficiency = 83.5%; standard curve range, 2,000–3.9 ng/ml; limit of detectability: ~1.0 ng/ml plasma). Inter-assay coefficient of variation was 19%, intra-assay coefficient of variation was 31%.

Statistical Analysis

Exp. 1: Data were analyzed by two separate multivariate analyses of variance (MANOVAs), one for females and one for males. In the MANOVAs, AP treatment level was used as the explanatory variable, and thyroidal T_4 and T_3 and thyroid gland mass were used as response variables. Subsequently, individual univariate analyses of variance (ANOVAs) on each response variable versus treatment level were examined. Differences between group means were examined using single degree of freedom contrasts ('contrast' command in SAS).

Exp. 2, 3: all data was first analyzed by MANOVA. AP treatment level was used as the explanatory variable, and thyroidal T_4 and T_3, thyroid gland mass, and pre and post-stress plasma concentrations of T_4, T_3 and corticosterone were used as response variables. Subsequently, individual ANOVAs on each response variable versus treatment level were run. Differences between group means were examined using single degree of freedom contrasts ('contrast' command in SAS).

Because the plasma hormone data were taken from the same animals at two sample times (pre and post-stress), differences in plasma corticosterone and plasma T_4 and T_3 concentrations due to AP treatment level, stressor, and treatment by stressor interaction were identified by repeated-measures ANOVAs.

For all MANOVAs in this study, the Wilks' Lambda test statistic was used to identify significant effects. All statistics were performed using SAS version 9.1 (SAS, SAS Institute, Cary, NC). Probabilities of $p < 0.05$ were considered indicative of statistically significant differences for all statistical tests.

Results

Dose/Response (Exp. 1)

In male birds, AP treatment significantly affected thyroid function (MANOVA: $F_{15, 34} = 3.75$, $P < 0.0007$). Thyroid gland T_4 content was decreased significantly compared to controls in response to increasing AP treatment (Fig. 1A; ANOVA: $F_{5, 14} = 3.62$, $P < 0.03$). Specifically, the 500 (contrast: $P < 0.02$), 1,000 (contrast: $P < 0.04$), and 2,000 (contrast: $P < 0.005$) mg AP/L treatment groups had significantly decreased thyroidal T_4 compared to controls (Fig 1A). Assays for plasma T_4 and T_3 were unsuccessful. Thyroid gland T_3 content did not respond to treatment (ANOVA: $F_{5, 14} = 1.87$, $P = 0.16$). Thyroid gland mass, an indicator of HPT axis activation, was increased significantly compared to controls in response to increasing AP treatment (Fig. 1B; ANOVA: $F_{5, 14} = 9.23$, $P < 0.0005$). Specifically, the 2,000 mg AP/L treatment group had significantly increased thyroid gland mass compared to controls (Fig. 1B; $P < 0.0001$). Females did not respond significantly to treatment (MANOVA: $F_{15, 34} = 1.44$, $P = 0.19$).

Effects of diminished thyroid function on corticosterone stress response I (Exp. 2)

AP treatment did not significantly affect thyroid function (MANOVA: $F_{21, 84} = 1.48$, $P = 0.1$). Plasma corticosterone was significantly elevated post-stressor for all birds combined across treatments (repeated measures ANOVA: $F_{1, 44} = 6.83$, $P <$

0.02), but no individual experimental groups responded significantly to the stressor when considered by themselves. Neither AP treatment (repeated measures ANOVA: $F_{1, 44} = .71$, $P = 0.55$) nor the interaction between AP treatment and stressor (repeated measures ANOVA: $F_{1, 44} = 0.74$, $P = 0.41$) significantly affected plasma corticosterone concentrations.

