

BEYOND CARRIER PROTEINS

Plasma binding proteins as mediators of corticosteroid action in vertebrates

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Abstract

Stressors elicit a complex but variable suite of endocrine events. Comparative studies of the stress response have focused primarily on the adrenocortical response to stress, in particular the measurement of plasma levels of glucocorticoids. However, a number of other factors contribute to and modify cellular and organismal responses to glucocorticoids. Notably, plasma corticosteroid binding globulins (CBGs) can regulate the general availability of steroid to tissues, and/or direct the delivery of hormones to specific sites. In this paper, we discuss possible functions of CBG and mechanisms of CBG action, review CBG characteristics among vertebrates, and discuss our recent studies indicating that CBG may indeed modulate responses to stressors. For example, in house sparrows, we found that basal and stress-induced concentrations of total corticosteroid (cortisol or corticosterone) (CORT) vary seasonally, but CBG concentrations change proportionally,

so that free CORT concentrations appear static year-round. In contrast, in white-crowned sparrows and tree lizards, CBG concentrations change under conditions when total CORT levels do not, resulting in significant changes in circulating free CORT. These differences in free CORT are masked if CBG is not accounted for. We have also found that the binding properties of CBG vary considerably between species and need to be determined empirically. Such studies led to the observation that CBG in several species may also serve as a functional androgen binding protein; this is especially important for birds, because previous studies had concluded that birds lack androgen binding globulins. We propose that consideration of CBG is paramount to understanding the role of glucocorticoids in mediating behavioral and physiological responses to stress.

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Introduction

The acute adrenocortical response to stress is highly conserved across vertebrate classes; with few exceptions, glucocorticoid levels increase significantly within a few minutes of a perceived stressor. Historically, the stress response was classified as a non-specific reaction to a multitude of stressors (Selye 1936). However, the magnitude of the adrenocortical response, and the resulting physiological and behavioral changes, can be modulated by a number of factors, such as body condition, reproductive state or environmental conditions. The plasticity in the stress response appears to allow for the appropriate level of behavioral or physiological response given the conditions at hand (Orchinik *et al.* 2002).

Investigations into the plasticity of the stress response have largely focused on the regulation of the hypothalamic–pituitary–adrenal (HPA) axis and plasma

corticosteroid (CORT; cortisol or corticosterone) levels. It is common to regard CORT release as the entire organismal response to a stressor. However, multiple factors ‘downstream’ of glucocorticoid secretion, such as receptor number or the abundance of plasma binding proteins, may be regulated to alter the organismal response to stress. CORT binding globulin (CBG) binds CORT with high affinity (Westphal 1971), and is regulated according to physiological and environmental conditions. This binding of CORT to CBG may serve as a tissue buffer against potentially deleterious effects of elevated CORT, regulate the availability of free hormone to target tissues, or alter CORT clearance rates. CBG may also regulate CORT actions by altering local concentrations of CORT or via interaction with signaling pathways initiated in cell membranes.

In this paper, we will outline some of the recent discoveries about CBG action. The primary goal of the

paper, however, is to illustrate how CBG may play an active role in regulating the plasticity of organismal responses to stress, particularly by altering the free levels of hormones. Our recent studies indicate that this is a dynamic component of CORT action with some unexpected outcomes.

CBG function

Free hormone hypothesis

According to the free hormone hypothesis, steroid bound to plasma binding globulins is unavailable to tissues; the 'free' (unbound) hormone is the biologically active fraction, able to enter cells, activate intracellular or membrane receptors, and also be available for metabolism in the liver. Within this hypothesis, the primary role of CBG is to regulate the bioavailability and metabolic clearance of glucocorticoids (Westphal 1983, Mendel 1989, Ekins 1990).

Two examples support the hypothesis that free CORT is the biologically active fraction (i.e. the fraction entering tissues). First, metabolic clearance rates are inversely correlated with percent bound hormone (i.e. the greater the fraction of bound hormone, the slower the metabolic clearance (Siiteri *et al.* 1982)). This relationship was tested in a human clinical trial; clearance rates of radioactively labeled CORT were lower in subjects with higher CBG capacity (Bright 1995). This indicates that CORT available for metabolism is from the free fraction. If CBG were delivering a significant portion of bound CORT to tissues (a hypothesis discussed in the next section), then the degradation rate would be unrelated to CBG capacity, as both free CORT reaching the liver, and bound CORT reaching tissues, would be degraded. Perhaps more compelling is the tight regulation of free CORT levels in humans with abnormal CBG. Three separate mutations in the CBG gene have arisen that decrease affinity for CORT (Van Baelen *et al.* 1982, Emptoz-Bonneton *et al.* 2000, Torpy *et al.* 2001). In patients carrying these mutations (the Lyon mutation or the null mutation), total CORT levels are lower than in control individuals. The net result is that free CORT levels are the same between groups. Therefore, total CORT secretion is regulated to maintain a given free CORT level; these data indicate that, in the regulation of total CORT levels, free CORT is the physiologically relevant fraction.

While there is continued support for this hypothesis, and most who work on CBG agree that free hormone may be the primary regulator of hormone action, there is evidence for more complex actions of CBG at the systemic and cellular levels. Many of these more complex actions allow for CBG to contribute to localization of CORT action, instead of solely regulating the amount of free hormone that is available to all tissues. The basic

components of the following discussion were originally informed by two excellent reviews (Rosner 1990, Hammond 1995), which offer more detailed discussions of each issue.

CORT reservoir, with localized vascular delivery?

