RESEARCH ARTICLE

Chronic stress in Lizards: Studies on the Behavior and Benzodiazepine Receptors in *Liolaemus koslowskyi* and *Cnemidophorus tergolaevigatus*

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ABSTRACT

Behavioral and physiological adaptive responses of animals facing chronic exposure to a single stressor may allow them to overcome its negative effects for future exposures to similar stressful situations. At chemical level, the $GABA_A$/benzodiazepine complex is considered one of the main receptor systems involved in the modulation of stress-induced responses. Here, we describe the behavioral responses of two different lizard species, *Liolaemus koslowskyi* and *Cnemidophorus tergolaevigatus* exposed to three potential chronic stressful treatments: (a) high temperature, (b) forced swimming, and (c) simulated predator. Additionally, we aimed to determine in those lizards whether the central-type benzodiazepine receptor (CBR; an allosteric modulator site of the $GABA_A$ receptor) is related to adaptive responses to those stressful stimulations. Our results revealed that the simulated predator was the stress condition that showed the largest difference in behavioral responses between the two species, resembling previously described strategies in nature. The basal affinity of CBRs (obtained from undisturbed animals) showed differences between both

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species, and the simulated predator was the only stressor that altered the affinity of CBRs. *L. koslowskyi* CBRs showed a decreased receptor affinity, whereas *C. tergolaevigatus* showed an increased receptor affinity in comparison to their respective control groups. We show for the first time the effects of different types of stressors upon behavioral responses and CBR biochemical parameters in two lizard species. Our findings suggest a potential GABA/benzodiazepine role in the ability of lizards to cope with a repeated exposure to a stressful (e.g., predator) condition. *J. Exp. Zool. 325A:713–725, 2016.* © 2017 Wiley Periodicals, Inc.

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INTRODUCTION

Animals can react to stressors by generating behavioral and physiological responses that appear to be mainly modulated by the neuroendocrine system (Wingfield and Kitaysky, 2002; Reeder and Kramer, 2005). In vertebrates, including lizards, the most common way to measure stress involves measuring the circulating levels of glucocorticoids (e.g., corticosterone) that result from the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Marin et al., 2002; Langkilde and Shine, 2006; Miles et al., 2007; French et al., 2008; Fraker et al., 2009; Cull et al., 2015; Monaghan and Haussmann, 2015). Even when physiological and behavioral responses may help animals to cope with stressors (Wingfield and Kitaysky, 2002; Reeder and Kramer, 2005; Cote et al., 2006; Amo et al., 2007), variations in the animal body condition as a consequence of stressors may reduce their abilities to cope with the surrounding changing circumstances, to successfully interact with congeners, and to exploit environmental resources (Badyaev, 2005; Ling et al., 2009; Papargiris et al., 2011), thus, potentially leading to a reduction of their fitness.

Previous studies found that experimental exposure of animals to acute stressors may generate negative effects, such as a decrease in motor coordination, a disruptive breathing rate, development of anxiety-like behavior, anorexia, and cognitive-impairing effects (Labra and Leonard, '99; Liu et al., 2007; Adamec et al., 2008; Chen et al., 2010; Verma et al., 2010; Béracochéa et al., 2011). In addition, chronic exposure to stress may produce a variety of outcomes, including reproductive inhibition, immunosuppression, reduction in parental investment, and decreased growth rates (Wingfield and Sapolsky, 2003; Angelier and Chastel, 2009; Dragoş and Tănăsescu, 2010; Skomal and Mandelman, 2012). However, repeated exposure to the same stressor may allow animals to overcome its negative effects by developing adaptive responses to future exposures to similar stressful situations (Romero and Wikelski, 2001; Wingfield and Sapolsky, 2003; Girotti et al., 2006). A repeated exposure to a single stressor may also induce changes that modulate dopamine, opioids, serotonin, adrenergic, and gamma-aminobutyric acid (GABA) receptors, including the central-type benzodiazepine receptor (CBR) (Haleem et al., 2007; Lucas et al., 2007; Chekina et al., 2009; Poulter et al., 2009).

The CBR is an allosteric modulator site of the $GABA_A$ receptor, and the $GABA_A$/benzodiazepine (BZD) complex is considered one of the main receptor systems involved in the modulation of stress-induced responses (Drugan and Holmes, '91; Drugan et al., '94; Caldji et al., 2000). Previous studies have also indicated that homeothermic animals exposed to stressor stimuli affects the $GABA_A$/BZD receptor functions, suggesting that the adaptive behavioral responses to stressors may be linked to an improvement in the $GABA_A$/BZD receptor activity through changes in either the receptor affinity or the number of binding sites (Biggio et al., '90; Kellog et al., '93; Marin and Arce, '95, '96, Turina et al., 2016). Furthermore, there is evidence indicating that strategies allowing animals to cope with an aversive experience involve the release of endogenous CBR agonists that protect the organism from stress pathologies (Drugan et al., '94). The lack of capacity to modulate GABAergic transmission following exposure to stress may lead to difficulties in the regulation of the stress-induced response, causing undesired consequences for the organism (Martijena et al., '97).