Effects of hypothyroidism on the corticosterone stress response II (Exp. 3)

AP treatment significantly affected thyroid function (MANOVA: $F_{16, 52} = 3.74$, $P < 0.0002$). Thyroidal T_4 decreased in response to increasing AP treatment (Fig 2B; ANOVA: $F_{2, 37} = 23.5$, $P < 0.0001$); specifically, the 2-week treatment group had lower thyroidal T_4 than controls (contrast: $P < 0.0001$), and the 5-week treatment group had lower thyroidal T_4 than both the controls (contrast: $P < 0.0001$) and the 2-week treatment group (contrast: $P < 0.05$). Thyroidal T_3 content was decreased significantly compared to controls (ANOVA: $F_{2, 37} = 19.07$, $P < 0.0001$). Thyroid gland mass increased in response to increasing AP treatment (Fig 2C: ANOVA: $F_{2, 37} = 15.45$, $P < 0.0001$); specifically, the 2-week treatment group had increased thyroid gland mass compared to controls (contrast: $P < 0.05$), and the 5-week treatment group had increased thyroid gland mass compared to both the controls (contrast: $P < 0.0001$) and the 2-week treatment group (contrast: $P < 0.001$). Plasma T_4 decreased in AP treated birds compared to controls (Fig 2A: ANOVA: $F_{2, 37} = 3.15$, $P < 0.05$); specifically,

the 5-week treatment group had decreased plasma T_4 compared to controls (contrast: $P < 0.02$). Plasma T_3 was not affected by AP treatment (ANOVA: $F_{2, 37} = 0.85$, $P = 0.26$).

Experimental animals responded to the stressor with highly significant increases in plasma corticosterone (repeated measures ANOVA: $F_{1, 40} = 101.07$, $P < 0.0001$) in all groups, but AP treatment significantly affected the magnitude of the corticosterone response to the stressor (Fig. 3A), as indicated by a significant treatment/stressor interaction (ANOVA: $F_{2, 40} = 4.51$, $P < 0.02$). The corticosterone response to the bag stressor was significantly blunted compared to controls in both the 2-week treatment group (by 38%, contrast: $P < 0.03$) and the 5-week treatment group (by 54%, LSD: $P < 0.004$). Baseline corticosterone levels were unaffected by AP treatment (contrast: control vs. 2-week: $P = 0.63$; control vs. 5-week: $P = 0.74$).

After the confinement and agitation stressor, mean plasma T_4 concentrations were increased significantly for all experimental animals compared to their pre-stress levels (Fig 3B; repeated measures ANOVA: $F_{1, 41} = 4.52$, $P < 0.04$). However, because of my *a priori* interest in treatment by stressor interaction, which approached significance (repeated measures ANOVA: $F_{2, 41} = 2.5$, $P = 0.094$), I did post-hoc analysis on the simple effects of interaction. Only the 5-week treatment group demonstrated a change in response to the stressor (contrast: $P < 0.005$): 5-week birds had significantly decreased plasma T_4 before the stressor, which then increased to euthyroid levels in response to the

stressor. The 2-week treatment group and control group showed no changes in plasma T_4 in response to the stressor (contrast: $P = 0.61$ and $P = 0.92$, respectively). Plasma T_3 concentrations did not change in response to the stressor (repeated measures ANOVA: $F_{1, 41} = 0.91$, $P = 0.32$).

Increasing thyroid gland mass (hypertrophy) was significantly correlated with declining stress-induced plasma corticosterone concentrations. (Fig 4A; linear regression: $r^2 = 0.145$, $P < 0.02$). However, plasma T_4 concentrations were not correlated with stress-induced plasma corticosterone concentrations (linear regression: $r^2 = 0.0351$, $P = 0.24$).

Discussion

There are three key results from this study. First, decreased thyroid function blunted the corticosterone stress response in both thyroid challenged and hypothyroid birds. Second, stress had an effect on thyroid function; specifically, plasma T_4 increased in hypothyroid birds in response to the stressor. To my knowledge, this stress-induced change in plasma T_4 has not been observed previously in hypothyroid animals. Third, there were sex-specific differences in the thyroid responses to AP treatment; males responded to AP treatment with significant thyroid hypertrophy and significant decreases in thyroidal T_4 content but females did not respond significantly to treatment. To my knowledge, this sex difference in the thyroidal effect of AP has not been previously observed.