Consistent with the free hormone hypothesis is the idea that CBG provides a reservoir of CORT in the plasma (CORT is hydrophobic; binding to CBG increases the solubility of CORT in plasma). Within this framework, CBG serves to store CORT in the plasma for release if it is needed. This release of CORT from CBG could affect all tissues, or it could be targeted at certain tissue sites. Since more than 90% of total CORT may be bound to CBG in plasma, and CBG may be dynamically regulated by diverse factors, it is important to consider the reservoir of CORT available under different physiological or environmental conditions. Multiple conditions have been identified which decrease total CBG capacity, increasing free CORT levels available to all tissues. Restraint or tail-shock stress, social stress and food deprivation have all been shown to decrease CBG capacity (Tinnikov 1993, Fleshner *et al.* 1995, Spencer *et al.* 1996, Marti *et al.* 1997, Alexander & Irvine 1998). While this does not appear to be a rapid mechanism for increasing free CORT, the regulation of CBG levels may enable an animal to maintain an elevated free CORT titer when total CORT levels are no longer elevated.

More intriguing is the possibility that CBG holds a reservoir of CORT that is released at localized sites in the vasculature. Mammalian CBG is a member of the serine protease inhibitor (serpin) superfamily (e.g. Pemberton *et al.* 1988, Hammond *et al.* 1991). One characteristic of these proteins is that they are cleaved by the serine proteases that they inhibit. Activated neutrophils at sites of inflammation carry and secrete serine proteases. These proteases can then cleave CBG, releasing CORT and increasing the local concentration of free CORT over what is available in the general circulation. Pemberton *et al.* (1988) hypothesized that CBG evolved as a carrier protein for CORT because of this characteristic, which allows for local delivery of free CORT to sites of inflammation.

Intracellular localization of CBG

CBG has been identified within multiple cell types (Werthamer *et al.* 1973, Perrot-Appanat *et al.* 1981, 1984, Siiteri *et al.* 1982, Kuhn *et al.* 1986). CBG may arrive at these locations through active cellular uptake across the cell membrane, or through local (extra-hepatic) synthesis. Cellular uptake of CBG is thought to be achieved through binding of CBG-CORT complex to a membrane CBG receptor, which then either transports the CBG-CORT complex across the membrane, or is internalized through

endocytosis. After internalization, CBG would be cleaved, increasing the amount of free CORT available to activate intracellular receptors. CBG membrane binding sites (possibly receptors) have been identified in human liver, decidual endometrium, placental syncytiotrophoblast and prostate, rat spleen and liver, and rhesus kidney, spleen, liver and uterus (Hryb *et al.* 1986, Singer *et al.* 1988, Strel'chyonok and Avvakumov 1991, Maitra *et al.* 1993); these receptors could serve to transport the CBG-CORT complex into the cells. In both human breast cancer and rat hepatoma cell lines, active internalization of CBG has been documented (Kuhn 1988, Nakhla *et al.* 1988). This mechanism could serve to locally increase free CORT levels above what could be achieved through simple diffusion of free CORT from the blood. With this mechanism it is also possible to locally enhance CORT action at a tissue or cell-specific level, by increasing the number of CBG transporters.

There is evidence for extra-hepatic CBG synthesis in lung, ovary, endometrium, trophoblast cells, neonatal kidney and fetal exocrine pancreas and pituitary (Hammond *et al.* 1987, Scrocchi *et al.* 1993a,b, Berdusco *et al.* 1995, Misao *et al.* 1995, 1999, Seralini 1996, Benassayag *et al.* 2001). In contrast to cellular internalization of the CBG-CORT complex, which serves to locally increase free CORT levels, cellular synthesis of CBG is thought to limit the accessibility of CORT to the intracellular receptors. CBG mRNA and protein have been localized to the corticotropes in the mammalian pituitary (Berdusco *et al.* 1995); in this case, Berdusco *et al.* hypothesized that CBG interferes with CORT's negative feedback on adrenocorticotropin (ACTH) secretion. Taken together, the localization of CBG to tissues may serve to locally enhance or inhibit CORT action in that tissue. This complex interaction of binding proteins, binding-protein receptors and hormone receptors has been well characterized in the insulin-like growth factor (IGF) system, where at least six different IGF binding proteins regulate IGF accessibility and entry into cells (for review, see Kelley *et al.* in this volume).

Biologically active CBG

There is evidence that when CBG binds to its membrane receptor, it activates intracellular second-messenger systems, increasing cAMP within the cell. The studies come from MCF7 cells (a mammary carcinoma line (Nakhla *et al.* 1988)), as well as trophoblast cells from a human embryo (Strel'chyonok & Avvakumov 1991). In both systems, CBG-cortisol complex binds to a membrane receptor with high affinity and causes accumulation of intracellular cAMP within minutes; in both systems, CBG without CORT has no effect. These data suggest that the CBG molecule is not just for storage or localized delivery of CORT, but has intrinsic biological activity.

Hence, there is widespread support for the free hormone hypothesis, but growing support for CBG playing a more active and directed role in mediating physiological responses. At the cellular level, it is likely that free CORT is the primary mediator of CORT action. However, delivery of free CORT may be mediated by CBG, especially at sites of inflammation. The general biological relevance of CBG-stimulated cAMP production has yet to be established; this may or may not be important for regular physiological function. Throughout the rest of this review, we primarily discuss the effects of CBG on free hormone levels, but we include consideration of alternative functions of CBG, and how they would affect glucocorticoid action during stress.