Stress exposure in lizards affects a wide variety of features, ranging from negative effects on locomotion (McMillan et al., 2011) to deeper morphological effects such as fluctuating asymmetry (Băncilă et al., 2010). Most studies dealing with stress in lizards analyzed glucocorticoids and brain monoamines as the physiological response to stressor stimuli (Grassman and Hess, '92; Øverli et al., 2007; Thaker et al., 2010; Trompeter and Langkilde, 2011). A recent study in the lizard *Tropidurus oreadicus* showed that when lizards received BZD-based drugs, defense responses, such as freezing and circling decreased, suggesting the involvement of CBRs (Maximino et al., 2014).

Lizard species in arid regions face different adverse situations, including extreme heat, dehydration, and sudden flooding of large dry riverbed areas during the rainy season (Fitzgerald et al., '99). In the Monte region of North West Argentina, there are two sympatric lizard species (*Liolaemus koslowskyi* and *Cnemidophorus tergolaevigatus*), which have different life histories, including prey capture strategy, behavior, and physiology (Videla

and Puig, '94; Martori, 2005; Cruz et al., 2012). The lizard *L. koslowskyi* (Iguania: Liolaemini) shows a cryptic dorsal color pattern that apparently helps this species to be less detectable by predators and is an ambush predator of insects (Videla and Puig, '94; Aun and Martori, '98; Martori, 2005). Conversely, *C. tergolaevigatus* (Teiidae) is a brightly colored cruiser, which actively searches for prey and has the ability to flee from predators. However, both species share the same environment and therefore face the same potential stressors.

The aim of the present study is to describe the behavioral responses of *L. koslowskyi* and *C. tergolaevigatus* to three potential chronic stressful conditions: (a) high temperature, (b) forced swimming, and (c) simulated predator. Additionally, we aimed to determine in these lizards whether GABA/BZD receptors are involved in adaptive responses to those stressful stimulations. Owing to the differences in the life history of these two lizard species, we expect to answer the following questions: (a) Do both species respond similarly to the stress exposure?, (b) Do the characteristics of stressors matter?, and (c) Are the behavioral responses consistent with the observed modulation at the CBR level? Expected CBR adaptive changes in response to a chronic stressor may be either an increase in the receptor affinity or an increase in the number of exposed sites that may represent an increased GABAergic transmission (Biggio et al., '90; Kellog et al., '93; Marin and Arce, '95, '96; Turina et al., 2016). To our knowledge, this is one of the few studies examining CBR characteristics in reptiles (Hebebrand et al., '87; Schlegel and Kriegstein, '87; Friedl et al., '98; Schmitz et al., '98) and the first one on stress exposure, behavioral patterns, and CBR changes in lizards.

MATERIALS AND METHODS

Animals

Lizards were studied at the mid-end of summer (February) when mating season has just finished (Cruz, '96; Martori, 2005; Martori and Aun, 2010). To avoid females in different reproductive stages (oviductal eggs, repose, or even yolked follicles), only adult males were used in this study. Forty-eight *L. koslowskyi* and 48 *C. tergolaevigatus* lizards were collected by using pitfall traps made from 20-L plastic buckets (Basso, '90; Cruz et al., '93) (Table 1). Traps were deployed 5 km east from the town of Anillaco, La Rioja, Argentina (28° 49′S, 66° 57′ W), where these species are abundant. Traps were visited daily for 10 consecutive days. Body size (snout-vent length) ranged from 53.6 to 68.3 mm for *L. koslowskyi* and from 48.8 to 58.3 mm for *C. tergolaevigatus*. The collected material was deposited at the Instituto de Biociencias, CRILAR (CONICET-Universidad Nacional de La Rioja).

The experimental procedures were performed under the Guidelines for the Treatment of Animals in Behavioral Research and Teaching (ASAB/ABS) and the Guidelines for Use of Live Amphibians and Reptiles in Field Research (ASIH, HL, SSAR).

Stress Experiments and Behavioral Evaluations

Immediately after capture, each animal was individually labeled with nontoxic ink on its back. Lizards from each species were randomly assigned in groups of four to one terrarium (24 lizards in total) containing a 0.02-m deep sand bedding. A 12:12 light–dark photoperiod cycle was used. Temperature gradually reduced from 29°C (± 1°C) during the day to 21°C (± 1°C) during the night. Lizards were offered daily access to mealworms, and water was spread over lizards twice a day. Although standard laboratory practice and guidelines were followed, it should be taken into account that we cannot rule out that this social housing could be stressful for the animals (DeNardo, 2006).