The observed blunting of the corticosterone stress response in both hypothyroid and thyroid challenged birds suggests there is a functional interaction between the HPT and HPA axes in birds. Overt hypothyroidism, with significantly lowered plasma T_4 and depleted gland T_4, induced a 54% decrease in stress-activated corticosterone release compared to controls. In addition, in thyroid challenged birds, which had depleted their gland stores of T_4 but still maintained euthyroid plasma T_4 levels, a 38% decrease in stress-activated corticosterone release was observed. (In contrast to plasma T_4, plasma T_3 did not respond significantly to treatment and thyroidal T_3 decreases were proportionally less than those of T_4). Previous studies of this thyroid - stress

interaction have examined only the effects of profound hypothyroidism on the HPA axis in rats (Murakami et al., 1984; Nolan et al., 2000; Tohei et al., 1998; Tohei 2004; and Shi et al., 1994). As indicated earlier, intermediate states of decreased thyroid function, like the thyroid challenged state used in this experiment, exist in nature, e.g., herring gulls exposed to a PCB-predominated mixture of pollutants in some areas of the Great Lakes (McNabb and Fox, 2003) and are present in human beings in the clinical setting (Benker, et al., 1990). The blunting effects of decreased thyroid function on the glucocorticoid stress response observed in this study thus may be of significant biological and clinical importance.

The results from this study suggest similarities in the effects of thyroid deficiency on HPA axis function in birds and mammals, indicating that this functional interaction between the HPT and HPA axes may be conserved across vertebrate classes. The Japanese quail in this study showed results similar to those in rats with chronic iodine insufficiency, whose hypothyroidism was moderate and whose stress-activated increases in corticosterone were blunted by 56% compared to controls (Nolan et al 2000). However, in studies of mammals with profound hypothyroidism due to thyroidectomy (Murakami et al., 1984) or thiouracil treatment (Nolan et al., 2000, Tohei et al., 1998, Tohei 2004, and Shi et al., 1994) corticosterone responses to stressors were completely abolished.

The interaction I observed between thyroid deficiency and the stress response is consistent with a hypothalamic or pituitary-level interaction between the HPT and HPA endocrine axes. I was unable to directly assess the HPT axis activation that results from decreases in thyroid function, as assays for avian thyroid-stimulating hormone (TSH) are not available. However, I did measure thyroid gland mass to indirectly assess HPT axis activation. Thyroid gland hypertrophy occurs in response to chronically elevated TSH, and this measurement has been shown to be a moderately sensitive indicator of HPT axis activation (McNabb et al. 2004). In this study, increases in thyroid gland mass (and therefore HPT axis activation) were correlated with declines in stress-induced plasma corticosterone concentrations (Fig. 4). This supports either a pituitary or hypothalamic level interaction between the HPT and HPA axes. These data do not support an adrenal-level interaction because there was no correlation between plasma corticosterone and plasma T_4 levels, as might be expected if circulating T_4 were acting directly on the adrenal glands. Also, the thyroid challenged birds, which had euthyroid levels of plasma T_4, had blunted corticosterone stress responses, which would not be expected if T_4 was directly affecting adrenal corticosterone release.

Current evidence for a mechanism by which hypothyroidism may affect the HPA axis is through a specific corticotropin-release-inhibiting hormone (CRIF) that functionally connects the HPT and HPA axes at the hypothalamic level (McGivern et al.,

1997, and Redei et al., 1995). In rats, this CRIF originates from one segment of the precursor to TRH, prepro-TRH 178-199, that has been shown to inhibit adrenocorticotropic hormone (ACTH) release *in vitro* (McGivern et al., 1995). Intra-cerebroventricular administration of this protein in rats resulted in decreases in stress-induced plasma ACTH and corticosterone concentrations, as well as behavioral changes consistent with a less stressed animal (Redei et al., 1995). It has been proposed (Engler et al., 1999) that this prepro-TRH-derived CRIF may be mediating the changes in HPA axis hormone profiles that occur during hypothyroidism. Hypothyroid animals respond to low circulating thyroid hormones by activating the HPT axis, during which the synthesis of prepro-TRH is increased, thereby increasing the production of CRIF. It has been noted that this CRIF effect is unlikely to be the only factor in these axes interactions, as stress-induced glucocorticoid release does not return to normal immediately after thyroid hormone replacement (Nolan et al., 2000, and Murikami et al., 1984), even when measured TRH transcripts, previously elevated during hypothyroidism, have returned to normal (Shi et al., 1994).