CBG diversity

Mammalian CBG is a member of the serpin superfamily of serine protease inhibitors (Hammond *et al.* 1987). Molecules in this superfamily have conserved sequences that serve as substrates for one of the serine proteases. CBGs are glycoproteins containing a partially conserved steroid binding site and several conserved glycosylation sites; changes in specific amino acids or the glycosylation state can alter steroid binding affinity (Avvakumov *et al.* 1993, Avvakumov & Hammond 1994a,b, Emptoz-Bonneton *et al.* 2000). However, overall, there is a rather low degree of structural similarity between CBGs in the mammalian species studied (Kato *et al.* 1988). Non-mammalian CBG molecules have not been studied, and the only cDNA sequence identified as a CBG in a non-mammalian species is from the zebrafish (GenBank Acc: BG727884; BM156495; AW421242). However, given that the mouse and human CBG amino acid sequences are only 48% identical and the mouse and rat CBGs are only 73% identical, it may be that the zebrafish sequence represents another member of the serpin family and not CBG. It is interesting to note that the divergence in CBG structure is paralleled by a surprising diversity in binding specificity between species. Although a functional CBG has been identified in birds, reptiles, fish and amphibians on the basis of binding studies, we use the term CBG loosely and have no assurance that the functional CBG in non-mammalian species is the same molecule as mammalian CBG.

In Table 1, we have organized studies by vertebrate class. Affinity estimates generated by the individual studies vary widely, even when CBG from the same species is being considered. The high interspecies variation is most likely an artifact of variability in the methods used to generate the data (for a discussion of methodology, see Orchinik *et al.* (1997, 2000) and Breuner & Orchinik (2002)). However, general differences can be seen between groups of vertebrates. The new world monkeys are an interesting example. In general, new world

Table 1 Continued

	Affinity (nM)	Hormone**	Capacity (nM)	Specificity***	Reference
Mammals (continued)*					
<i>Rattus norvegicus</i> (laboratory rat)	—	B	—	B>F=P ₄ >>ALDO>DEX>>E	Feldman et al. (1979)
Male Holtzman Sprague—Dawley	0.6–0.72	B	750	—	Fleshner et al. (1995)
Female Sprague—Dawley	—	—	1750–2050	—	Hsu & Kuhn (1988)
Long—Evans	—	B	525	—	Spencer et al. (1996)
<i>Microtus ochrogaster</i> (prairie vole)	14.7	B	716	F=B>>P ₄ >>DEX>>ALDO	Orchinik et al. (1997)
<i>Ovis aries</i> (sheep)	9.2	F	78	—	Gayrard et al. (1996)
<i>Bos taurus</i> (cow)	13	F	110	—	Gayrard et al. (1996)
<i>Equus caballus</i> (horse)	19	F	220	—	Gayrard et al. (1996)
<i>Canis domesticus</i> (dog)	7.9	F	155–192	—	Alexander & Irvine (1998)
<i>Petaurus breviceps</i> (sugar glider—marsupial)	10.5	F	82	—	Gayrard et al. (1996)
Birds*					
<i>Zonotrichia leucophrys</i> (white-crowned sparrow)	3.6–5.1	B	—	—	Breuner (unpublished observations)
	1.65	B	—	—	Lynn et al. (In Press)
	2.4	B	76	P ₄ >F>B>T=E>DHT	Wingfield et al. (1984)
	460–525	B	90–160	—	Romero & Wingfield (1998)
<i>Zonotrichia albicollis</i> (white-throated sparrow)	40	B	1520	—	Meier et al. (1977)
<i>Junco hyemalis</i> (dark-eyed junco)	2.74–3.08	B	117–365	P ₄ >B>DEX>T=DHT>>E	Deviche et al. (2001)
	20–50	B	30–170	—	Klukowski et al. (1997)
	490	B	60–100	—	Romero et al. (1998b)
	639	B	60–88	—	Romero et al. (1998a)
<i>Carduelis flammica</i> (redpolls)	456–794	B	65–110	—	Romero et al. (1998c)
<i>Passer domesticus</i> (house sparrow)	1.8	B	60–220	—	Breuner & Orchinik (2001)
<i>Ficedula hypoleuca</i> (pied flycatcher)	25	B	8–117	B=P ₄ >>E=T>DHT	Silverin (1986)
<i>Coturnix coturnix</i> (Japanese quail)	40–80	B	60–730	P ₄ >B>T>E>F=DHT	Wingfield et al. (1984)
<i>Columba livia</i> (pigeon)	478	B	77	P ₄ =B>>E=T=F	Wingfield et al. (1984)
<i>Anas platyhynchos</i> (mallard)	7.5	B	3	B>P ₄ >F>T>E	Wingfield et al. (1984)
<i>Gallus domesticus</i> (chicken)	55.9	B	14.4	B>P ₄ >T>DHT>F>>E	Wingfield et al. (1984)
Chicks, adults	1.95	B	45–75	—	Fassler et al. (1986)
<i>Larus occidentalis</i> (western gull)	3.8–5.0, 4.5	B	—	T>B	Savu et al. (1986)
	44.7	B	35.5	P ₄ =B>>F=E>T=DHT	Wingfield et al. (1984)
Reptiles*					
<i>Urosaurus ornatus</i> (tree lizard)	K _i =527 nM	B	1900–3250	DHT>T>P ₄ >>B>>E	Jennings et al. (2000)
Amphibians*					
<i>Rana temporaria</i> (common frog)	1.2	B	130	B>P ₄ >T>>E	Martin & Ozon (1975)
<i>Discoglossus pictus</i> (painted frog)	4.5	B	640	T=P ₄ =B>>E	Martin & Ozon (1975)
<i>Salamandra salamandra</i> (fire salamander)	6.7	B	480	T>B=P ₄ >E	Martin & Ozon (1975)
<i>Ambystoma tigrinum</i> (tiger salamander)	2.7	B	70	DHT=D<DEX>CORT=T>P ₄ >E>ALDO	Orchinik et al. (2000)
Bony fish*					
Idler & Freeman (1968) identified specific binding of cortisol to plasma of six species of salmon, cod, and trout.					Idler & Freeman (1968)
Cartilaginous fish*					
<i>Psycyllorhinus canicula</i> (dogfish)	15.4–33.3	B	1000–1300	E=P ₄ =DHT>T>B>F	Martin (1975)