Within each terrarium, each lizard was randomly assigned to one of the following four experimental treatments: (a) high-temperature exposure, (b) forced swimming, (c) simulated predator, and (d) a control condition where they remained undisturbed during the whole study (control group only for CBR determinations) (Table 1). Along 12 consecutive days, lizards were taken daily from their terraria and subjected individually to one of the three assigned experimental conditions, and the behavioral variables were recorded (see below). Once a lizard was set in one of the treatments, it repeated the same treatment during the 12 days.

High-temperature exposure. A 20-L plastic bucket with a 0.02-m layer of sand on the bottom was heated with an incandescent 75-W lamp placed 0.45 m above the sand floor until the sand surface reached 50 ± 1°C. Once that sand temperature was reached, a lizard was removed from a randomly selected terrarium, transferred to a separate room, and placed in the center of the heated ground for 5 min. During this time, we recorded the following data: (a) the time that the animal remained immobile on the sand (immobile time; IT) and (b) the number of escape attempts (EA), including jumps or sand excavations. The same procedure was repeated every 24 hr during the 12 consecutive days. The order in which each lizard was set on the heated ground was randomly assigned. For each lizard, we used a designated "same" bucket to avoid potential interference of chemical cues between animals. The escape response was studied because lizards show this behavior once they reach a certain temperature (the "voluntary escape temperature"; Carothers et al., '98), when they turn uncomfortable and start attempting to escape; this behavior shows that temperature is near critical or even lethal temperatures leading lizards to escape to thermally viable spots. We do not know of previous experimental studies evaluating the IT of lizards exposed to high temperatures; therefore, we have no a priori prediction on the IT response of lizards in our study.

Forced swimming. Lizards were removed from their terrarium, transferred to a separate room, and placed into a 20-L bucket containing 20 cm deep water at a temperature of 25°C (± 1°C). The water column was set to prevent lizards from standing on the floor and thus forcing them to swim or float. The same

Table 1. Number of lizards from each species used for behavioral tests (and control group) and number of binding assays for CBR analyzed for each experimental group

	L. koslowskyi		*C. tergolaevigatus*	
Condition	$N_{lizards}$	$N_{binding\ assays}$	$N_{lizards}$	$N_{binding\ assays}$
CG	12	3	12	3
HT	12	3	12	3
FS	12	3	12	3
SP	12	3	11	3

Abbreviations: CBR: central-type benzodiazepine receptor; CG: control group; FS: forced swimming; HT: high temperature; SP: simulated predator.The three binding assays were performed using pooled brains from four lizards from behavioral tests.

procedure was repeated daily every 24 hr during the 12 trial sessions. Each trial took 6 min. The bucket was washed, and the water was changed between animals to avoid potential interference of chemical cues. The behavioral variables recorded were (a) the time that an animal remained motionless while floating (IT) and (b) the number of EA, including the number of attempts of wall climbing or underwater swimming. It is important to note that none of the lizards exhibited signs of exhaustion or drowning during the testing sessions. The forced swimming test had not been previously used in lizards; therefore, we had no a priori prediction on the response of lizards in our study.

Simulated predator. A wooden open-field cylinder of 0.90 m in diameter and 0.20 m high fence of black cardboard was used as an experimental arena. Lizard behavior was recorded while a black falcon silhouette made of cardboard (0.20 m × 0.25 m) passed over the arena repeatedly describing a circle at a speed of 0.25 m/sec. The figure was attached to a small crane on a 15° angle from the horizontal plane and moved on the circular shape trajectory with the aid of a motor placed 10 cm apart from the experimental arena. The floor of the experimental arena was divided into 44 square sectors of approximately 0.015 m^2 to monitor lizards´ ambulation. We used a Falconidae shape as a model because it is one of the most important lizard predators among birds (Jaksić et al., '82; Stellatelli et al., 2015).

During testing, each lizard was placed in the midpoint of the experimental arena and remained there for 6 min, during this period the lizard´s behavior was monitored. During the first 3 min and at 10 sec intervals, the falcon-shaped figure appeared over the arena (at a height of 30 cm from the ground) being visible to the lizard for approximately 5 sec. During the following 3 min, the motor was switched off and the figure was no longer visible. The same procedure was repeated each of the 12 trial days. During each trial, we recorded (a) the IT, as described previously, and (b) the number of sectors entered. A similar raptor model has been previously used to study antipredator responses in *L. monticola* (Labra and Leonard, '99). In antipredator response, the IT is commonly called freezing response, since it is assumed that it is due to fear. Here, we used the term IT to make it consistent through all the tests.

