Available online at www.sciencedirect.com
ScienceDirect
General and Comparative Endocrinology 152 (2007) 82–88
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Characterization and quantification of corticosteroid-binding globulin in a southern toad, *Bufo terrestris*, exposed to coal-combustion-waste

Chelsea K. Ward [a,*], Cristiano Fontes [a], Creagh W. Breuner [b], Mary T. Mendonça [a]

[a] *Department of Biological Sciences, Auburn University, Auburn, AL 36849, USA*
[b] *Division of Biological Sciences, Organismal Biology and Ecology, University of Montana, Missoula, MT 59812, USA*

Received 25 October 2006; revised 27 February 2007; accepted 28 February 2007
Available online 12 March 2007

Abstract

Corticosteroid-binding globulin (CBG) is a plasma protein that binds corticosterone and may regulate access of hormone to tissues. The role of CBG during a stress response is not clear. At least two hypotheses have been proposed: 1) CBG levels may increase in response to a stressor, thereby decreasing the amount of circulating free corticosterone, or 2) CBG levels may decline, making corticosterone available for its role in increased metabolic needs during stress. In this study, southern toads, *Bufo terrestris*, were exposed to a chronic pollutant (coal-combustion-waste), to determine changes in CBG and free corticosterone levels. Since toads exposed to chronic pollutants in previous studies did not exhibit the predicted changes in metabolic rate and mass, but did experience a significant elevation in total corticosterone, we hypothesized that CBG would likewise increase and thus, mitigate the effects of a chronic (i.e. 2 months) pollutant stressor. To conduct this study, we first characterized the properties of CBG in southern toads. Toad CBG has a $K_d = 20.6 \pm 1.0$ nM and a $B_{max} = 332.2 \pm 5.1$ nmol/L plasma. The rank order potencies for steroid inhibition of tritiated corticosterone are: dihydrotestosterone > corticosterone $\gg$ progesterone = testosterone $\ggg$ estrogen = dexamethasone. After characterization, we monitored the changes in CBG, total corticosterone, and free corticosterone in male toads that were exposed to either coal-combustion-waste or control conditions. CBG increased in all groups throughout the experiment. Total corticosterone, on the other hand, was only significantly elevated at four weeks of exposure to coal-combustion-waste. The increase in CBG did not parallel the increase in total corticosterone; as a result, free corticosterone levels were not buffered by CBG, but showed a peak at four weeks similar to total corticosterone. This finding indicates that, in this species, CBG may not provide a protective mechanism during long-term pollution exposure.

Keywords: Amphibian; Corticosteroid-binding globulin; Corticosterone; Stress; Metal

1. Introduction

Corticosteroid-binding globulins (CBG) are plasma proteins that affect the activity, transport, and availability of glucocorticoids in the body. The free hormone hypothesis posits that while a hormone is bound to a binding globulin, it is not biologically active (Breuner et al., 2006; Mendel, 1989). However, there is evidence that CBG can deliver corticosterone to specific tissues (Mendel, 1989), or have its own biological activity (reviewed in Rosner, 1990). Levels of CBG cycle seasonally and parallel changes in corticosterone in animals not confronting a stressor (reviewed in Breuner and Orchinik, 2000). Thus, as the concentration of corticosterone in the blood rises, the concentration of CBG also rises, binding excess corticosterone and maintaining the level of unbound (free) corticosterone relatively constant. When an organism is exposed to a stressor, however, the concentrations of CBG and corticosterone diverge and the level of free corticosterone rises above baseline

* Corresponding author. Fax: +1 334 244 3826.
E-mail address: cward3@mail.aum.edu (C.K. Ward).

0016-6480/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2007.02.031

(Breuner and Orchinik, 2000). Although a potentially important component in the stress response, the extent of corticosterone–CBG interaction has not been fully documented and the exact function of CBG in the regulation of corticosterone is still not understood (reviewed in Breuner and Orchinik, 2000).

The characteristics of CBG have been widely studied in humans, rats, and birds (e.g. Vogeser et al., 1999; Tinnikov et al., 1996; Armario et al., 1994; Tinnikov, 1993a; Deviche et al., 2001; Lynn et al., 2003, respectively). However, very little information exists about CBG characteristics and function in lower vertebrates, such as amphibians. Martin and Ozon (1975) have examined limited binding properties of numerous amphibians and Orchinik et al. (2000) have thoroughly described corticosteroid-binding in plasma of the tiger salamander (*Ambystoma tigrinum*). There are only three studies that have characterized CBGs in anurans from four families (Jolivet-Jaudet et al., 1984; Jolivet-Jaudet and Leloup-Hatey, 1996; Martin and Ozon, 1975). The actual role of CBG in glucocorticoid regulation in lower vertebrates, to our knowledge, has not been studied.