*Seal & Doe (1965) published estimates of capacity in 131 species, with multiple vertebrate classes represented. However, they did not assess affinity, and used only one concentration of steroid, so their methods could grossly underestimate capacity. The data (not shown here) are most useful to determine if there is specific binding of B or F to plasma in that species.

**Hormone used to assess affinity: F=cortisol, B=corticosterone.

***ALDO, aldosterone; DEX, dexamethasone; DHT, dihydrotestosterone; T, testosterone; P₄, progesterone; E, estrogens.

monkeys have much higher levels of total cortisol than old world monkeys and primates (Klosterman *et al.* 1986). This is thought to be due to a mutation in the glucocorticoid receptor gene, resulting in this receptor having unusually low affinity for glucocorticoids (Hammond *et al.* 1994); more recent studies, however, indicate that it may be a change in the interaction of the glucocorticoid receptor with chaperone proteins (Scammell *et al.* 2001). CBG in new world monkeys (squirrel monkey, dusky titi and owl monkey from the Table) also has lower affinity for cortisol, possibly allowing for more cortisol to reach tissues. It is difficult to assess actual affinity and capacity from the wide variety of results in the Table, but in studies that assess cortisol's affinity for CBG in both new world and old world monkeys (Siiteri *et al.* 1982, Pugeat *et al.* 1984, Klosterman *et al.* 1986), new world monkeys generally have 5- to 20-fold lower affinity for cortisol. As for capacity, Pugeat *et al.* (1984) consistently reported lower capacity in new world monkeys (squirrel and owl monkeys) than in the other primates (human, chimpanzee, rhesus macaque, crab-eating macaque and guinea baboon) and Robinson *et al.* (1985) could not detect any CBG in new world primates, whereas Klosterman *et al.* (1986) showed no differences between new world monkeys and other primates.

Pregnancy also can affect CBG. In humans, CBG capacity approximately triples as estrogen levels rise (Brien 1981, Hammond & Lahteenmaki 1983, Robinson *et al.* 1985). However, the effect is not seen in rhesus macaques or baboons, where CBG capacity increases in the first half of pregnancy, but then declines during the second half (Oakey 1975, Stanczyk *et al.* 1986).

Across classes, specificity of CBG varies. In mammals, CBG generally has high affinity for glucocorticoids and progesterins, and low affinity for androgens, estrogens and mineralocorticoids. The pattern is similar in birds, except that multiple species also appear to have relatively high affinity for androgens. This is noteworthy given that birds do not have a sex-steroid binding globulin, and will be discussed in more detail at the end of the next section. In amphibians, 'CBG' binds glucocorticoids and androgens with similar affinities. There is not enough information from reptiles and fish to note any general tendencies; the one species that has been well characterized in reptiles does not have a true CBG. It has an androgen binding globulin that also binds glucocorticoids with relatively low affinity. It seems that in multiple classes of vertebrates, CBG is a misnomer for the binding globulin that binds CORT.

Changes in either CBG capacity or affinity can alter the amount of CORT available to cells. Several equations have been used to estimate the amount of free hormone in plasma; using the equation of Barsano & Baumann (1989), it can be seen that the amount of free hormone (H_{free}) is dependent upon the amount of total hormone (H_{total}), the binding capacity of CBG in the plasma (B_{max}), and the affinity of hormone for CBG (K_a ; here expressed as $1/K_a$):

$$H_{\text{free}} = 0.5 \left[H_{\text{total}} - B_{\text{max}} - \frac{1}{K_a} \pm \sqrt{\left(B_{\text{max}} - H_{\text{total}} + \frac{1}{K_a} \right)^2 + 4 \left(\frac{H_{\text{total}}}{K_a} \right)} \right]$$

In the absence of other changes, an increase in CBG capacity would decrease the amount of free CORT in plasma. So while less CORT may be immediately available to target cells, increased CBG capacity would presumably decrease the metabolic clearance rate of CORT. In many species, the binding capacity of CBG is far greater than the concentration of total CORT in plasma, although this is not always the case (Deviche *et al.* 2001). If the CBG is present in concentrations far in excess of steroid, then small changes in the fraction of CBG occupied could have a large effect on the amount of free vs bound CORT. It is more likely to see changes in the binding capacity of CBG, rather than affinity, but differences in the glycosylation state of CBG may lead to changes in the affinity of CBG for steroid (Avvakumov *et al.* 1993, Avvakumov & Hammond 1994a).

How can CBG alter CORT action in vertebrates? Case studies

When CBG was first identified, there was a fair amount of work done elucidating the role of CBG in regulating free levels of hormone. Since that time, CBG has been studied primarily in the clinical arena (especially concerning pregnancy and obesity), and in basic studies using rodents (especially in relation to chronic stress). Few comparative studies have addressed the role of CBG in the stress response. Here we present four recent case studies in which CBG capacity, affinity and/or specificity alter the estimated level of hormone reaching tissues, and therefore the apparent magnitude of the organismal response to stress.