Biochemical Analyses

Twenty-four hours after the end of the last stressor session, all lizards from all treatments were sacrificed by fast decapitation to avoid the stress of the procedure itself and immediately after removing the brain, it was placed on ice and then stored in a −80°C freezer for its later CBR biochemical analysis (see below).

Preparation of membrane tissues. All the procedures involved in preparing the membranes were carried out at 4°C as described by Sábato et al. ('81) and modified by Martijena et al., '92). Brains from three/four lizards of the same species and treatment were pooled and homogenized in 50 vol of 50 mM Tris–HCl buffer (pH 7.4) using a Potter-S glass Teflon homogenizer and then centrifuged at 35,000×*g* for 15 min (Awad and Gavish, '89). In all, three treatment replicates per species were obtained (Table 1). Each pellet was then resuspended and homogenized in this buffer to obtain a final concentration of about 0.3 mg protein/mL. Protein determination was made as described by Lowry et al. ('51).

CBR binding assays. Binding of [^{3}H] flunitrazepam (FNZ) to CBR in membranes of the whole brain of lizards was established by using the filtration technique. The binding was made at [^{3}H] FNZ concentrations ranging from 0.25 to 15.00 nM. The nonspecific binding was evaluated in the presence of 10 μM diazepam. To minimize a procedure error, each sample was evaluated in triplicate in a final volume of 250 μL (0.075 mg protein). After incubation for 60 min (0–4°C), samples were filtered under vacuum over Whatman GF/B filter using a Brandel M-24R filtering manifold. Samples were washed three times with 4 mL of 50 mM Tris buffer, and the radioactivity was counted with a LKB-121PRack-Beta Counter at 48% efficiency. Maximal number of binding sites (B_{max}) and equilibrium dissociation constant (K_D) values for CBR were determined by computer-aided nonlinear regression analysis of the experimental data. These parameters were analyzed for each experimental group (see below).

Data Analysis

Behavioral Evaluation. As shown in Table 1 for each stress condition and control group, we used 12 lizards of each species except for the simulated predator condition in the case of *C. tergolaevigatus*, where we used 11 individuals due to an escape during placement in the arena. Behavioral variables in each of the three experimental conditions were analyzed using mixed repeated measurements analysis of variances (ANOVAs) with lizard species as a factor (*L. koslowskyi* and *C. tergolaevigatus*) and day number as repeated measures (1–12). When the data from the simulated predator condition were analyzed, the mixed ANOVA also included predator (presence or absence) as a within group (nonindependent) measure. To fit ANOVA assumptions, some variables (number of EA in the high-temperature test; the IT in the forced swimming test; and the IT and the number of sectors entered in the simulated predator test) were transformed to ranks (Shirley, '87; Lábaque et al., 2007). Fisher's least significant mean test was used for post hoc pairwise comparisons. A P value of < 0.05 was considered to represent significant differences.

Determination of CBR-Binding Parameters. Within each species, CBR measurements derived from each of the three replicates per treatment were analyzed with Wilcoxon nonparametric tests for independent samples. B_{max} and K_D values were evaluated as dependent variables and each experimental condition (high temperature, forced swimming, simulated predator, and nonstressed control group) as an independent variable.

To compare the effect of each experimental condition between species, we also ran the Wilcoxon test, but using B_{max} or z-score K_D as a dependent variable and lizard species (*L. koslowskyi* and *C. tergolaevigatus*) as the independent variable. We used K_D z-scores relative to the control group (Abate et al., 2001) because controls showed significant differences between species in K_D basal values ($W = 15.00$, $P < 0.05$). This z-score was calculated as follows:

$$z = \frac{(\text{Individual } K_D \text{value from a given species in a given experimental condition} - \text{CG mean } K_D)}{\text{CG standard deviation}}$$

where CG is the control group.

Negative z-scores indicated that K_D values in a given experimental condition were lower than those from the control group, whereas positive z-scores indicated the opposite. However, two criteria had to be met to determine a significant difference between species in a given experimental condition (Abate et al., 2001): (a) the difference between the species had to be statistically significant and (b) within each experimental condition, z-scores of at least one species had to differ significantly from 0 (indicative of a K_D value different from that of the control group).