During stressful situations, the concentration of plasma glucocorticoids in vertebrates increases. In the short-term, this increase in glucocorticoids is adaptive and contributes to the survival of the organism by decreasing unnecessary physiological activities and increasing available metabolic energy (Bonga, 1997). However, if the stressor persists the effects of increased glucocorticoids can become detrimental. CBG is hypothesized to modulate the stress response by controlling the concentration of plasma free corticosterone. Most studies measuring changes in CBG during a stress response focus on short-term stressors (those lasting a few minutes to a few days) in mammalian (Fleshner et al., 1995; Tinnikov, 1993a; Viau et al., 1996; Vogeser et al., 1999) or avian (Breuner and Orchinik, 2000; Lynn et al., 2003; Silverin, 1986) systems with mixed results. Some studies found that CBG decreased with stress (e.g. Fleshner et al., 1995) and others found that CBG increased, mirroring total plasma corticosteroid concentrations (e.g. Breuner and Orchinik, 2000). For example CBG decreases with tail shock but increases during the stress of breeding (Fleshner et al., 1995; Breuner and Orchinik, 2000). It has been proposed that animals exposed to a chronic stress experience an increase in CBG. Two studies have shown that animals chronically exposed (1 month) to increased corticosterone levels by corticosterone implant experienced an increase in CBG that mirrored the increase in corticosterone levels (Breuner et al., 2003; Jennings et al., 2001). However, the organisms in these studies were not exposed to an actual stressor and, thus, would probably not have the same energy needs or responses as a stressed organism. Alternatively, CBG may decline due to persistent stressors. Several mammalian studies have demonstrated that stressors lasting several days actually cause a decline in CBG (see Breuner and Orchinik, 2002 for review). The role of CBG in response to a persistent (e.g. >1 month), low-level stressor is still unclear. The role of CBG in the stress response of lower vertebrates, in either acute or persistent situations, has also not been evaluated.

Given the current concern regarding amphibian decline, which some attribute to long-term environmental degradation (e.g. pollution, ultraviolet radiation, habitat destruction, Pounds and Crump, 1994; Carey and Alexander, 2003), further research on how amphibians respond to chronic stressors seems warranted. This study first characterizes the properties of corticosteroid-binding globulin (CBG) in the southern toad, *Bufo terrestris*, and then documents changes in CBG levels in control toads versus those chronically exposed (2 months) to coal combustion waste, a contaminant stressor. Toads exposed to this contaminant have been shown to have contradictory physiological responses in light of the stress response, indicating that CBG may be buffering circulating corticosterone (Ward et al., 2006). CBG levels were measured and compared to total plasma corticosterone concentrations, and then used to calculate free plasma corticosterone levels. These data will help to determine if CBG may function in a protective role, by reducing free corticosterone levels, or exacerbate stress reactivity, by buffering less of the corticosterone in plasma.

2. Materials and methods

2.1. Animal capture

Male southern toads were captured by hand at the Savannah River Site, Aiken, SC, USA, in early spring: March and April, 2001 ($n = 96$) and 2002, ($n = 168$). Toads were collected from either a coal-combustion-waste storage area, ash basin, (see Gutherie and Cherry, 1979 for description) or a control site, ~15 km from the ash basin. Mass was measured on a digital balance (0.01 g accuracy) and an initial blood sample was taken (see Section 2.3). All toads were then given an individual identification number by toe clip.

2.2. Housing and experimental design

Within two days of capture, toads were transported to Auburn University, Auburn, AL, USA, and transferred into mesocosms (208L Rubbermaid containers with screen lids). Each mesocosm contained 70% sediment (coal-combustion-waste, ash, collected from the capture site or play sand purchased from a local hardware store) and 30% water by area, 6 cm deep. Toads were placed in mesocosms with the sediment type equivalent to the type on which they were captured. There were 60 total mesocosms (30 ash and 30 sand controls) each housing three toads. Sediment was covered with pine straw, 5 cm deep, and a 10×25 cm piece of pine bark was included for shelter. All mesocosms were located outdoors under a shade-cloth tent and subject to ambient conditions.

Toads were acclimated in mesocosms containing their capture sediment for one month before being transferred to the experimental sediment. Toads were assigned into one of four groups ($n = 40$ per group). Toads acclimated to control (C) or ash (A) sediments were then transferred to mesocosms containing control ($C \rightarrow C$, $A \rightarrow C$) or ash ($C \rightarrow A$, $A \rightarrow A$) sediments. Toads were fed weekly (~10 crickets per toad *ad lib.*) with crickets raised on either a control diet of cat food or dry cat food chow contaminated with coal fly ash (50/50 mixture by volume), depending on treatment sediment. All toads were euthanized after four months by immersion in an aqueous solution of MS222 (300 ppm) (Andrews, 1993).

