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Plasma-binding Globulins and Acute Stress Response

Abstract

Within studies of acute stress physiology an increase in glucocorticoid secretion is thought to be the primary mediator of tissue response to stress. Corticosteroid-binding globulin may regulate tissue availability of steroids, but has not been considered a dynamic component of the acute stress response. Here, we examined CBG level over the common 60-minute time frame in an acute capture and handling protocol to investigate whether CBG capacity is dynamic or static over short stressors. Using a comparative approach, we measured CBG response to capture and handling stress in nine species of birds, representing five orders and nine families. CBG capacity significantly declined within 30 – 60 minutes of capture in five of the nine species examined. This decline may serve to significantly increase the level of corticosterone reaching tissues during acute stress.

Key words
Corticosterone · free CORT · birds

Introduction

The stress response functions to mobilize glucose and redirect physiology and behavior to avoid chronic stress [1 – 3]. However, the mechanistic basis and functional outputs of the response depend on life-history strategy and current environmental conditions. Within stress physiology the comparative approach has provided a powerful tool in explaining individual or population variation in the stress response such as glucocorticoid secretion, corticosteroid-binding globulins, or corticosteroid receptors in relation to factors such as parental care, body condition, length of breeding season, latitude of breeding site, developmental stage, or stage in the annual cycle [4 – 11]. Here, we use a comparative approach to investigate corticosteroid-binding globulins (CBG), glycoproteins present in plasma that bind corticosterone (CORT) specifically and reversibly.

Plasma CBG is thought to reduce the amount of CORT available to cells – that only free (unbound) steroid enters tissues, while bound steroids remain in circulation (the "free hormone hypothesis": [12,13 – 15]). Hence, plasma CBG regulates both the bioavailability and the metabolic clearance of glucocorticoids. Additionally, the presence of CBG in tissues [16 – 19] may also regulate CORT actions by altering local concentrations of CORT or via interaction with membrane-mediated signaling pathways.

Changes in CBG capacity in response to stress apparently require hours, if not days, to occur [20 – 22]. The most detailed study in birds shows no change in CBG capacity in response to 1, 2, or 6 hours of food deprivation; only after 18 hours does CBG capacity decline [21]. This relatively extended period suggests a role for plasma CBG once the stressor has passed from acute (minutes to hours) to chronic (hours to days). During chronic stress, an an-

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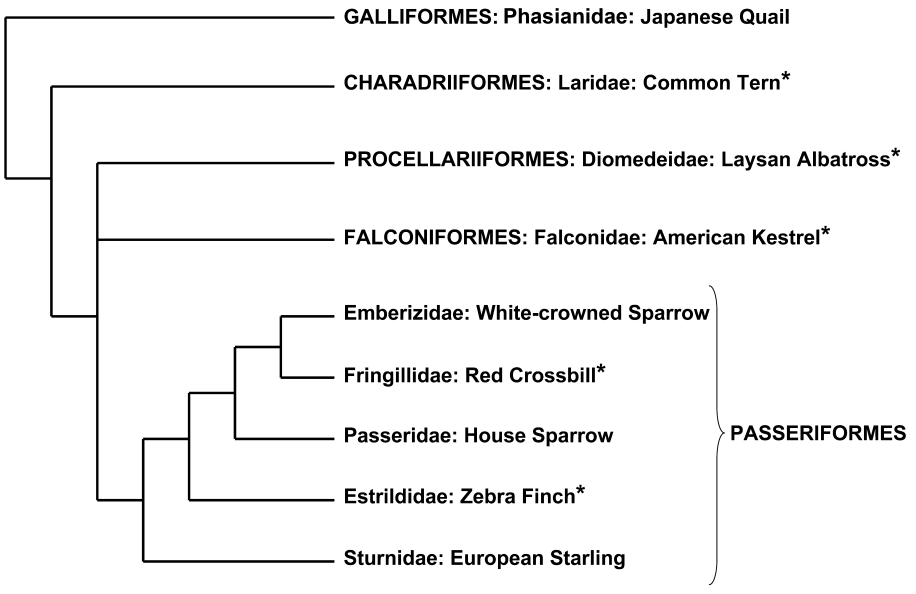

Fig. **1** Phylogeny of species adapted from Fain and Houde, 2004, with Passeriformes phylogeny adapted from Sorenson & Payne 2001. Asterisks denote species with significant decline in CBG capacity within 60 minutes.

imal is often incapable of increasing total CORT levels further; a decrease in CBG at this point may allow for larger quantities of CORT to reach target tissues without increased CORT secretion. Hence, the few studies to date suggest that CBG responds primarily to chronic stressors, but does not play a role in the acute response to stress.