Although the blunting of the corticosterone stress response observed in this study is consistent with the CRIF mechanism described above, other mechanisms could also mediate the blunting effect. Direct down-regulation of CRH synthesis by increasing HPT axis products during hypothyroidism is possible as decreased CRH mRNA transcripts have been observed during hypothyroidism (Shi,

et al., 1994; Nolan et al., 2000). Increased HPA axis sensitivity to negative feedback also has been suggested as a potential mechanism for reduced HPA axis activation during hypothyroidism (Nolan et al., 2000). It also has been proposed that the adrenal glands are directly suppressed during hypothyroidism due to decreased circulating thyroid hormones or increased TSH (Tohei et al., 1998). Some findings support this hypothesis, for example, CRF and ACTH levels were increased in hypothyroid rats while stress-induced corticosterone secretion was attenuated, suggesting direct adrenal suppression followed by compensatory activation of the HPA axis (Tohei et al., 1998; and Tohei, 2004).

Associated with changes in plasma corticosterone, hypothyroid birds had significant changes in plasma T_4 in response to the stressor. Specifically, hypothyroid quail with low plasma T_4 pre-stressor had euthyroid plasma T_4 concentrations post stressor. Presumably this was due to increased release of T_4 from thyroidal T_4 stores. This demonstrates that the thyroid glands can respond to acute stressors with increased thyroid hormone release, although this may only occur when animals are hypothyroid; there was no evidence of this response in control or thyroid-challenged birds. These data also suggests that T_4 may play a permissive role in mounting a stress response because lowered levels of pre-stress circulating T_4 may be metabolically insufficient to mount an effective response to a stressor.

The observed increase in plasma T_4 in hypothyroid animals in response to the stressor may be mediated by CRH. As mentioned

earlier, stressors (and/or CRH release) can induce thyroid hormone release in adults or juveniles of all non-mammalian vertebrate classes (fish: Larsen et al., 1998; amphibians: Denver, 1988; reptiles: Denver et al., 1989; juvenile chickens: Geris et al., 1996). However, this mechanism does not account for the fact that only hypothyroid birds responded to the stressor with elevated plasma T_4 levels. It is possible that hypothyroid birds are more sensitive to the thyrotrophic effects of CRH than euthyroid birds. More research is needed to determine the mechanism that mediates the elevated plasma T_4 observed in this particular case.

In the dose/response experiment (Exp 1), only males responded to AP with signs of developing hypothyroidism (decreases in thyroidal T_4 and increases in thyroid gland mass). Females, unexpectedly, showed no significant effects on thyroid function after two weeks of AP treatment. The lack of response in females may be the result of the perchlorate ion being actively sequestered by the ovaries and subsequently deposited in eggs (Pena et al., 1975). Perchlorate has goitrogenic effects because it competitively binds to the Na/I symporter in the thyroid glands and reduces iodine uptake. Female birds transport iodide into their ovaries (Pena et al., 1975) for deposition in eggs. Quail hens treated with perchlorate actively concentrate it in their eggs (bobwhite quail: Gentles et al., 2005, and Japanese quail: Pena et al., 1975), just as they normally concentrate iodide (Pena et al 1975). I speculate that the hens in the

present experiment sequestered some perchlorate in their ovaries and eggs and thus lowered their effective thyroidal dose of perchlorate compared to the male birds.

In conclusion, the blunted corticosterone stress responses observed in this study in birds with decreased thyroid function suggest a functional interaction between the HPT and HPA axes in birds, and that this interaction may be conserved across vertebrate taxa. My data also suggest that the mechanism of interaction between axes exists at the hypothalamic or pituitary level. In addition, this study demonstrates that overt hypothyroidism is not required to induce alterations in HPA axis function, because blunted corticosterone stress responses also were visible in thyroid challenged birds (with euthyroid plasma T_4 and depleted gland T_4). These results have potential ecological and clinical implications, as intermediate and overt states of decreased thyroid function are present in wild populations of animals and in humans in the clinical setting, and altered glucocorticoid stress responses may contribute to the total impact of decreased thyroid function. Additionally, the observed increase in plasma T_4 in hypothyroid animals in this study suggests that the thyroid glands may be able to respond to stressors with increased thyroid hormone release, at least during hypothyroidism, and that there may be a potential permissive role for T_4 in mounting a stress response in birds.