RESULTS

High-Temperature Exposure

Immobile Time. The mean IT values per day were 273.62 ± 6.18 sec and 261.76 ± 9.87 sec for *L. koslowskyi* and *C. tergolaevigatus*, respectively; therefore, the percentage of active time of each species was about 10% and 15% of test time (300 sec), respectively. The two-way mixed ANOVA revealed a significant main effect corresponding to the variable day of test ($F_{(11,242)} = 3.15$, $P < 0.000$) and a two-way interaction between species and day of test ($F_{(11,242)} = 1.90$, $P < 0.039$; Fig. 1A). The *post hoc* test indicated that on day 2, 5, 6, 7, and 10, *L. koslowskyi* showed a higher IT than that of *C. tergolaevigatus*. However, the IT on the first and the last day of the test did not differ between lizard species. When *L. koslowskyi* behavior was examined throughout the days, the IT values on the last day of the test (day 12) were similar to those on day 1. For *C. tergolaevigatus*, the IT on day 1 was significantly lower than that on day 12.

Number of Escape Attempts. The two-way mixed ANOVA showed neither significant main effects nor an interaction between the variables studied ($P > 0.1$ in all cases).

Forced Swimming

Immobile Time. The mean IT values considering the 12 days were 300.37 ± 7.90 sec and 320.55 ± 16.27 sec for *L. koslowskyi* and *C. tergolaevigatus*, respectively. Therefore, the percentage of active time of each species was about 20% and 12% of test time (360 sec), respectively. The ANOVA showed a main effect of day ($F_{(11,242)} = 4.21$, $P < 0.000$) and a two-way interaction between species and day of test ($F_{(11,242)} = 2.60$, $P < 0.003$) (Fig. 1B). The post hoc test revealed that on day 3, 4, and 9, *L. koslowskyi* showed a lower IT than *C. tergolaevigatus*. However, the IT on day 1 or 12 of the test did not differ between lizard species. When examining *L. koslowskyi* behavior throughout the days, the post hoc test showed that lizards increased the IT after day 5, and that the IT on day 1 was significantly higher than that on day 12. Although the post hoc test showed some differences in *C. tergolaevigatus* behavior across days, there were no significant differences between the first and the last day of the trials.