However, the Tinnikov study cited above [22] briefly suggests a role for CBG in the acute response to stress: in female rats, 10 minutes of ether stress decreased CBG capacity by ~12% (samples taken 10 minutes after termination of stressor). A decline in CBG during the acute stress response would enable greater mobilization of free CORT to tissues. Hence, the ability to rapidly alter CBG titers would enable a second level of regulation of the acute stress response: a post-secretion mechanism that may relate to physiological or environmental conditions.

In this study, we examine the timing of CBG responses within a broad phylogenetic context. We examine possible changes in CBG capacity over the first ~60 minutes of a standardized capture and handling stressor in 9 species of birds distributed in 5 orders and 9 families (Fig. **1**). A rapid change in plasma CBG would necessitate a re-evaluation of the dynamic factors regulating the acute stress response.

Materials and Methods

All methods were approved by local animals care committees: University of Texas at Austin (protocols #01010901, #04032904, and #04040503 to CW Breuner), Simon Fraser University (protocol #499B to TD Williams), University of California at Davis (protocol #10111 to TP Hahn), Indiana University (protocol #04–049 to B Heidinger), The College of Wooster (protocol #506 to S Lynn), and Boise State University (protocol #006–05–003 to AM Dufty).

Animals

Male house sparrows (*Passer domesticus*, n = 7) were caught in nest box traps in Titus County, TX (33°09'N, 94°58'W), in March of 2002. In captivity, sparrows were housed individually in animal rooms under short day lengths (8L: 16D) with food and water available *ad libitum*. Blood samples were taken after animals had been in captivity for more than 2 weeks.

Female European starlings (*Sturnus vulgaris*, n = 24) were caught in nest-boxes at the Pacific Agri-Food Research Center in Agassiz, British Columbia, Canada (49°14'N, 121°46'W). Captures generally took place between 8 PM and midnight. Tarsus and bill length measures were taken at capture.

Male Japanese quail (*Coturnix japonica*, n = 12) were sampled from a captive population kept by Dr. Michael Domjan at the University of Texas at Austin (16L: 8D). All samples were collected February 16th, 2005, between 10 AM and midday.

Male and female incubating common terns (*Sterna hirundo*, n = 10) were captured on the nest using treadle traps on Bird Island, Buzzards Bay, MA (41°40'N, 70°43'W). All captures occurred between 6:30 AM 12:30 PM in June, 2004. Blood samples, body mass and head to bill length were collected on capture, and birds were then released.

Male and female red crossbills (*Loxia curvirostra*, n = 10) were caught in mist-nets (using live decoys) in the Olympic National Forest (Grays Harbor County, WA, 47°20'N, 123°40'W). Captures took place between 7 and 11 AM (n = 4) or 2 and 4 PM (n = 3) throughout the year (no birds were molting). Blood samples were collected at capture and birds were then released.

Adult male white-crowned sparrows (*Zonotrichia leucophrys oriantha*, n = 11) were caught in mist-nets (using song playback) on their breeding grounds at Tioga Pass, CA (Inyo County, 37°56'N, 119°15'W). All captures occurred between 6 and 9 AM in the first week of June, 2002. Blood samples were taken at capture, and the birds were then released.

Table **1** Binding globulin methods

Common name	*Blood sampling times (minutes)*	*All CBG assays*	*Point sample assays*	
		Plasma dilution	*[^{3H}CORT]*	*% Bound in assay*
European starling	0, 30	1:720	15.5 nM	82%
house sparrow	0, 30	1:900	15.8 nM	90%
zebra finch	0, 30, 60	1:1500	15.4 nM	91%
red crossbill	0, 10, 30, 60	1:900	16.5 nM	88%
white-crowned sp. adult	0, 60	1:900	18.2 nM	83%
white-crowned sp. nestling	0, 15, 30, 60	1:900	16.5 nM	91%
American kestrel	0, 30, 60	1:180	21.4 nM	88%
Laysan albatross	0, 30, 60	1:500	13.6 nM	80%
common tern	0, 10, 30, 50	1:1089	18.1 nM	83%
Japanese quail	0, 30, 60	1:1000	15.1 nM	94%

Table **2** Affinity estimates of CORT for CBG

Species	*K d ± SE (nM)*
European starling [23]	3.46 ± 0.09
house sparrow [10]	1.8 ± 0.19
zebra finch	1.48 ± 0.08
red crossbill	2.3 ± 0.17
white-crowned sparrow-adult [21]	3.68 ± 0.31
white-crowned sparrow-nestling	1.67 ± 0.14
American kestrel	3.03 ± 0.54
Laysan albatross	3.3 ± 0.24
common tern	3.57 ± 0.26
Japanese quail	1.03 ± 0.07

Nestling white-crowned sparrows (*Zonotrichia leucophrys oriantha*, n = 9) were caught in their nests at Tioga Pass, CA (Inyo County, 37°56'N, 119°15'W). All captures occurred between 9 AM and 1 PM between June 26 and 30, 2004. Blood samples were taken at capture, and nestlings were returned to their nests.