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Figure legends:

Figure 1. The effects of 2 weeks of ammonium perchlorate (AP) exposure (0 - 2,000 mg/L) on thyroid function in adult Japanese quail. (A) T_4 content per thyroid gland pair and (B) mass of thyroid gland pair. Males (black bars) and females (white bars). Bars are means ± standard error (n = 4 for control and 2,000 mg AP/L groups, n = 3 for other groups). Asterisks indicate significant difference from same-gender controls ($p < 0.05$).

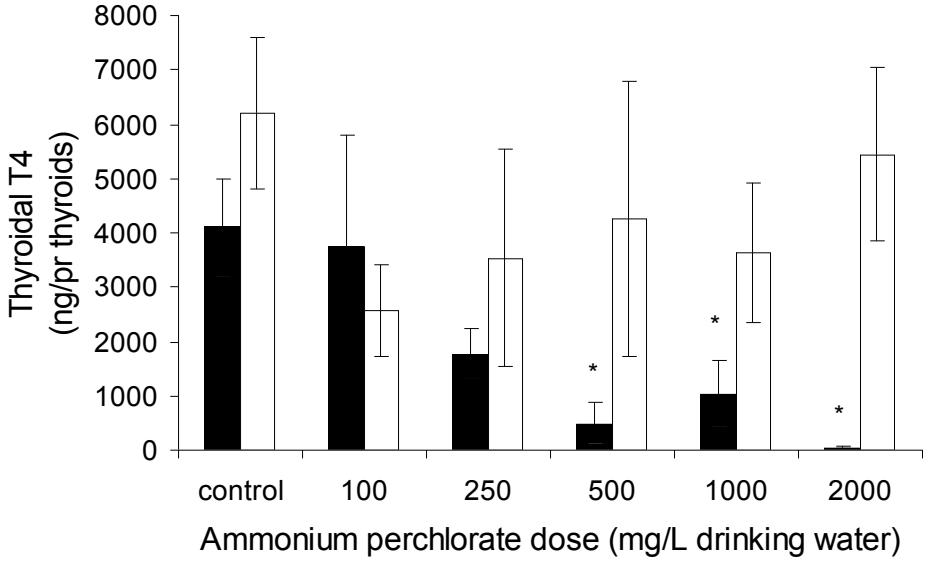
Figure 2. The effects of duration of ammonium perchlorate (AP) treatment on thyroid function in adult male Japanese quail. (A) plasma T_4 concentrations (B) T_4 content per thyroid gland pair and (C) mass of thyroid gland pair. Bars are means ± standard error (sample sizes listed above bars). Different letters indicate significant differences between means ($p < 0.05$).

Figure 3. Plasma corticosterone and plasma T_4 in Japanese quail treated with ammonium perchlorate (AP) to induce decreased thyroid function. (A) plasma corticosterone and (B) plasma T_4 concentrations, both basal (0 min) and stress-induced (30 min), in controls (diamond symbols; n = 16), 2-week thyroid challenged group (square symbols; n = 16), and 5-week hypothyroid group (triangle symbols; n = 14). All points are means ± standard error. Different letters indicate significant differences between means ($p < 0.05$).

Figure 4. Correlation between thyroid gland mass and stress-induced plasma corticosterone concentrations, with regression line, regression equation, and correlation coefficient (R^2) included.

Fig. 1

A.


8000
7000
6000
5000
4000
3000
2000
1000
0
Thyroidal T4
(ng/pr thyroids)
*
*
*
control
100
250
500
1000
2000
Ammonium perchlorate dose (mg/L drinking water)

B.