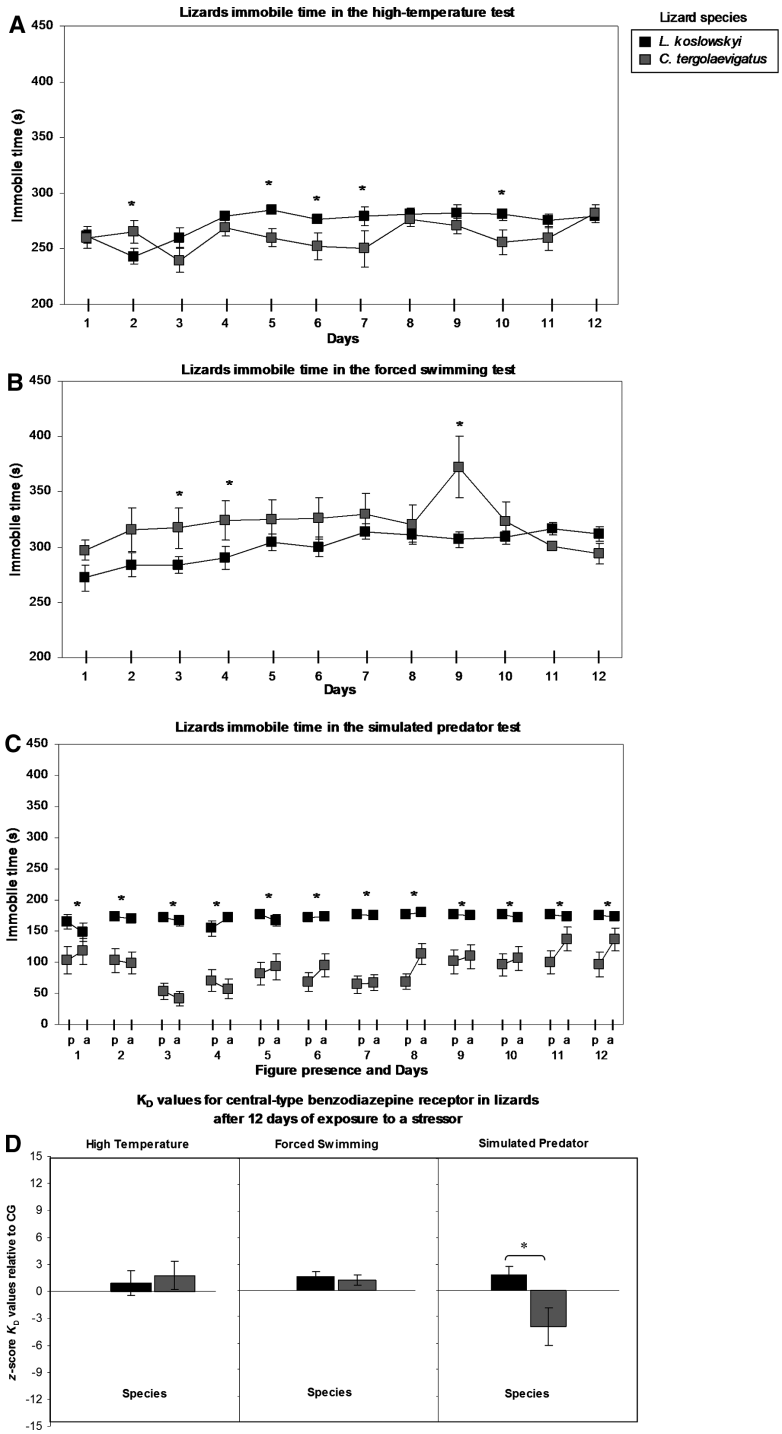

Figure 1. Behavioral (A–C) and biochemical (D) studies. Mean time of immobility in lizards during the daily exposure to (A) high temperature, (B) forced swimming, or (C) simulated predator (falcon figure p: present, a: absent). (D) *z*-score K_D values relative to the control group (CG) as a function of species in each stressor condition. * indicate significant differences between species (A–D) and the days of those differences (A–C). Simulated predator is the stressor showing main behavioral changes throughout the study (C) and the only stressor altering the affinity of CBRs (panel D). Vertical lines indicate standar error of the mean.

Number of Escape Attempts. The two-way mixed ANOVA showed neither significant main effects nor an interaction between the variables studied ($P > 0.05$ in all cases).

Simulated Predator

Immobile Time. The ANOVA revealed significant main effects for species ($F_{(1,21)} = 112.96$, $P < 0.000$), day of test ($F_{(11,231)} = 2.53$, $P < 0.005$) and trials (with or without the presence of the predator figure) ($F_{(1,21)} = 4.42$, $P < 0.047$). The analysis also showed two-way interactions between species and day of test ($F_{(11,231)} = 2.27$, $P < 0.012$), species and trials ($F_{(1,21)} = 8.05$, $P < 0.009$), and day of test and trials ($F_{(11,231)} = 1.92$, $P < 0.038$). A three-way interaction among species, day of test, and trials was also found ($F_{(11,231)} = 2.30$, $P < 0.010$). The post hoc test showed that the IT differed significantly during days (Fig. 1C). *L. koslowskyi* remained motionless most of the time during the 12 trials, whereas *C. tergolaevigatus* showed changes between days and/or trials. Thus, on day 6, 8, 11, and 12, *C. tergolaevigatus* showed significant differences between trials, remaining more time motionless when the predator figure was not present. When examining the activity throughout days (within each species), the first and last day showed no significant differences in either lizard species.

Number of Sectors Entered. This analysis showed a pattern similar to that observed for the IT. The ANOVA revealed main effects of species ($F_{(1,21)} = 63.32$, $P < 0.000$) and trials ($F_{(1,21)} = 11.0$, $P < 0.003$). The analysis also showed two-way interactions between species and day of test ($F_{(11,231)} = 1.85$, $P < 0.047$), species and trials ($F_{(1,21)} = 14.55$, $P < 0.001$), and day of test and trials ($F_{(11,231)} = 2.14$, $P < 0.018$). The ANOVA revealed a three-way interaction among species, day of test and trials ($F_{(11,231)} = 1.85$, $P < 0.046$). The post hoc test indicated that *C. tergolaevigatus* ambulated through a higher number of sectors than *L. koslowskyi*. Also, at day 6, 8, 11, and 12 *C. tergolaevigatus* showed differences within trials, ambulating through a higher number of sectors when the predator figure was visible over the arena. When examining the activity throughout the days (within species), no differences were found between the first and last day of testing (data not shown).

Central Benzodiazepine Receptor

Differences within Species. None of the stress conditions (high temperature, forced swimming, simulated predator) appeared to affect *L. koslowskyi* B_{max} or K_D values when compared with the control group values (Table 2). However, the results of the *z*-score analysis showed that the effects of the chronic stress would be considerable depending on the stressor (see the next section).

The analysis of B_{max} in *C. tergolaevigatus* showed no significant differences for high temperature, forced swimming, or simulated predator conditions when compared to the control group. However, the K_D parameter was lower in the lizards that were exposed to a simulated predator than in their control nonstressed counterparts (Table 3).