Adult male zebra finches (*Taeniopygia guttata*, n = 9) were sampled from a captive population (14L:10D) kept by S Lynn at the College of Wooster. Blood samples and body weight were collected between 10:30 AM and 1 PM in the first week of August, 2005. Body size measures (tarsus, wing length, and culmen) were taken September 23, 2005.

Juvenile American kestrels (*Falco sparverius*, n = 10) were caught before fledging, while still in nest boxes (between May and July, 2005; the site covers both Ada and Canyon counties, ID, 43°33'40"N, 116°28'25"W). Birds were held in captivity on natural day length, and sampled between 9 AM and midday in mid-September, 2005.

Incubating Laysan albatross (*Phoebastria immutabilis*, n = 10) were captured on their nests on Sand Island, Midway Atoll National Wildlife Refuge (28°13'N, 177°22'W) in the Northwestern Hawaiian Islands. All samples were collected on January 27th, 2005 between 1:30 and 3:30 PM.

Capture and handling stress

Baseline blood samples were taken within three minutes of capture (nestling white-crowned sparrows within 4 minutes [9]). All birds (except terns) were held in cloth bags between serial sampling (sampling times in Table **1**). Blood samples in all passerines, quail and kestrels were collected by alar vein puncture with a 26 gauge needle, with 40 – 80 µl of blood (150 – 200 µl taken from kestrels) collected into heparinized microcapillary tubes. Common terns were restrained in a holding tube for the hour of capture. The initial blood sample in terns was collected through jugular vein puncture with a 25 gauge 5/8" needle, with 200 µl of blood collected with a syringe. Subsequent blood samples were taken from the alar vein with a 28 gauge needle, with 100 µl blood collected with a syringe. Albatross samples were collected from the alar vein using a 26 gauge 3/8" needle, with 400 – 500 µl blood collected into an uncoated syringe. Blood was immediately expelled into a BD Microtainer tube coated with EDTA. Blood samples from all birds were kept on ice until plasma was separated by centrifugation and stored at – 20°C.

Corticosteroid-binding globulin assay

General Methods: CBG affinity and capacity were estimated using radioligand binding experiments. Incubation time, temperature, and plasma dilution was optimized separately for each species. Charcoal-stripped plasma samples were incubated in triplicate for 2 hours at 4 °C with or without 1 µM unlabeled CORT to determine non-specific and total binding. Separation of bound and free steroid was achieved by rapid vacuum filtration over glass-fiber filters presoaked in a polycationic solution (see [8] for further detail). Final plasma dilution for each species is listed in Table **1**.

Affinity: Three of the 9 species (house sparrows, adult white-crowned sparrows, and European starlings) have published accounts of the dissociation constant (K_d, [10,21,23]). For the remaining 6 species (plus nestling white-crowned sparrows) equilibrium saturation binding analysis ([^{3H}CORT] = 0.2 – 15 nM) was run as part of the optimization for each species.

Capacity: Individual hormone binding capacity was estimated using point sample analysis (for specific assay conditions: [^{3H}CORT] and plasma dilution, see Table **1**). All samples within a species were completed in the same assay. Each species was run in a separate assay, and we did not make statistical comparisons among species. Percentage CBG bound in the assay (reported in Table **1**) is estimated using concentration of ^{3H}CORT in the assay and affinity estimates (Table **2**) from saturation analysis (% bound = [^{3H}CORT]/([^{3H}CORT] + K_d)).

Corticosterone assay

Zebra Finch corticosterone levels were detected using Enzyme Immunoassay (EIA) kits (cat # 901 – 097, Assay Designs, Ann Arbor, MI). In this assay, raw plasma is added directly into wells without extraction; steroid displacement buffer (SDB-containing proteases) is added prior to the assay to degrade binding globulins. Plasma dilution and SDB concentration were optimized