Seasonality in house sparrows

Many species show seasonal changes in either baseline or stress-induced total CORT levels (Licht *et al.* 1983, McLeese *et al.* 1994, Romero *et al.* 1997, Tyrrell & Cree 1998, Kenagy & Place 2000). Hypotheses generated to explain these seasonal changes differ, but generally it is thought that a higher level of stress-induced CORT represents a greater sensitivity to stressors, and therefore will result in a greater change in the behavior or physiology in response to the given stressor. For total CORT levels to represent free CORT levels, CBG levels would need to stay fairly constant throughout the year.

House sparrows (*Passer domesticus*) show a robust seasonal cycle in CORT secretion (Fig. 1). Baseline and

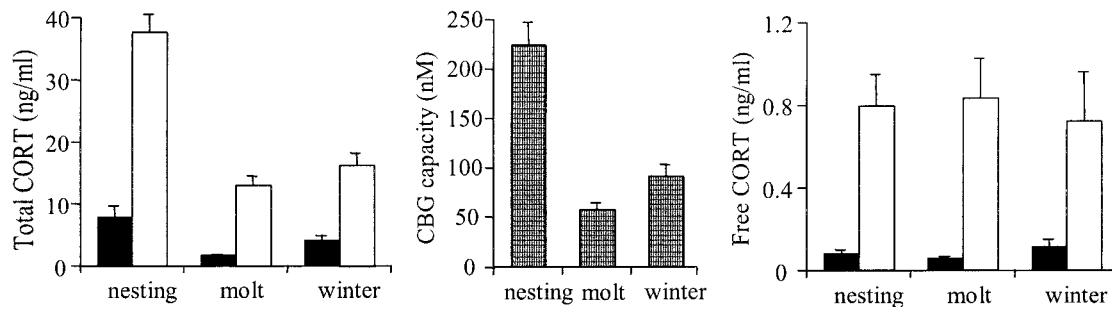


Figure 1 Total CORT, CBG capacity and free CORT titers (means \pm S.E.) in house sparrows ($n=16$, males and females combined) from three different periods in the annual cycle. Baseline (black bars) and stress-induced (white bars) total CORT and CBG titers changed seasonally, but there was no significant difference in estimations of baseline or stress-induced free CORT (ANOVA, $P=0.85$). Free CORT titers were estimated for each individual, calculated from that data. Redrawn with permission from Breuner *et al.* (2001). In each study presented in this paper, CBG capacity was measured using point sample radioligand binding experiments (bound and free fractions separated using rapid vacuum filtration over glass fiber filters, all samples run in triplicate), as described in Breuner & Orchinik (2001); however, incubation time, temperature, radioligand concentration and plasma concentration were optimized for each species.

stress-induced (following 30 min of capture and handling) total CORT levels were highest during nesting, and lower during molt and winter. However, CBG capacity changed in a similar manner. An equation (Barsano & Baumann 1989) was used to estimate free CORT levels based on total CORT, CBG capacity and the affinity of CORT for CBG. Because of the seasonal changes in CBG capacity, there was no seasonal difference in free CORT (Fig. 1; Breuner & Orchinik (2001)). Hence, despite large seasonal variation in total CORT levels, it appears that the amount of biologically active CORT was static throughout the year.

Why alter total CORT levels if free CORT levels are going to be static? These data suggest that it is not just free CORT that is important, but also CORT bound to CBG. As CORT and CBG increase from winter to the breeding season in house sparrows, free CORT levels are static, but CBG-bound CORT levels double. It may be that during the breeding season, house sparrows need a much larger reservoir of CORT: energetic needs are greater, metabolic rates are higher; a large reservoir of CORT allows for an increase in free CORT to regulate energy availability without activation of the HPA axis, or after the HPA axis has shut down. Alternatively, CBG may be actively delivering CORT to specific tissues during the breeding season, increasing the local concentration of free CORT at specific sites.

Food deprivation in white-crowned sparrows

Free-living animals can experience rapid decreases in food availability during severe storms, floods or extreme temperature fluctuations. Food restriction and food deprivation activate the HPA axis, increasing circulating glucocorticoid levels over hours to days (Woodward *et al.* 1991). The increase in CORT is thought to increase food searching behavior and foraging rates, until conditions deteriorate to

the point where abandonment of the home range is necessary (Wingfield & Ramenofsky 1997, Wingfield *et al.* 1997). But food availability and energetic state have been shown to influence CBG capacity as well (Woodward *et al.* 1991, Tinnikov 1993), which could alter the amount of CORT available to enter tissues. Lynn *et al.* (In Press) measured both CORT and CBG responses to food deprivation in wild-caught, captive white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Total CORT levels were elevated within 1 h of food deprivation, and remained elevated after 2 h. By 22 h without food (the beginning of the next 'day'), total CORT levels were no longer elevated over controls. However, CBG capacity was reduced by 22%, so that free CORT levels continued to be elevated (Fig. 2; Lynn *et al.* In Press). There are interesting implications of these data for free-living animals. For example, under low food conditions, free-living animals may show enhanced glucocorticoid secretion, leading to increased foraging and food searching behaviors. As the food deprivation continues, eventually CBG levels will drop, presumably eliminating the 'buffer' between adrenal secretions and CORT-sensitive tissues. Any further increase in total CORT would result in an unusually large surge in free CORT, which could induce territory abandonment and/or irruptive migration.