Differences between Species. Regardless of the treatment condition, no differences were detected in the CBR B_{max} parameter between species (control group: $W = 8$, high temperature: $W = 11$, forced swimming: $W = 9$ and simulated predator: $W = 9$; P values > 0.2). Mean *L. koslowskyi* and *C. tergolaevigatus* B_{max} values are shown in Tables 2 and 3, respectively. Analyses of *z*-score K_D values revealed significant differences between species when exposed to the simulated predator condition ($W = 6$, $P < 0.049$), showing that the K_D parameter in *C. tergolaevigatus* was lower than in *L. koslowskyi* (Fig. 1D). Also, in the simulated predator condition, the *z*-score of both species differed significantly from their respective control group (from zero). Neither the high temperature nor the forced swimming conditions induced significant effects on the z-score K_D values ($W = 12$ and $W = 9$; P values > 0.5; Fig. 1D).

DISCUSSION

In the present study, we show for the first time the effects of different types of stressors upon behavioral responses and CBR biochemical parameters in two lizard species. When exposed the lizards to high temperature, the teiid *C. tergolaevigatus* showed a higher activity than the liolaemid *L. koslowskyi*. These results are consistent with the fact that *C. tergolaevigatus* is usually active at a mean body temperature of 38.24°C (±1.9; $n = 36$; Cruz unpublished data), higher than the mean 34.8°C of *L. koslowskyi* (Videla and Puig, '94; Moreno Azócar et al., 2013). Also, the range of body temperatures known for teiids is greater than that for iguanian lizards (Cei, '93; Cruz et al., '93; Pough et al., '98). The number of escape attempts was similar in both species, what is expected since *C. tergolaevigatus* and *L. koslowskyi* have similar voluntary escape temperatures (40.06 and 38.87°C, respectively; Cruz et al., unpublished data).

During the forced swimming test, *C. tergolaevigatus* remained motionless (floating without expending energy) for longer periods of time than *L. koslowskyi* but the analysis reveals differences only during three out of 12 days of testing. These results may be related to the nature of each lizard species lineage (Teiidae and Liolaemidae). For example, teiid lizards, such as *Salvator merianae*, achieve more than 5 min of apnea by continuous immersion in water (Achaval and Langguth, '72) and can also be seen swimming in rivers or even in the sea; other teiid species such as *Crocodilurus* and *Dracaena* are semiaquatic, seeking refuge and food in the water (Pough et al., '98). Further explanations inherent to this test are difficult to provide because the forced swimming test was seldom used in reptiles, except to measure speed reptation in snakes (Finkler and Claussen, '99). Nevertheless, we can infer from our results that a longer time floating may be an indicator of less discomfort in water. However, a longer time floating can also be considered

Table 2. Specific [^{3}H] FNZ-binding parameters in *L. koslowskyi* brains subjected or not to a chronic stress situation and summary of Wilcoxon statistical results

	B_{max} (fmol/mg prot)			K_D (nM)		
Condition	Mean ± S.E.	*W*	*P value*	Mean ± S.E.	*W*	*P value*
CG	935.15 ± 79.82	–		1.02 ± 0.12	–	
HT	847.40 ± 176.45	10	0.827	1.21 ± 0.30	9	0.512
FS	891.45 ± 115.31	11	0.827	1.35 ± 0.13	7	0.126
SP	868.39 ± 146.65	11	0.827	1.40 ± 0.19	8	0.275

Abbreviations: CG: control group; FS: forced swimming; HT: high temperature; SP: simulated predator. *W*: Wilcoxon statistic.

Table 3. Specific [^{3}H] FNZ-binding parameters in *C. tergolaevigatus* brains subjected or not to a chronic stress situation and summary of Wilcoxon statistical results

	B_{max} (fmol/mg prot)			K_D (nM)		
Condition	Mean ± S.E.	*W*	*P value*	Mean ± S.E.	*W*	*P value*
CG	719.83 ± 162.90	-		1.55 ± 0.07	-	
HT	949.70 ± 198.21	9	0.512	1.75 ± 0.18	9	0.512
FS	894.42 ± 3.70	9	0.512	1.69 ± 0.07	7	0.126
SP	791.06 ± 120.08	10	0.827	1.08 ± 0.24*	15	0.049

Abbreviations: CG: control group; FS: forced swimming; HT: high temperature; SP: simulated predator. *W*: Wilcoxon statistic.
*denotes a significant difference in the K_D parameter when compared to the control group.

a better strategy for survival because it allows saving energy while surviving in a potentially dangerous novel environment.