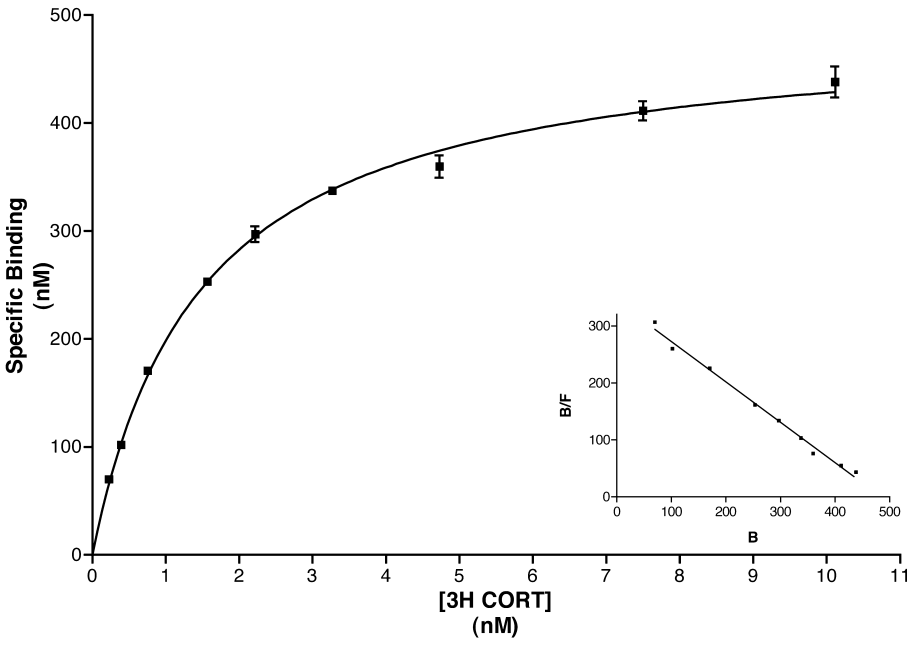

Fig. **2** Equilibrium saturation binding of [^{3H}CORT] to zebra finch plasma. Data shown are specific binding (means ± SE at each concentration). Data are best fit by a one-site model with a K_d of 1.48 ± 0.08 nM. Inset: Scatchard-Rosenthal replot of the data.

using stripped, spiked plasma (stripped with 1% norit A Charcoal and 0.1% dextran in ddH_2O, then spiked to 500 pg/ml with CORT standard from the assay). In the optimization, stripped, spiked samples were run against a standard curve at 1 : 20, 1 : 40, 1 : 60, and 1 : 80 dilutions (diluted into a buffer of known CORT concentration: 500 pg/ml), each with 0, 1.5% and 3% SDB (percentage of raw plasma volume). In this optimization, every sample should read 500 pg/ml; this allows us to look for plasma interference on CORT measures. For the CORT EIA of Zebra Finch plasma, a plasma dilution of 1 : 40 or greater with 1.5% SDB (per raw plasma volume) eliminated effects of plasma on the assay. Optimizing SDB concentration is critical, as higher concentrations interfere with antibody activity in the assay, artificially increasing estimated CORT amounts in wells.

To determine CORT levels, 7 µl 1.5 : 100 dilution of SDB buffer was added to 7 µl plasma. After 5 minutes, 266 µl assay buffer was added to each sample, vortexed, and aliquoted to individual wells in the assay plate (100 µl sample per well, each in duplicate). The standard curve was measured in triplicate, with six standards ranging from 2000 pg/ml to 15.63 pg/ml (100 µl per well). For the first incubation with conjugated CORT and antibody, the plate was shaken for two hours at 26 °C. For the second incubation with substrate solution, the plate was incubated at the same temperature for one hour without shaking. After adding stop solution, the plate was read immediately on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. All samples were run on one plate; intra-plate variability (coefficient of variation) was 6.3%.

Common tern corticosterone levels were determined using radioimmunoassay in Dr. Ellen Ketterson's laboratory at Indiana University [24]. Briefly, samples were extracted with 4 ml diethyl ether; ether was evaporated, samples were resuspended in assay buffer and run against a standard curve (2000 – 3.9 pg/ml). Recoveries were used to determine extraction efficiency, and final results corrected to reflect loss of CORT during extraction. Recovery ranged from 68 – 93%. Inter-assay variability was 13%; intra-assay variability was 19%.

Body condition index

Estimates of body condition were made by dividing body mass by body size. For terns, zebra finches and starlings, body size values were estimated by multiplication of each body measure obtained. So, for example, body condition index equals body mass divided by the product of bill length times tarsus in the starlings. This value gives a general estimate of body weight per body size given constant measures taken from each individual.