Morph-type regulation

Among fish, lizards, and mammals, alternative male reproductive tactics are common. These morphological and behavioral types (dominant vs subordinate males, territorial vs sneaker males, or site-faithful vs nomadic males) often show differences in steroid hormone patterns that relate to their breeding strategy (Moore *et al.* 1998), but little is known about patterns of binding globulins associated with these morphs. Subordinate males have lower CBG levels than dominant males in both horses and rats (Spencer *et al.*

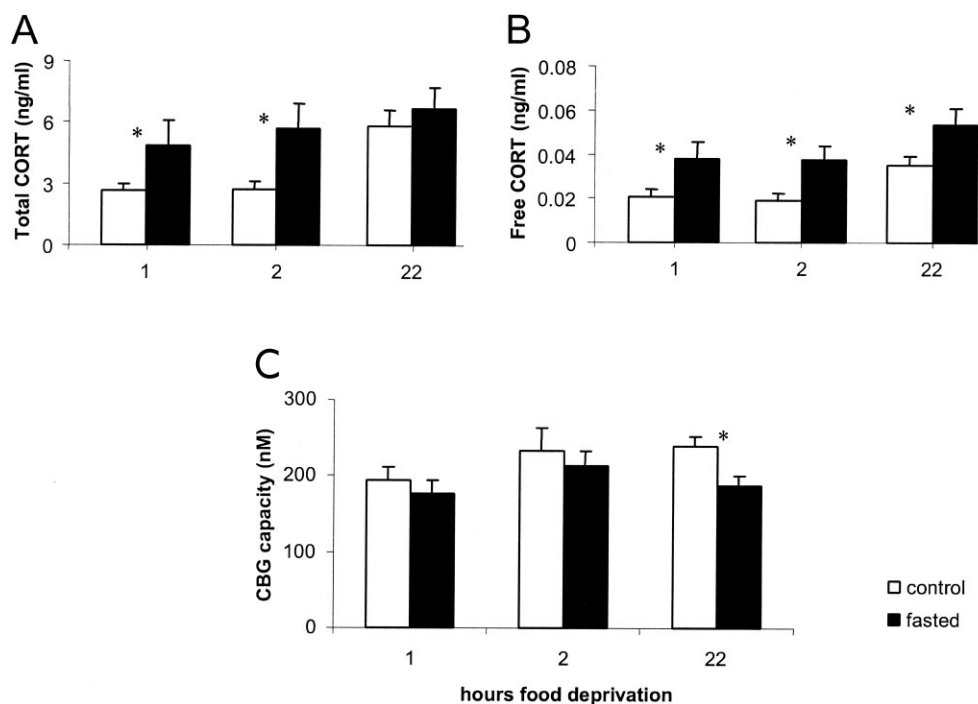


Figure 2 Total CORT, CBG capacity and free CORT titers in control and food-deprived captive white-crowned sparrows ($n=12$, means \pm s.e.). (A) Total CORT levels were measured within 3 min of entering the environmental chamber. Birds held under fasted conditions for 1 and 2 h had elevated total CORT compared with control conditions ($*P<0.05$). There were no differences in total CORT levels after 22 h. (B) Free CORT levels were estimated from total CORT, CBG capacity and the known affinity of CORT for CBG in this species. Free CORT levels were higher in fasted birds after 1, 2 and 22 h ($*P<0.05$). (C) CBG capacity was measured from the same samples. There were no differences in specific binding after 1 or 2 h of fasting, but specific binding decreased after 22 h fast ($*P<0.05$). Redrawn with permission from Lynn *et al.* (In Press).

1996, Alexander & Irvine 1998), but this seems to be a result of the agonistic interaction itself, not part of the baseline phenotype of the animal. Jennings *et al.* (2000) measured binding globulin levels in two male morphs of the tree lizard (*Urosaurus ornatus*), which show alternative reproductive strategies associated with different dewlap color patterns. Orange-blue males are aggressive, territorial, and site-faithful; orange males are less aggressive, non-territorial and nomadic (Moore *et al.* 1998). Baseline CORT and testosterone levels are similar between the two morphs; however, they differ in feedback effects of CORT on testosterone: testosterone levels are more sensitive (show a greater decline) to an increase in CORT in the orange, non-territorial morph than in the orange-blue, territorial morph (Knapp & Moore 1996, 1997). Jennings *et al.* (2000) hypothesized that differences in testosterone sensitivity could be due to differences in binding globulin levels between male morph types. Initially, Jennings *et al.* (2000) characterized the steroid binding globulins present in tree lizard plasma, and found two binding globulins: a sex-steroid binding globulin (which bound both estrogens and androgens), and a second binding globulin that bound primarily

androgens, but also glucocorticoids (see Table 1). This 'androgen-glucocorticoid binding globulin' (AGBG) binds CORT with low affinity ($K_i \sim 500$ nM), but is present in high enough capacity (~ 3 μ M) to bind a significant proportion of total CORT (78–95%) in the plasma. This 'AGBG' is therefore the functional CBG in this system. In fact, orange (subordinate) males have significantly less AGBG capacity than orange-blue males, leading to 2-fold higher stress-induced free CORT levels in orange males when total CORT levels are similar between morphs (Fig. 3; Jennings *et al.* 2000). Thus, the higher free CORT could have driven the decrease in testosterone seen in response to stress in the orange males, but not the orange-blue males.

Androgen binding globulin?