2.3. Blood sampling

Toads were monitored from 26 April to 19 June 2002. Blood sampling began the day after transfer into the experimental sediment. Each experimental group of toads (C → C, A → C, C → A, A → A) was divided into four sampling subgroups ($n = 10$/subgroup for a total of 40 per experimental group). Blood samples were taken from each subgroup consecutively, so that alternating subgroups of toads were bled every day for the first eight days and every week for the next four weeks. Two subgroups were combined and the new, larger, subgroups ($n = 20$/subgroup, for a total of 40 per experimental group) were bled on consecutive weeks, no blood sample was taken on the third week (Fig. 1).

All blood samples were taken via cardiac puncture using a 26 gauge heparinized needle in less than 2 min. Blood samples were stored on ice (less than 2 h) until they were centrifuged at 7000 rpm for 5 min. The plasma was then removed by pipette and frozen at −20 °C until further analysis.

2.4. Corticosteroid-binding globulin characterization

Three adult male toads were captured in May at a control site at the Savannah River Site and euthanized (as described above). The toads were exsanguinated, the blood immediately pooled and centrifuged, and the plasma was separated and frozen at −20 °C. All hormones were then removed from the plasma with 2 volumes (2:1) of dextran-coated charcoal (1% Norit A charcoal, 0.1% dextran solution in 50 mM Tris, pH 7.4). Charcoal was added to the samples, vortexed, and allowed to equilibrate at room temperature for 30 min. Charcoal was pelleted by centrifugation at 3000 rpm at 4 °C. The supernatant was then aliquotted and frozen.

For saturation analysis (to determine K_d), all plasma was diluted (1:54-final dilution, empirically determined) with assay buffer (50 nm Tris buffer). Fifty μl plasma was added to either 50 μl assay buffer or 50 μl of 1 μM unlabeled corticosteroid (non-specific binding) and 50 μl of increasing concentrations of labeled corticosterone ([^{3}H]CORT). Each point of the saturation curve was analyzed in triplicate. Sample tubes were incubated for 2 h at 4 °C (empirically determined). Bound radioligand was separated from unbound ligand by vacuum filtration through a Whatman GF-B glass fiber filter (retention size 1.0 μl using a Brandel tissue harvester (Gaithersberg, MD, USA). After filtration, filters were rinsed with 9 ml of ice-cold rinse buffer (25 nm Tris buffer) and radioactivity bound to the filter was measured using a standard liquid scintillation spectrometer. Filters were soaked in 25 mM Trisma buffer (pH 7.4) with 0.3% PEI for 60 min prior to use. Assay protocols adapted from Orchinik et al. (2000).

CBG binding specificity was determined by incubating plasma with a set concentration of labeled corticosterone (empirically determined) and increasing concentrations (1×10^{-6} to 1×10^{-10} nM) of corticosterone, dexamethasone, testosterone, estrogen, dihydrotesosterone, or progesterone. Assay conditions (temperature, plasma dilution, and separation of bound and free fractions) were completed as described for saturation analysis.

2.5. Corticosteroid-binding globulin quantification

Corticosteroid-binding globulin binding capacity was measured in all toad samples collected. The assay parameters were optimized to maximize binding. As above, stripped plasma was incubated for 30 min at room temperatures, 1 μM unlabelled corticosterone was used to determine non-specific binding, bound and free fractions were separated by vacuum filtration. In this assay each sample was incubated with 30 nM of [^{3}H]CORT. Based on affinity estimates from CBG saturation assays, this concentration should occupy 54% of total binding sites. All individual samples were run in triplicate.

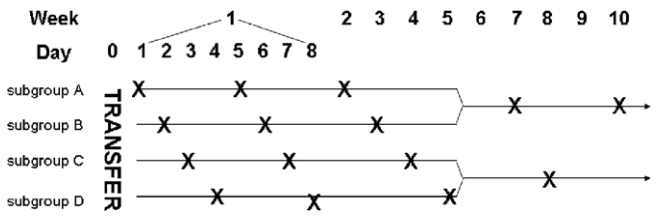

Fig. 1. Time line for blood sampling for subgroups (A–D) of each experimental group, X indicates sampling.

2.6. Hormone quantification

Plasma corticosterone levels were quantified using a standard competitive binding radioimmunoassay as described in Mendonça et al. (1996). Anhydrous diethyl ether was used to extract steroid hormones from the plasma. Corticosterone antibodies were purchased from Esoterix (Calabasas Hills, CA, USA). The assay utilized a standard charcoal–dextran separation technique. Intra-assay variation is 3.71% and inter-assay variation is 13.0% for corticosterone (Chard, 1995).