Statistics

Binding parameter estimates from the saturation analyses were obtained by fitting untransformed data to appropriate equations using iterative, least-squares curve-fitting techniques (GraphPad Prism, San Diego, CA). Point sample data were analyzed with repeated measures ANOVA (StatView 5, SAS Institute Inc., Cary, NC) followed by Tukey's HSD test. Relationships between body condition (body weight/body size) and maximum CBG change were evaluated by linear regression (GraphPad Prism) within each species. Free corticosterone levels in zebra finches and common terns were calculated from total CORT and 100% CBG capacity, using the equation of Barsano and Baumann [25]. Free CORT estimations were made assuming static CBG (no change in CBG over the capture and handling period) and compared to free CORT estimations using dynamic CBG (the CBG measured from each time point). Free CORT results from the two methods (static *vs.* dynamic CBG) were compared by examining the x-fold increase in CORT (maximal free CORT/baseline free CORT) for each individual, and then comparing them statistically with repeated measures ANOVA (StatView 5). A familywise value of $\alpha = 0.05$ significance level was used for all tests.

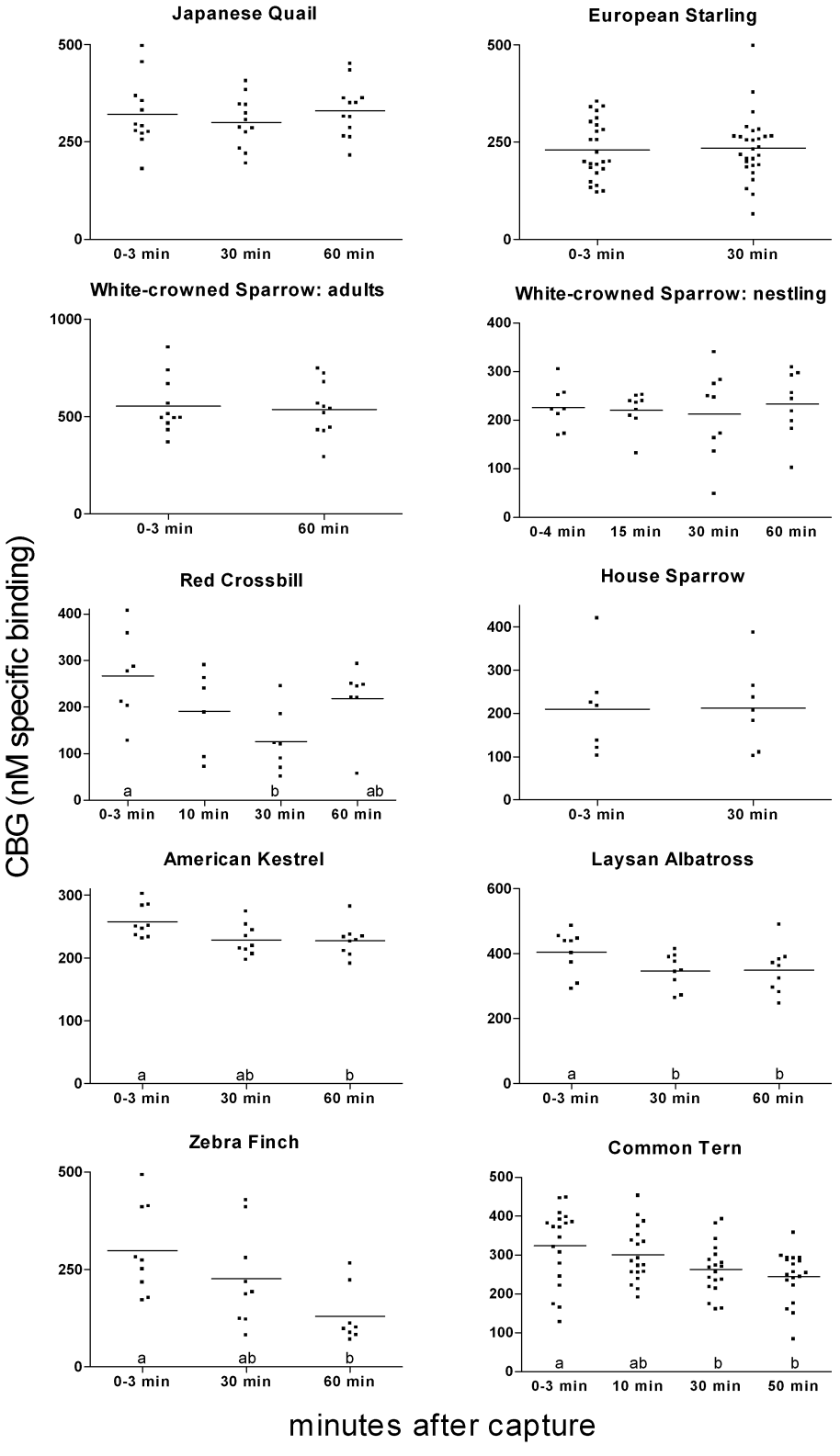

Fig. **3** Point sample analysis within 60 minutes of capture estimating hormone binding capacity through the stress series. CBG capacity shows a significant decline in albatrosses, terns, kestrels, red crossbills and zebra finches. Significant differences within the 60 minute sampling period in these species are noted by different letters (as detected by Tukey's HSD *post hoc* analysis). Time points within a species were assayed in the same experiment, but each species was assayed at a different time. Therefore, these data are meant to illustrate patterns across species, not comparison of absolute levels among species.