CBG in many species also has high affinity for androgens (see above). This high-affinity androgen binding could have different biological importance depending on the species concerned. In birds, androgen binding characteristics of CBG are especially relevant as no sex-steroid binding globulin has been characterized in this class. In

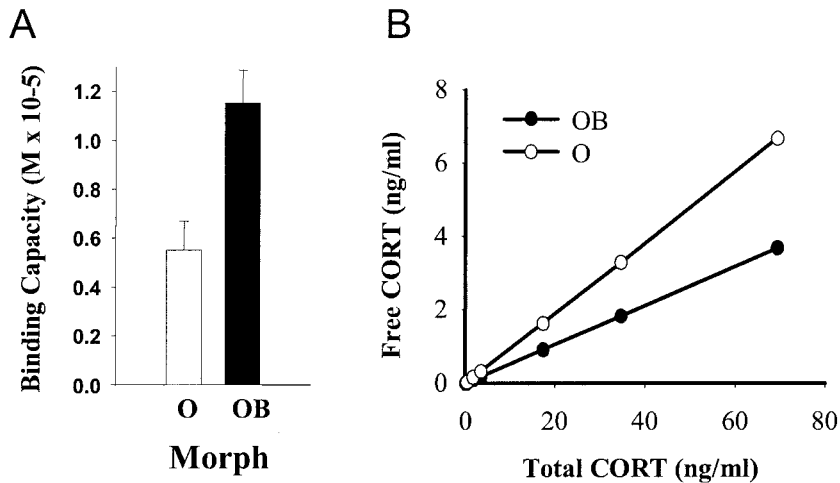


Figure 3 AGBG capacity in two male morphs of *Urosaurus ornatus*. (A) Orange ('O') males (non-territorial and nomadic) have lower AGBG capacity (means \pm s.e.) than orange-blue ('OB') males (aggressive, territorial and site-faithful; ANOVA: $P < 0.05$, $n = 15$ for each morph). (B) Estimates of free CORT levels in response to stress, based on known total CORT levels for each morph, average AGBG capacity for each morph, and the affinity of CORT for AGBG. Redrawn with permission from Jennings *et al.* (2000).

recent specificity studies, both dark-eyed juncos (*Junco hyemalis*) and white-crowned sparrows show affinities for CORT ranging from 1 to 5 nM, and affinities for testosterone about 10-fold lower ($K_i = 18.3$ nM; Fig. 4). Despite the lower affinity for CBG, Deviche *et al.* (2001) estimated that $\sim 90\%$ of testosterone was bound to CBG at baseline levels of CORT. Hence, CBG is a physiologically relevant androgen binding globulin, and may play a role in

regulating free testosterone levels during development and adulthood. In fact, in many avian studies, CBG capacity is greater when testosterone is naturally elevated (i.e. the breeding season: Romero & Wingfield (1998), Romero *et al.* (1998*b,c*), Deviche *et al.* (2001)) and experimentally elevated (i.e. via testosterone implant: Klukowski *et al.* (1997), Deviche *et al.* (2001)). These data suggest that a primary function of CBG during the breeding season may

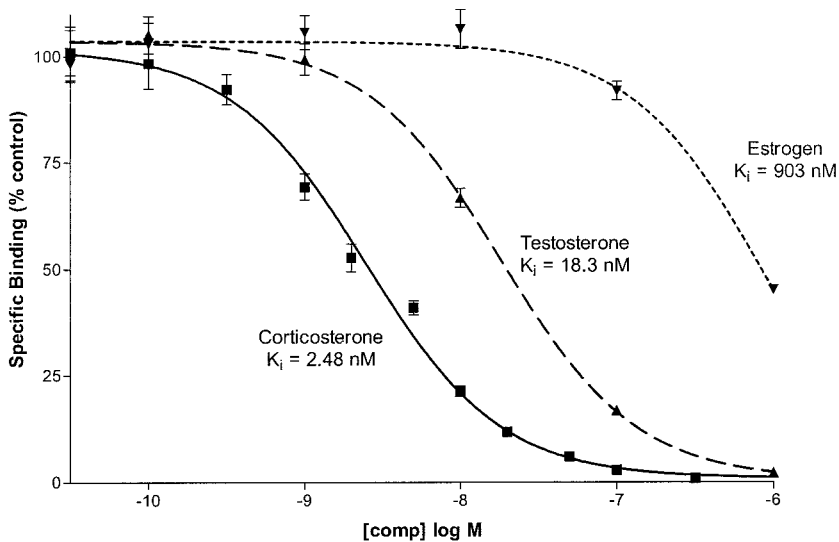


Figure 4 Specificity of white-crowned sparrow CBG. Inhibition of 2 nM [3 H]CORT binding to house sparrow plasma by unlabeled CORT (solid line), testosterone (dashed line) and estrogen (dotted line). Shown are specific binding data, expressed as the percentage of [3 H]CORT specific binding in the absence of competitor. K_i = the concentration of competitor required to inhibit 50% of [3 H]CORT binding.

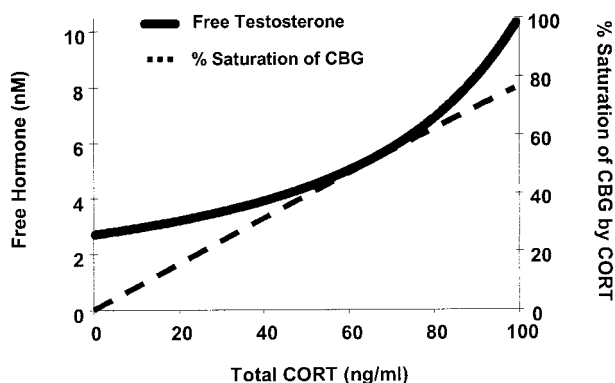


Figure 5 Estimated change in percent saturation of CBG (dashed line) and resulting increase in free testosterone (solid line) as total CORT increases (x-axis). Total CORT range represents baseline to maximum CORT levels reached in free-living dark-eyed juncos (from Deviche *et al.* 2000). As CORT increases in response to stress, testosterone is displaced from CBG, resulting in an increase in free testosterone. Redrawn with permission from Deviche *et al.* (2001).

be to regulate free testosterone. However, in each of these cases, CORT is also elevated during periods of high testosterone secretion or testosterone implant, so we are unable to evaluate if CBG functions primarily in regulating availability of CORT or testosterone.