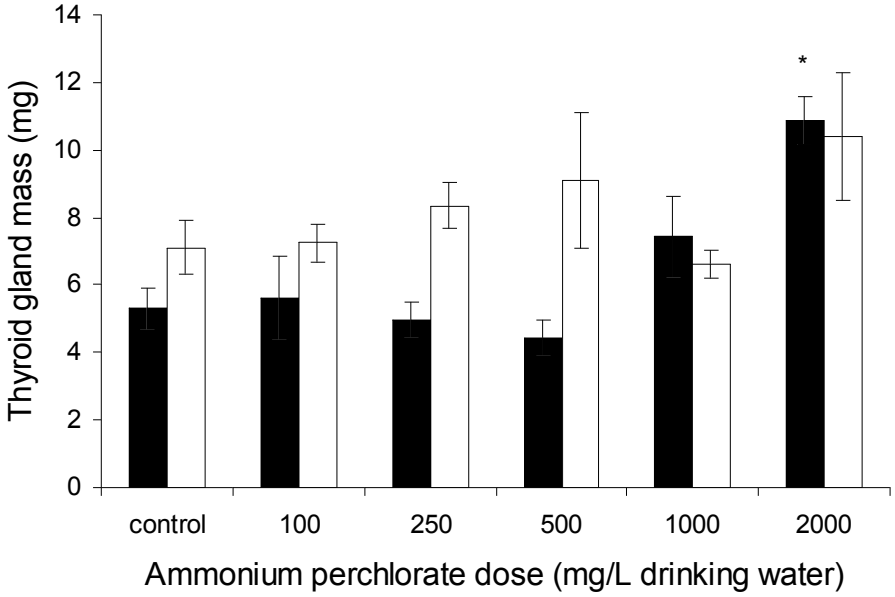
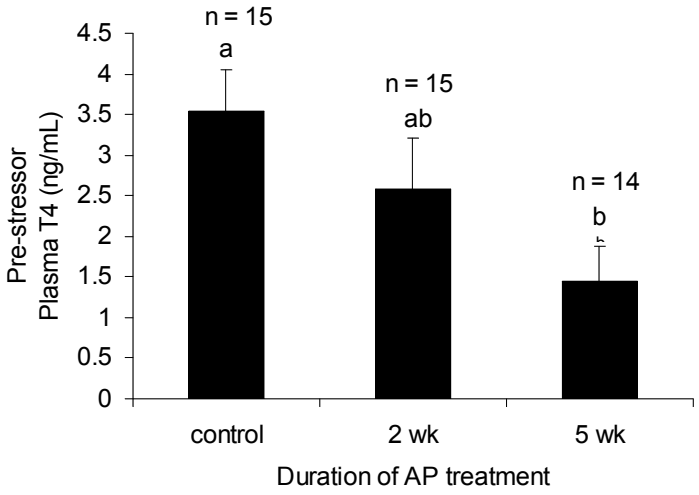
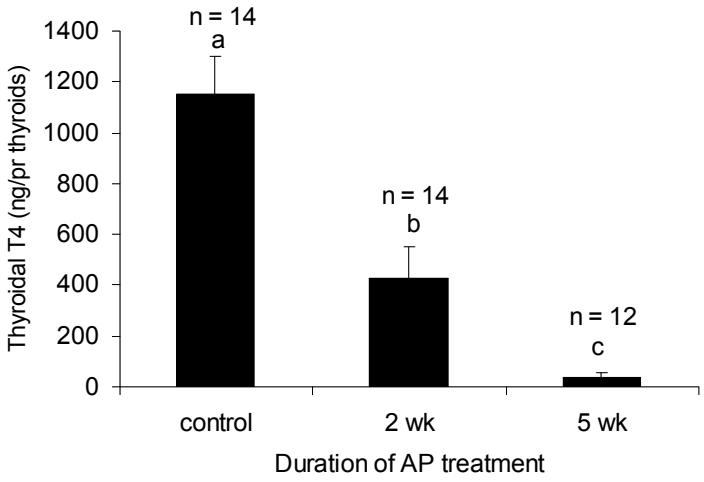

14
12
10
8
6
4
2
0
Thyroid gland mass (mg)
*
control
100
250
500
1000
2000
Ammonium perchlorate dose (mg/L drinking water)

Fig. 2

A.


n = 15
a
n = 15
ab
n = 14
b
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Pre-stressor
Plasma T4 (ng/mL)
control
2 wk
5 wk
Duration of AP treatment

B.


n = 14
a
n = 14
b
n = 12
c
1400
1200
1000
800
600
400
200
0
Thyroidal T4 (ng/pr thyroids)
control
2 wk
5 wk
Duration of AP treatment

C.