Results

CBG characterization

Binding characteristics of CBG have been previously published in three of the species studied here. In the remaining species, radioligand binding experiments detected a single binding site for corticosterone with K_d ranging between 1 and 3.5 nM (see Table **2**). A representative saturation curve from zebra finches is shown in Fig. **2**.

CBG capacity during acute stress

Estimates of hormone binding capacity show significant changes in 5 of the 9 species studied (Fig. **3** and Table **3**). CBG capacity estimates are significantly lower than baseline measures by 30 minutes in common terns, 30 minutes in Laysan albatrosses, 30 minutes in red crossbills, 60 minutes in American kestrels and 60 minutes in zebra finches (for repeated-measures ANOVA statistics, see Table **3**).

Table **3** ANOVA table for point sample assays shown in Fig. **3**

Species	*DF**	*MST*	*MSE*	*F value*	*P value*
European starling	1, 23	990	2401	0.41	$p > 0.05$
house sparrow	1, 6	27	5894	0.005	$p > 0.05$
zebra finch	2, 14	41813	8802	4.75	$p < 0.05$
red crossbill	2, 12	36519	4920	7.42	$p < 0.01$
white-crowned sp. adult	1, 10	1303	9488	0.14	$p > 0.05$
white-crowned sp. nestling	3, 24	685	3807	0.18	$p > 0.05$
American kestrel	2, 14	2356	639	3.69	$p = 0.05$
Laysan albatross	2, 16	12903	1797	7.18	$p < 0.01$
common tern	3, 54	24571	4479	5.49	$p < 0.005$
Japanese quail	2, 22	2820	2081	1.36	$p > 0.05$

*DF listed are for treatment (time) and error (t,e).

Body condition

Previous studies in Laysan albatross indicate baseline CBG varies with body condition [26]. If CBG reflects body condition, individual variation in body condition may explain individual differences in CBG change over the 60 minute capture and handling protocol. However, linear regression models detected no significant relationship between body condition index (measures of body weight/body size) and individual change in CBG capacity in terns, zebra finches, and starlings (data not shown, F and p result from test of slope being significantly different from zero; terns: $F = 0.131$, $p = 0.72$, $r^2 = 0.008$; zebra finches: $F = 0.01$, $p = 0.92$, $r^2 = 0.002$; starlings: $F = 0.829$, $p = 0.37$, $r^2 = 0.037$).

Total and free CORT levels – zebra finches

Changes in CBG capacity may affect free CORT levels. To evaluate this, free CORT was estimated in two species – one showing the largest change in CBG (zebra finch) and one showing a more moderate decline (common tern). In both species, total CORT increased significantly over the capture and handling period (zebra finches: 0 – 3 min = 2.29 ± 0.23 ng/ml; 30 min = 7.18 ± 2.12 ng/ml; 60 min = 9.45 ± 1.76 ng/ml; repeated measures ANOVA: $F = 6.48$, $p < 0.02$; common terns: 0 – 3 min = 8.89 ± 1.04 ng/ml; 10 min = 25.36 ± 1.57 ng/ml; 30 min = 26.93 ± 2.62; 50 min = 29.62 ± 2.84 ng/ml; repeated measures ANOVA: $F = 27.61$, $p < 0.0001$). Free CORT was then calculated under two separate CBG conditions. First, prior studies usually assume that CBG does not change during acute stressors [10,23], and use the 0 – 3-minute CBG capacity to estimate free CORT for the entire stress series. To examine this assumption, we estimated free CORT using total CORT values from each time point and the 0 – 3-minute CBG capacity (static CBG). Second, we estimated free CORT using total CORT and CBG measured from each time point (dynamic CBG). Results are shown in Fig. **4**.

For statistical analysis, we determined the x-fold increase for each individual; that is, maximal free CORT value was divided by baseline value for each individual, and the mean ± SEM is reported here. In zebra finches (Fig. **4a**), the estimated free CORT increase in response to capture and handling stress was significantly greater when CBG decline was accounted for (5.49 ± 1.09-fold increase with static CBG *vs.* 19.77 ± 6.8-fold increase with dynamic CBG; repeated measures ANOVA; $F = 5.455$, $p < 0.05$). In common terns with a less robust decline in CBG, Fig. **4b**, the estimated free CORT increase in response to capture and handling stress was also significantly greater when CBG decline was accounted for (5.75 ± 0.7-fold increase with static CBG *vs.* 8.0 ± 1.22-fold increase with dynamic CBG; repeated measures ANOVA; $F = 7.714$, $p < 0.015$).