2.7. Metal quantification

Toads were sacrificed in May 2001 and 2002, after five weeks of exposure ($n = 5$/yr) and examined for whole-body metal content. This time point corresponds to the increase in corticosterone seen after four weeks of exposure. Toad carcasses were homogenized and freeze dried. A sub sample of homogenized toad tissue (250 mg) was digested with 5 ml HNO_3 and then 1 ml H_2O_2 and brought to 25 ml volume with deionized water. The digested samples were analyzed using an ELAN 900 Perkin–Elmer Inductively Coupled Plasma – Mass Spectrometry (Shelton, CT, USA) (USEPA method 200.8) at the University of Georgia, Athens, GA. Samples were analyzed for Al, As, Ba, Cd, Co, Cr, Cs, Cu Fe, Ni, Pb, Rb, Se, Sr, Tl, U, V, and Zn. Quality control checks were done every 15 samples and a certified standard reference material (TORT-2, Lobster hepatopancreas reference material for trace metals, National Research Council, Canada) was digested with the samples. The variation of the standard reference material was within the published standard error of the material.

2.8. Statistics

All hormones and CBG data were tested for heterogeneity of variance and determined homogenous by a F max test ($p < 0.01$) and, thus, untransformed ANOVAs were used for statistical analysis (Sokal and Rohlf, 1995). Samples from days 1–8 did not differ significantly and were pooled as a single sample for further analysis. Samples taken over time within the same experimental treatment (hormone, CBG, and metal) were analyzed with repeated measures ANOVA. Time point to time point comparisons were calculated using Student–Newman–Keuls Test. CBG capacity was corrected to 100% and free corticosterone was calculated using the equation described in Barsano and Baumann (1987). Data are presented as means ± SE. Statistics were calculated with Statview vs. 5.0.1 (SAS institute, Cary, NC, USA), SAS v. 9.1 (SAS institute, Cary, NC, USA), and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. CBG assay validation

The binding of toad CBG was best explained with a one-site model, $K_d = 20.6 \pm 1.0$ nM and a $B_{max} = 332.2 \pm 5.1$ nmol/L plasma (Fig. 2). Specificity analysis determine a rank order potency of dihydrotestosterone > corticosterone ≫ progesterone = testosterone ⋙ estrogen = dexamethasone (see Table 1).

3.2. Heavy metal body concentrations

Toads kept on ash sediment, in our experiment, significantly accumulated a variety of trace metals within four

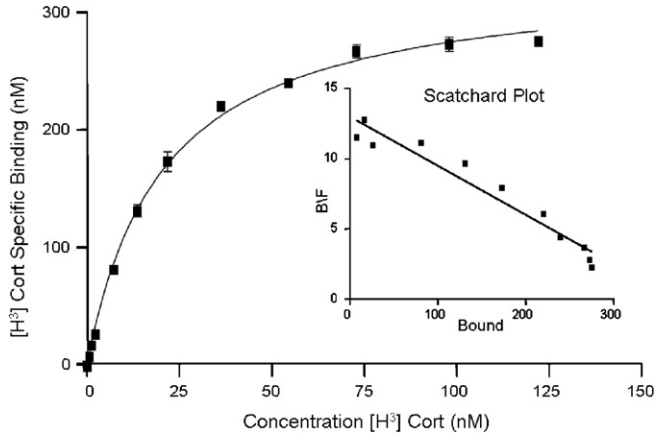

Fig. 2. Equilibrium saturation binding of [^{3}H]CORT to southern toad corticosteroid-binding globulin. Data are shown are specific binding (means ± SE at each concentration) of [^{3}H]CORT. This data was best fit with a one-site model K_d of 20.61 ± 1.04. Inset: Scatchard–Rosenthal replot of the data.

Table 1
Steroid displacement of [^{3}H]CORT from binding sites in plasma

Hormone	K_i ± SE (nM)	% Inhibition at 1 (μM)
Dexamethasone	<400	41
Estrogen	312.0 ± 63.6	44
Testosterone	104.6 ± 14.5	84
Progesterone	75.14 ± 18.9	89
Corticosterone	16.26 ± 1.88	100
Dihydrotestosterone	1.08 ± 0.71	46

K_i is the dissociation constant for each hormone.

weeks of exposure compared to control toads. The top four metals that increased in control to ash-exposed toads at four weeks were Al, U, As, and V (573%, 871%, 1883%, 1980% increase over capture, respectively). The majority of metals (11 of the 18 measured) were significantly increased by the 4th week of exposure to coal fly ash ($p \leqslant 0.1$).

3.3. Corticosterone and CBG response

Four weeks post transfer control-to-ash toads (C → A) experienced a significant increase in total corticosterone (week 2 vs. week 4, 8.82 vs. 22.86 ng/ml, $p = 0.009$, $df = 11$, $t = -3.14$), which was significantly elevated above control (C → C) toads ($p = 0.01$) at the same sample period (4 weeks) (Fig. 3). Corticosterone levels then returned to baseline concentrations by the 5th week of exposure (e.g. 7.35 ± 1.25 ng/ml) (Fig. 3). There was no other significant elevation in total corticosterone throughout the rest of the sampling period, nor did corticosterone differ significantly among the four groups at any other time point ($p > 0.5$).