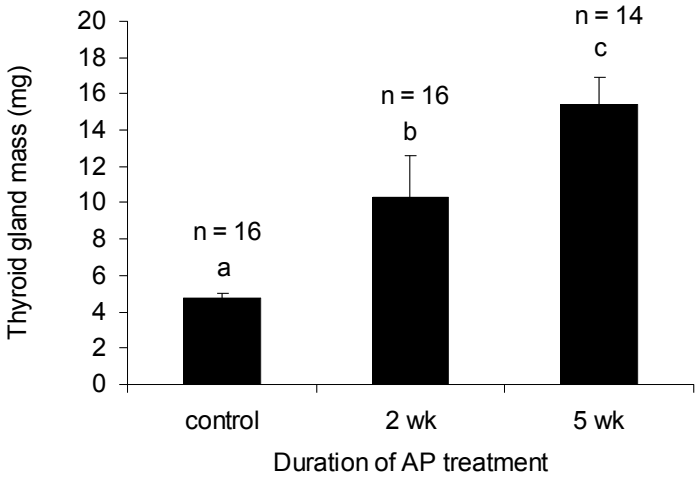
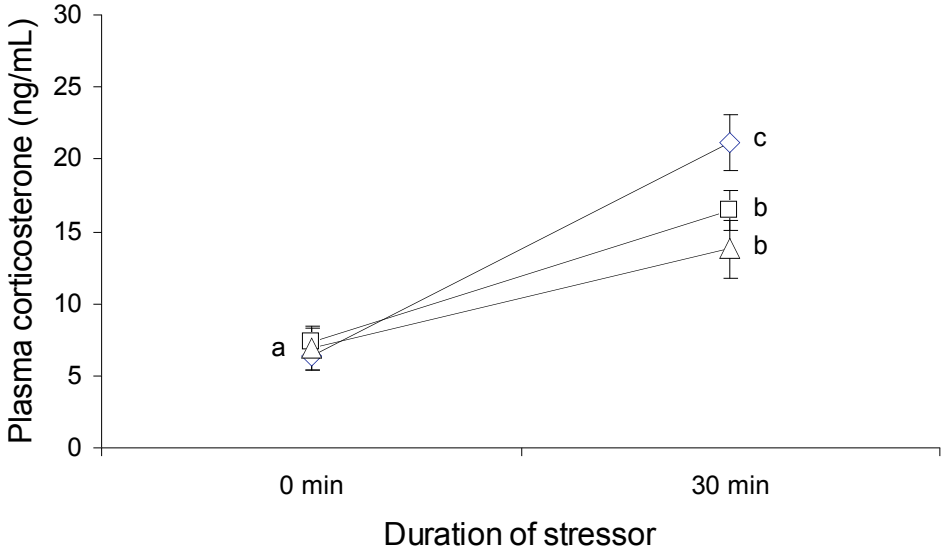

n = 14
c
n = 16
b
n = 16
a
20
18
16
14
12
10
8
6
4
2
0
Thyroid gland mass (mg)
control
2 wk
5 wk
Duration of AP treatment

Fig. 3

A.


Plasma corticosterone (ng/mL)
30
25
20
15
10
5
0
a
b
b
c
0 min
30 min
Duration of stressor

B.