Discussion

CBG binding capacity rapidly decreased in five of nine species examined. Our results have important implications for both understanding the physiology of the stress response and designing and implementing studies of CBG. Declining CBG will elevate free CORT levels in plasma, and likely the amount of CORT reaching tissues. From a functional standpoint, this CBG decline then may mobilize a much greater stress response (if the free-hormone hypothesis is true). Many physiological and behavioral effects of CORT show non-linear dose response curves [27 – 32] – the organismal output can be very different given intermediate *vs.* high tissue levels of CORT. Hence, a stress response with static CBG could produce one outcome, whereas CBG decline during stress could mobilize a qualitatively different response.

In zebra finches, CBG levels are almost 67% lower after 60 minutes of capture and handling stress. This decrease in CBG results in free CORT levels 20 times as high as baseline (static CBG levels only produce a 6-fold increase; see Fig. **4**). However, CBG decline in zebra finches was the most extreme change detected. What is the outcome for a less drastic decline in CBG? Common tern CBG capacity only decreases 24% on average over 50 minutes, and this in the face of large individual variation in absolute CBG capacity. However, the slight decline in CBG still results in a significantly greater elevation of free CORT than if CBG were static. Hence, even non-dramatic changes in CBG capacity may significantly alter the stress response.

This paper only considers plasma levels of binding globulin. However, CBG is thought to be in the extracellular fluid (ECF), and CBG mRNA or CBG immunoreactivity has been identified in the cytoplasm of several tissues [33,34 – 36]; for review, see [13]. ECF CBG may originate in the plasma: CBG-specific pumps in capillary beds may actively move CBG out of the vasculature. Vasculature-specific pumps have not been discovered, but CBG binding sites – possibly transport pumps – have been identified in cell membranes of several tissues; see [37 – 39]. Alternatively, capillaries may become leaky during stress [40 – 42], and larger proteins may exit, increasing flow of CBG from plasma into ECF. If ECF CBG is related to plasma levels, then this plasma decline in CBG during acute stress could have very interesting effects on cellular actions of CBG [43,44] outside of regulating free CORT in the plasma.

The movement of CBG from plasma to the ECF also describes two possible mechanisms for the decline in plasma CBG during stress. Leaky capillaries may allow for greater exit of CBG, causing a decline in total plasma CBG if liver production is constant. Additionally, the active movement of CBG from the plasma fraction (e.g. through CBG-specific pumps in the blood brain barrier)