There was no significant difference in plasma CBG concentrations among the four groups over the course of the experiment ($p = 0.83$). CBG concentrations continually increased in all toads between the first week post transfer to the fourth week post transfer ($\bar{x} = 164.2 \pm 7.9$ vs. 255.1 ± 16.7 nM, $p = 0.03$, $df = 15$, $t = -2.4$) (Fig. 4). However, because all four groups exhibited this elevation, there was no significant difference among the four groups at the fourth week of exposure ($p = 0.3$, $df = 27$, $F = 1.2$), although this four week time point was when total corticosterone increased significantly in C → A males.

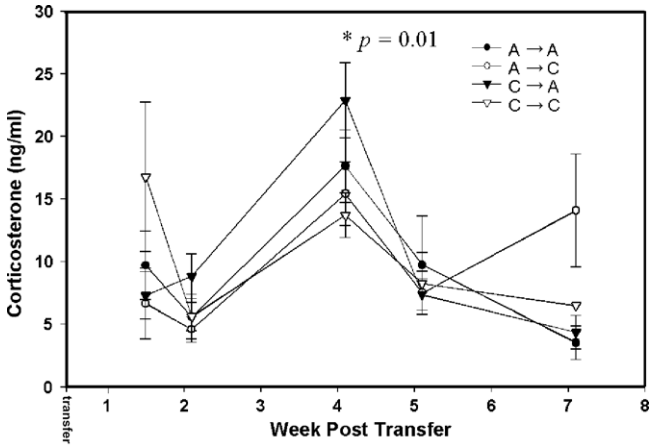

Fig. 3. Circulating total (bound and unbound) corticosterone concentrations of male southern toads moved from control (C) or ash (A) environments to control (C) or ash (A) mesocosms, A → A (closed circle), A → C (open circle), C → C (open triangle), C → A (closed triangle). Values are expressed as means ± 1 SE. *Denotes a significant ($p = 0.01$) difference.

CBG elevations were not great enough to buffer the increase in corticosterone seen in the C → A group. Hence, free corticosterone levels also rose significantly with transfer to ash (3.79 ± 1.13 vs. 1.67 ± 0.29 ng/ml, $p = 0.05$) at this critical four weeks post transfer (Fig. 5). There were no other significant elevations in free corticosterone during the experiment and the free corticosterone concentrations did not differ significantly among the four groups at any other time point ($p = 0.22$).

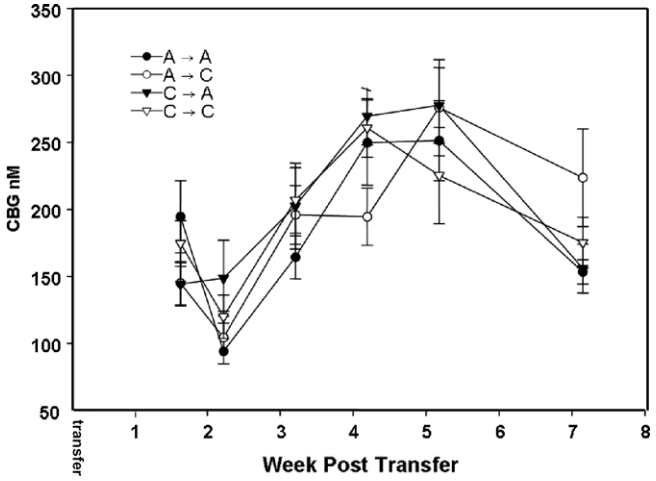

Fig. 4. Circulating corticosteroid-binding globulin concentrations of male southern toads moved from control (C) or ash (A) environments to control (C) or ash (A) mesocosms, A → A (closed circle), A → C (open circle), C → C (open triangle), C → A (closed triangle). Values are expressed as means ± 1 SE.

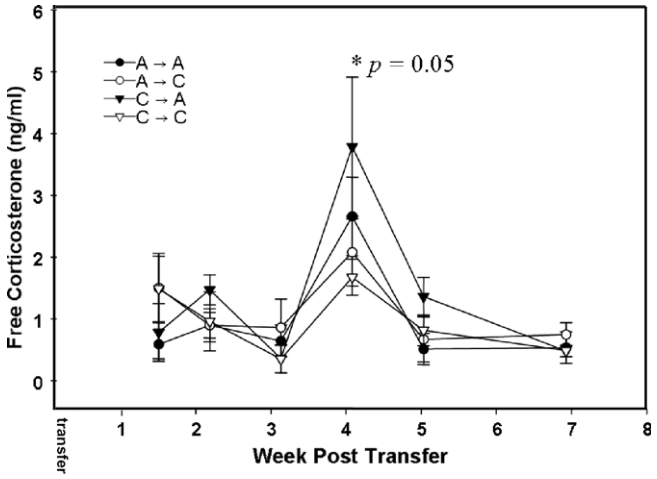

Fig. 5. Circulating unbound corticosterone concentrations of male southern toads moved from control (C) or ash (A) environments to control (C) or ash (A) mesocosms, A → A (closed circle), A → C (open circle), C → C (open triangle), C → A (closed triangle). Values are expressed as means ± 1 SE. *Denotes a significant $p = 0.05$.