The dual binding characteristic of CBG has interesting implications for the interaction of CORT and testosterone in breeding birds. Under non-stressed conditions in juncos, CORT occupied only 12% of total CBG capacity. Under these conditions, it is estimated that 90–95% of testosterone was bound to CBG. However, if the animal becomes stressed, total CORT levels can increase 5-fold, and should displace testosterone from CBG (due to the higher affinity for CORT). Therefore, an increase of CORT in response to stress will cause a surge in free testosterone (Deviche *et al.* 2001; Fig. 5). This displacement of testosterone from CBG could have a number of interesting effects. Primarily, the increase in free testosterone may have behavioral effects during stressful encounters (such as a territorial intrusion); part of the aggressive response to a territorial intrusion, or the maintenance of aggressive behaviors after the encounter is over may be due to an increase in free testosterone. Secondly, the increase in free testosterone will decrease overall testosterone levels through increased testosterone clearance, and increased negative feedback on the hypothalamo-pituitary-gonadal axis. This decrease in testosterone in response to stress occurs in many avian species, but has not been previously attributed to changes in CBG availability.

In these case studies CBG capacity appears to play a significant role in regulating CORT action. In general, what factors regulate CBG capacity? The results are a resounding 'it depends.' Hormonal manipulations have a wide variety of effects: estrogen and thyroid hormones can

increase CBG capacity (Gala & Westphal 1965, Spangler *et al.* 1969, Feldman *et al.* 1979, Brien 1981, D'agostino & Henning 1982, Stanczyk *et al.* 1986), but effects are dependent on sex and developmental stage; testosterone can increase or decrease CBG capacity, depending on the species (Gala & Westphal 1965, Assenmacher *et al.* 1975, Klukowski *et al.* 1997, Deviche *et al.* 2001); growth hormone can increase or decrease CBG capacity, depending on whether it is given tonically or cyclically (Jansson *et al.* 1989); interleukin-6 decreases CBG capacity (Tsigos *et al.* 1998); and altering CORT levels (through implant, injection or adrenalectomy) can increase or decrease CBG capacity, depending on the species or developmental stage (Feldman *et al.* 1979, Vallette *et al.* 1982, Cohen *et al.* 1990, Zao *et al.* 1997).

Season can also affect CBG capacity, but not predictably across vertebrates. In mammals, CBG capacity is lower during the breeding season (Bradley & Stoddart 1992, Boonstra *et al.* 2001). In birds, however, CBG capacity is elevated during all or part of the breeding season (Assenmacher *et al.* 1975, Daniel *et al.* 1981, Silverin 1986, Romero & Wingfield 1998, Romero *et al.* 1998*b,c*, Breuner & Orchinik 2001). This may be related to the differential effects of testosterone among species. In birds, testosterone increases CBG capacity, whereas in mammals, testosterone decreases CBG capacity.

Alternatively, stress effects on CBG capacity appear similar among most studies to date. Social stress (Spencer *et al.* 1996, Alexander & Irvine 1998), septic shock (Savu *et al.* 1981), immobilization (Tinnikov 1993, Marti *et al.* 1997), inescapable tail shock (Fleshner *et al.* 1995, Deak *et al.* 1999), food deprivation (Tinnikov 1993, Lynn *et al.* submitted), and surgery (Tinnikov *et al.* 1996, Vogeser *et al.* 1999) all decrease CBG capacity, although the effect is usually not apparent for at least a few hours. Given that stress tends to decrease CBG capacity, whereas CORT manipulations have no generalizable effect, the decrease in CBG capacity during stress may be due to another stress hormone (such as epinephrine or ACTH), or as a result of metabolic changes at the liver in response to one of the hormones released during stress.

Conclusions

Mammalian CBG is a member of the serpin superfamily of proteins, with high affinity for glucocorticoids and progestins. As one moves out of the mammalian class, little is known about the molecular structure of the molecule, and specificity varies widely.

Whether free or CBG-bound CORT is the biologically active fraction, CBG appears to play a significant role in regulation of plasticity of the stress response. In house sparrows, changes in CBG covary with total CORT, so that free CORT levels are static, but the pool of available CORT changes seasonally. In white-crowned sparrows

and tree lizards, CBG levels change when total CORT levels are static, causing significant changes in free and bound CORT. In each example, CBG alters free and bound CORT in ways that could not be predicted based on total CORT levels alone.

Given that CBG regulation cannot be predicted from total CORT, measurement of CBG is paramount to understanding the control of the behavioral and physiological response to stress. Future research directions in this field should include experimental manipulation of both total CORT and CBG capacity, measuring covariation of physiological or behavioral output with total CORT, free CORT or CORT-bound CBG. These types of experiments would allow for determination of which fraction is driving the changes that are measured. Additionally, molecular and cellular studies are needed in non-mammalian species to provide the tools to localize CBG and CBG receptors, and identify biological activity of the CBG-CORT complex.

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