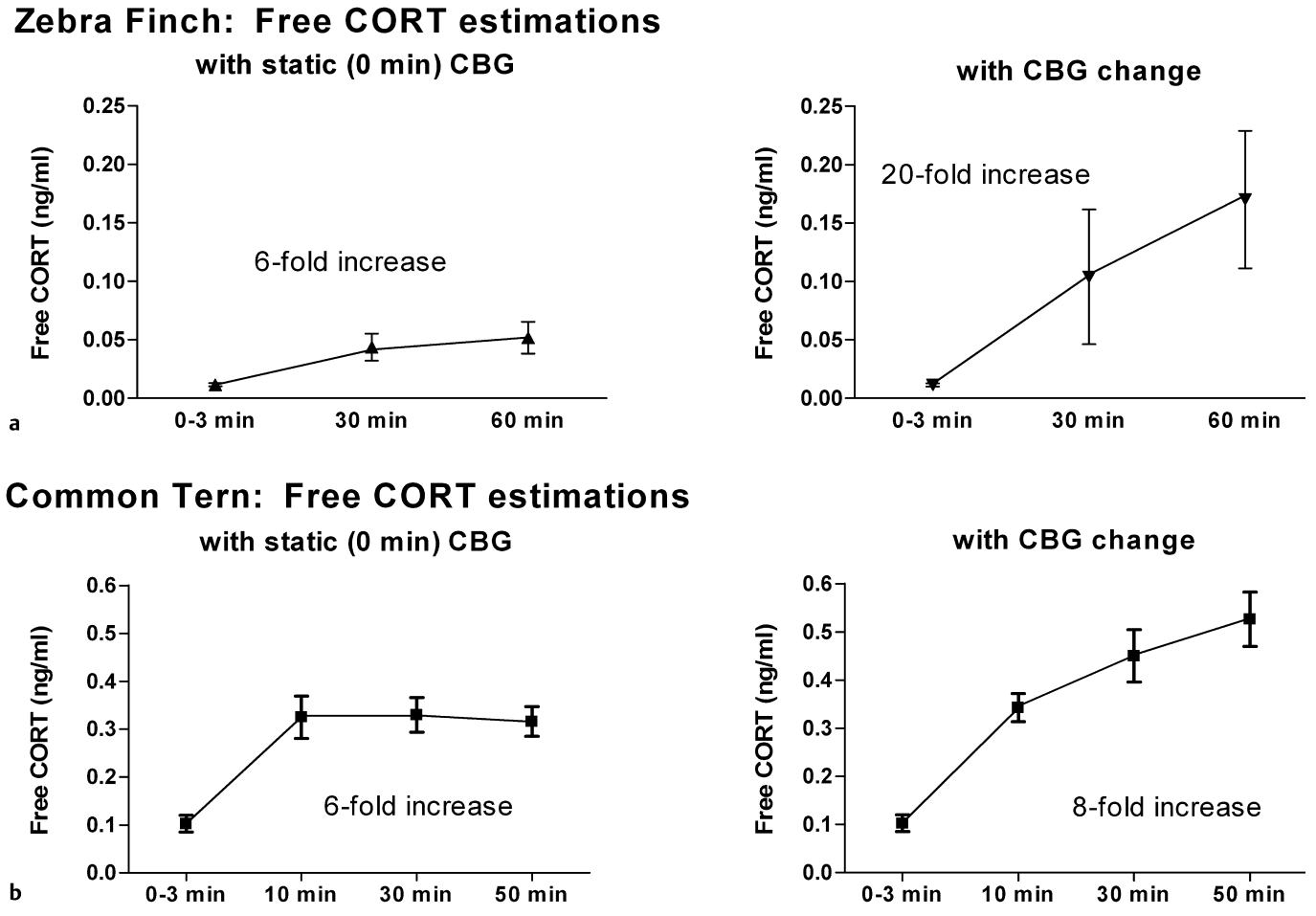

Fig. **4** Estimates of free CORT change during acute stress in zebra finches (**a**) and common terns (**b**) using two methods: 1. using the 0–3 minute sampling point (static CBG: to estimate what free CORT levels would have been if CBG had not changed), and 2. using the appropriate CBG level at each time point (dynamic CBG). The increase from baseline to maximal CORT level was calculated for each individual as an x-fold increase; the averages are shown for each method. The increase in free CORT was significantly greater with dynamic CBG in both zebra finches and terns.

may be increased during stress. Two simpler, alternative hypotheses are that CBG production may decline at the liver, or CBG degradation may increase. Stress may cause a global degradation of plasma proteins, or the decline may be specific to breakdown of CBG. Measurement of an alternative protein during a stressor such as albumin would help answer that question.

One of the goals of this study was to help determine the appropriate methods for incorporating measures of CBG into studies of acute stress physiology. If CBG levels change during capture and handling, then it is necessary to measure CBG at each sampling time for correct evaluation of free CORT. Alternatively, if CBG is static, then CBG would need to be measured only at the first time point. The reduction in plasma needed could be critical, especially in studies that measure CORT levels at five time points with small passerines. However, with the variation in CBG response across species, it appears critical that CBG change in each new species be determined empirically. Additionally, it is important to note that glucocorticoid physiology acts in a context-specific manner [45]; changes in CBG during the parental phase (such as in terns) may not occur during molt or migration. Hence, a primary outcome of this paper is a call for inclusion of CBG measures in each new species tested, to determine the species and stage-specific responses.

Emerging roles for binding globulins: an organismal perspective

The focus of binding globulin research has been highly variable dependent on vertebrate class. Mammalian/biomedical research has focused on the cellular action of binding globulins, revealing their presence and action within the intracellular and extracellular space in multiple tissue types [16,17,46,47]. Unfortunately, it is often difficult to project from these cellular studies to eventual organismal response. Comparative research has focused on plasma binding globulin levels, comparing hormone carrying capacity among individuals, phenotypes and populations [8,23,48]. The main goal of comparative studies has been to gain organismal, ecological, and evolutionary perspectives on reproductive or stress physiology. Unfortunately, the search for patterns in total CORT or plasma CBG may have obscured important cellular or tissue mechanisms affecting CORT's actions. In general, those interested in mammalian models tend to take a bottom-up approach; those in comparative studies take a top-down approach. We propose that a productive way forward lies in merging these strategies (for an elegant example, see Hammes et al, 2005 [46]).

This hybrid approach would allow for an organismal perspective based on well-understood mechanisms at the molecular, cellular, and plasma levels.

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