4. Discussion

There are very few characterizations of amphibian CBG; several early studies identified specific binding, but did not further characterize the hormone-binding protein interaction (Jolivet-Jaudet and Leloup-Hatey, 1986; Martin and Ozon, 1975). CBG has been well characterized in *Ambystoma tigrinum* (Orchinik et al., 2000). *A. tigrinum* CBG binds corticosterone, testosterone, and progesterone with a higher affinity than *B. terrestris* CBG and in a slightly different rank order (dihydrotestosterone > dexamethaxone > corticosterone > testosterone > progesterone vs. dihydrotestosterone > corticosterone ≫ progesterone = testosterone ⋙ dexamethaxone for *B. terrestris*). Interestingly, the greatest difference between the two proteins was in their binding of dexamethasone. *A. tigrinum* CBG bound dexamethasone with very high affinity, while *B. terrestris* CBG did not bind dexamethasone at all. However, both binding proteins bind androgens and endogenous glucocorticoids with high affinity. In *B. terrestris*, CBG affinity for dihydrotestosterone is high, but the percent inhibition of corticosterone was relatively low (only 46%). This result could potentially indicate the presence of two classes of corticosterone binding sites, wherein dihydrotestosterone only binds half of the potential corticosterone sites. Unfortunately, we are unable to confirm this hypothesis with our current data.

It is not surprising that CBG in anurans exhibits different affinities from species to species. It has been shown that, although CBG has conserved steroid binding sites, there is only a small amount of structural similarity among mammalian species (Kato, 1988: Kato et al., 1988). If this large degree of variation is also the case in anurans, the structural dissimilarity may explain the observed differences in hormone specificity. Structural dissimilarity in anuran CBG is also supported by the varying K_d's. Known K_d's for amphibian CBG's range from 1 nM in *Pleurodeles waltlii* to 82 nM in *Rana temporaria* (Martin and Ozon, 1975), *B. terrestris* falls in the middle of the range with a K_d of 20.6 nM.

In this study we exposed toads to a persistent, low-level, chronic stressor (coal-combustion-waste) that, in previous studies, have induced chronic, significantly elevated levels of total plasma corticosterone (Hopkins et al., 1997). Our experimental treatment significantly increased body loads of 11 trace metals four weeks into exposure, and metal levels remained elevated for the remainder of the experiment (Ward, Hassan and Mendonça, submitted). However, coal-combustion-waste-exposed toads did not exhibit any of the expected metabolic abnormalities, such as increased standard or exercise metabolic rate, abnormal glucose levels, or weight loss (Ward et al., 2006), which are common in other coal-combustion-waste-exposed organisms (Hopkins et al., 1999; Rowe, 1998a; Rowe et al., 1998b). Due to these observed lack of dysfunction, we hypothesized that, in toads exposed to a persistent stressor (elevated body loads of heavy metals), CBG may increase, binding excess corticosterone, and, thus, buffering the actual amount of free corticosterone available. Thus, an elevation of CBG could function in a protective role and shield the organisms from the deleterious effects imposed by chronic stress.

When we measured CBG in response to exposure, we did not see the predicted protective response. First, we determined that there was no significant difference in CBG levels ($p > 0.1$) among the 4 treatment groups at the only time point during the treatment. that total plasma corticosterone was elevated in the C → A group (i.e. the 4th week of exposure). The C → A group was the only group to exhibit a significant increase in corticosterone, indicating that the increase in CBG seen in all groups was not specific to the ash exposure.

Second, when CBG levels were evaluated in this C → A group, we found that there was enough CBG present to bind 83% of circulating significantly elevated total corticosterone, reducing this significant increase in total corticosterone (e.g. 22.6 ng/ml total corticosterone vs. an estimated 3.79 ng/ml of free (unbound) corticosterone for C → A toads at four weeks). However, paradoxically CBG appeared to accentuate the difference in corticosterone levels between control toads and exposed C → A. That is, *total* corticosterone levels of C → A toads were only elevated 60% above that of controls, while *free* corticosterone levels in the same group increased 211% above controls. Therefore, CBG was not circulating in high enough concentrations to buffer the elevated levels of plasma corticosterone associated with the stress response at the time point (i.e. the 4th week of exposure) that C → A toads experienced a significant elevation of corticosterone at four weeks.