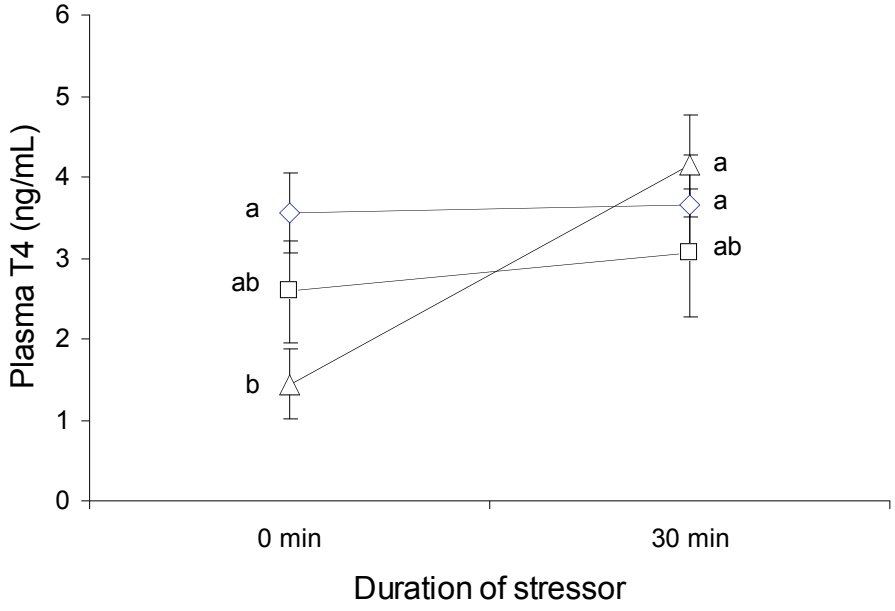

Plasma T4 (ng/mL)
6
5
4
3
2
1
0
a
ab
b
a
a
ab
0 min
30 min
Duration of stressor

Fig. 4

A.

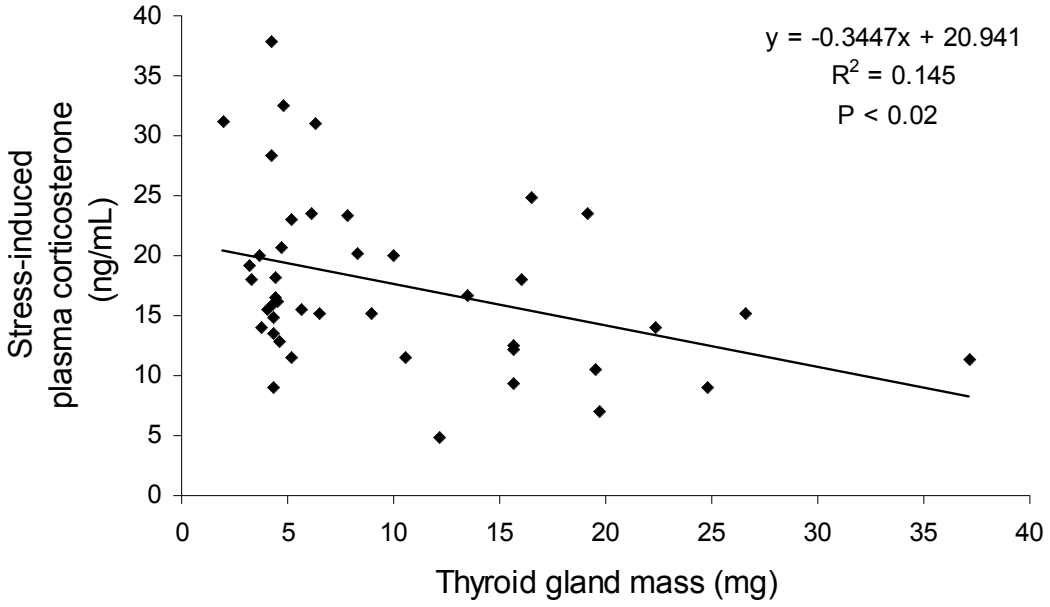

y = -0.3447x + 20.941
R2 = 0.145
P < 0.02
Stress-induced plasma corticosterone (ng/mL)
Thyroid gland mass (mg)
0
5
10
15
20
25
30
35
40

Table 1

Exp. 1 (males)	F	df	P	
MANOVA	3.75	15, 34	0.0007	*
ANOVA				
Thyroidal T_4	3.62	5, 14	0.0261	*
Thyroidal T_3	1.87	5, 14	0.16	
Thyroid gl. mass	9.23	5, 14	0.0005	*
Exp. 2				
MANOVA	1.48	21, 84	0.105	
Exp. 3				
MANOVA	3.74	16, 52	0.0002	*
ANOVA				
Plasma T_4	3.15	2, 37	0.0438	*
Plasma T_3	0.15	2, 37	0.86	
Thyroidal T_4	20.63	2, 37	< 0.0001	*
Thyroidal T_3	19.07	2, 37	< 0.0001	*
Thyroid gl. mass	20.05	2, 37	< 0.0001	*
Corticosterone	0.7	2, 37	0.503	