CBG seems to play a different role than we first predicted. CBG does not appear to be acting as a protective mechanism by buffering the corticosterone response. It is possible that CBG may be playing other roles in the stress

response, such as transporting corticosterone to effector tissues or by CBG's own biological action, binding to membrane receptors and initiating cellular processes (Rosner, 1990). If CBG actively delivers corticosterone and/or initiates its own cellular response, than the lack of increase in CBG in response to pollutants could reduce the cellular effect of corticosterone. Further research is needed to determine if alternative roles of CBG exist in this system.

In contrast, CBG has been found to mediate stress responses in other organisms such as mice (D'Elia et al., 2005) and humans (Ho et al., 2006). In both of these studies, CBG decreased in response to acute stress, allowing for free corticosterone to remain high, which may increase available energy. We did not observe a decrease in CBG in any group from our study. CBG was elevated in all groups, regardless of the circulating corticosterone levels or treatment. It is likely that circulating CBG in an organism changes depending on the energetic demands of the organism and the stressors to which they are exposed. Stressors themselves may also affect the structure and, therefore, the binding capacity of CBG; to compensate CBG concentrations could increase or decrease. For example, mice experience changes in CBG binding capacity after thermal injury as a result of a possible conformational change (D'Elia et al., 2005). It is possible that exposure to heavy metals changed the structure of the CBG molecule, and, therefore, the binding capacity of CBG, allowing for free corticosterone to remain high.

Additional research needs to be done to explore the roles of CBG in organisms exposed to both acute and chronic stressors to better elucidate its role in stress mechanisms and its interaction with corticosterone. By understanding CBG and other mechanisms associated with toxicant exposure, we may be better able to understand the complex interaction between the pollutants in an environment and the physiology of an organism. This level of understanding may give us insight into some of the factors leading to large-scale population declines.

Acknowledgments

We thank A. Appel, C. Guyer, K. Navara, P. Kahn, C. Schmaeman and M. Grilliot for their comments on this manuscript. We also thank W. Hopkins for all of his help. M. Carpenter, R. Birkhead, T. Folk assisted with statistics. C. Guyer and N. Liu assisted with project design. R. Birkhead, N. Hall, T. Smith, J. Thill, E. Carnahan, J. Pitchford, and D. Rues assisted in running this project. Thank you as well to P.West and B. Staub for their help with metal analysis. Partial funding provided by the ASIH Gaige Fund. Auburn University's Animal Care and Use Committee approved all studies (#2003-0467, 0211-R-2424).

References

Andrews, E.J., 1993. 1993 report of the AVMA panel on euthanasia. J. Am. Vet. Med. Assoc. 202, 229–249.

Armario, A., Giralt, M., Marti, O., Gavalda, A., Hidalgo, J., 1994. The effect of acute and chronic ACTH administration on pituitary–adrenal response to acute immobilization stress. Relationship to changes in corticosteroid-binding globulin. Endocrine Res. 20, 139–149.

Barsano, C.P., Baumann, G., 1987. Editorial: Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? Endocrinology 124, 1101–1106.

Bonga, S.E.W., 1997. The stress response in fish. Physiol. Rev. 77, 591–626.

Breuner, C.W., Lynn, S.E., Julian, G.E., Cornelius, J.M., Heidinger, B.J., Love, O.P., Sprague, R.S., Wada, H., Whitman, B.A., 2006. Plasma-binding globulins and acute stress response. Hormone Metab. Res. 38, 260–268.

Breuner, C.W., Orchinik, M., 2000. Downstream from corticosterone: seasonality of binding globulins, receptors and behavior in the Avian stress response. In: Dawson, A., Caturvedi, C.M. (Eds.), Avian Endocrinology. Narosa Publishing House, New Delhi, India, pp. 385–399.

Breuner, C.W., Wada, H., Shyu, J., Love, O.P., 2003. Corticosteroid-binding globulin capacity responds to chronic hormone treatment but not acute stressors. Integrat. Comp. Biol. 43, 1013.

Carey, C., Alexander, M.A., 2003. Climate change and amphibian decline: is there a link? Divers. Distrib. 9, 111–121.

Chard, T., 1995. An Introduction to Radioimmunoassay and Related Techniques. Elsevier, New York.

D'Elia, M., Patenaude, J., Hamelin, C., Dominique, R.G., Bernier, J., 2005. Corticosterone binding globulin regulation and thymus changes after thermal injury in mice. Am. J. Physiol. Endocrinol. Metab. 288, E852–E860.

Deviche, P., Breuner, C., Orchinik, M., 2001. Testosterone, corticosterone, and photoperiod interact to regulate plasma levels of binding globulin and free steroid hormone in dark-eyed juncos, *Junco hyemalis*. Gen. Comp. Endocrinol. 122, 67–77.

Fleshner, M., Deak, T., Spencer, R.L., Laudenslager, M.L., Watkins, L.R., Maier, S.P., 1995. A long-term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. Endocrinology 136, 5336–5342.

Gutherie, R.K., Cherry, D.S., 1979. Trophic level accumulation of heavy metals in a coal ash basin drainage system. Water Resour. Bull. 15, 244–248.

Ho, J.T., Al-Musalhi, H., Chapman, M.J., Quach, T., Thomas, P.D., Bagley, C.J., Lewis, J.G., Torpy, D.J., 2006. Septic shock and sepsis: a comparison of total and free plasma cortisol levels. J. Clin. Endocrinol. Metab. 91, 103–114.

Hopkins, W.A., Mendonça, M.T., Congdon, J.D., 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Gen. Comp. Endocrinol. 108, 237–246.

Hopkins, W.A., Rowe, C.L., Congdon, J.D., 1999. Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. Environ. Toxicol. Chem. 18, 1258–1263.

Jennings, D.H., Ruys, J.D., Moore, M.C., Orchinik, M., 2001. Corticosterone regulation of plasma steroid-binding globulin levels and free steroid hormone levels in Tree lizards, *Urosaurus ornatus*. Amer. Zool. 41, 1485–1486.

Jolivet-Jaudet, G., Leloup-Hatey, J., 1986. Corticosteroid-binding in plasma of *Xenopus laevis*. Modifications during metamorphosis and growth. J. Steroid Biochem. 25, 343–350.

Jolivet-Jaudet, G., Inoue, M., Takada, K., Ishii, S., 1984. Circannual changes in corticosterone plasma levels and binding of corticosterone to plasma in *Bufo japonicus formosus*. Zool. Sci. 1, 317–325.

Kato, E.A., Hsu, B.R.S., Kuhn, R.W., 1988. Comparative structural analyses of corticosteroid-binding globulin. J. Steroid Biochem. Mol. Biol. 29, 213–220.

Lynn, S.E., Breuner, C.W., Wingfield, J.C., 2003. Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird. Horm. Behav. 43, 150–157.

Martin, B., Ozon, R., 1975. Steroid-protein interactions in nonmammalian vertebrates. II. Steroids binding proteins in the serum of amphibian; a physiological approach. Biol. Reprod. 13, 371–380.

Mendel, C.M., 1989. The free hormone hypothesis: a physiological based mathematical model. Endocr. Rev. 10, 232–274.

Mendonça, M.T., Chernetsky, S.D., Nester, K.E., Gardner, G.L., 1996. Effects of gonadal sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus*, upon arousal from hibernation. Horm. Behav. 30, 153–161.

Orchinik, M., Matthews, L., Gaasser, P.J., 2000. Distinct specificity for cotricosteroid binding sites in amphibian cytosol, neuronal membranes and plasma. Gen. Comp. Endocrinol. 118, 284–301.

Pounds, J.A., Crump, M.L., 1994. Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. Conserv. Biol. 8, 72–85.

Rosner, W., 1990. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocr. Rev. 11, 80–91.

Rowe, C.L., 1998a. Elevated standard metabolic rate in a freshwater shrimp (*Palaemonetes paludosus*) exposed to trace element-rich coal combustion waste. Comp. Biochem. Physiol. 121A, 299–304.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998b. Elevated maintenance costs in an Anuran (*Rana catesbeinana*) exposed to a mixture of trace elements during embryonic and early larval periods. Physiol. Zool. 71, 27–35.

Silverin, B., 1986. Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. Gen. Comp. Endocrinol. 64, 67–74.

Tinnikov, A.A., Legan, M.V., Sheveluk, N.A., Cvetovkaya, G.A., Naumenko, S.E., Sidelnikov, S.G., 1996. Corticosteroid and immune responses to cardiac surgery. Steroids 61, 411–415.

Tinnikov, A.A., 1993a. Corticosteroid-binding globulin levels in the rat serum under conditions of starvation and restriction of motions. Horm. Metab. Res. 25, 88–89.

Viau, V., Sharma, S., Meaney, M.J., 1996. Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. J. Neuroendocrinol. 8, 1–8.

Vogeser, M., Felginger, T.W., Kilger, E., Roll, W., Fraunberger, P., Jacob, K., 1999. Corticosteroid-binding globulin and free cortisol in the early postoperative period after cardiac surgery. Clin. Biochem. 32, 213–216.

Ward, C.K., Appel, A.G., Mendonça, M.T., 2006. Metabolic measures of male southern toads (*Bufo terrestris*) exposed to coal combustion waste. Comp. Biochem. Physiol. A 143, 353–360.