The simulated predator exposure was the stress condition that showed substantial differences in behavioral responses between these two species. Apparently, the observed differences are consistent with the type of predator avoidance strategies used by each species. While *L. koslowskyi* usually relies on crypsis, *C. tergolaevigatus* is considered to use speed and constant movement (Videla and Puig, '94; Aun and Martori, '98; Martori, 2005). Additionally, *Liolaemus* species are mostly sit and wait predators, whereas *Cnemidophorus* are typical cruisers (Cei, '93). In our experimental arena, *L. koslowskyi* was motionless for most of the duration of the test, both in the presence and in the absence of the falcon-shaped figure. These results are in agreement with a previous study in other *Liolaemus* species, *L. monticola,* where a raptor model induced freezing response with long time to recover the activity after the figure disappeared (Labra and Leonard, '99). In contrast, *C. tergolaevigatus* not only ambulated more than *L. koslowskyi* throughout the test, but also showed to be more active while the falcon figure was present than when the figure was absent. The loss of an antipredator response even when the predator figure disappears suggests that an active avoidance may be more expensive than a passive avoidance. Losses of antipredator responses when the risk is removed should depend in part on the costs incurred by the antipredator strategy (Blumstein and Daniel, 2002). In both species, the activity during the first and last days of the test was similar, which is predictable as the daily presence of a predator even for just 3 min should always be considered risky. In summary, what is observed in these two species is that concatenated factors such as physiology, foraging mode, and predator avoidance type may play an important role when considering the type of response to the exposure to a fake predator. The species with higher metabolism, active foraging mode, and ability to flee in the presence of a predator such as *C. tergolaevigatus* will move constantly, with an increase in movement in the presence of a risk of predation (Cullum, '98). Conversely, a species with lower metabolism, with a sit and wait foraging mode that is cryptic in the environment will remain motionless under the risk of predation and then may move at a moderate rate, as the case of *L. koslowskyi* (Cruz et al., 2011).

The GABAergic system is widely considered to be involved in integrating physiological and behavioral aspects of the stress response (Øverli et al., 2007), to the extent that drugs that target the $GABA_A$ receptor (i.e., BZD) have been traditionally used as anxiolitics (Griffin et al., 2013). For example, modulation of the stress response in vertebrates includes glucocorticoid feedback at the hippocampal and hypothalamic levels (De Kloet et al., '98; Moore and Jessop, 2003) and regulation by neuronal pathways, including the GABAergic inhibitory system (Jessop, '99; Tokarz and Summers, 2011). The neurotransmitter GABA, which

is the main inhibitory neurotransmitter in the vertebrate central nervous system, in mammals can inhibit the HPA axis. Furthermore, GABA inhibits the ceruleus norepinephrine system locus (Tsigos and Chrousos, 2002). Conversely, stress-derived steroid hormones regulate the expression and function of $GABA_A$ receptors (see review by Mody and Maguire, 2012). The GABA system has also been proposed as a plausible candidate for modulating the magnitude of the stress response and has even been associated with specific stress coping styles in vertebrates (Øverli et al., 2007). The $GABA_A$ receptor complex is composed of five glycoprotein subunits (i.e., α, β, γ) each with multiple isoforms and can be composed of different subunits with individual subunits of the $GABA_A$ receptor, exhibiting distinct pharmacology as well as regional and cellular distribution (Darlison et al., 2005). For example, in mammals, α subunit isoforms 1,2,3, and 5 possess high affinity for BZD (Kelly et al., 2002), whereas in the rat brain, receptors containing the isoform $\alpha 6$ subunits show a low affinity for BZD (Nutt and Malizia, 2001) and are expressed almost exclusively within the cerebellar granule cells (Whiting et al., '99). In humans, a single nucleotide polymorphism in the $\alpha 6$ gene has been associated with specific personality characteristics, as well as a marked attenuation in HPA axis and blood pressure response to psychological stress (Uhart et al., 2004). Although characterization of subunit distribution in the lizard species evaluated has not been established, it is evident that even a small mutation in a subunit of the $GABA_A$ receptor or variations in the composition of subunits could be reflected both at behavioral and physiological level. Herein, the basal affinity of CBRs (undisturbed animals) for the benzodiazepine flunitrazepam showed differences between both species whereas no differences were observed in B_{max}. It is important to recall that the affinity of the receptor for its ligand is inversely related to the calculated K_D, whereas B_{max} is associated with the number of binding sites. In the specific case of FNZ, binding is dependent both on the molecular environment (i.e., membrane cholesterol content (Turina et al., 2012), as well as the subunit composition of the $GABA_A$ receptor. For example, in mammals, since $\gamma 2$ subunit confers the highest degree of sensitivity for BZD binding (Wafford et al., '93, 2004) and [^{3}H] FNZ exhibits no selectivity for the different α-subunits, a weaker binding affinity of [^{3}H] FNZ in ventral compared to dorsal rat hippocampus has been proposed to be due to the lower expression levels of $\gamma 2$ subunit (Sarantis et al., 2008). Moreover, considering that FNZ enhances the effects of GABA at its receptor by increasing the frequency of chloride ion channel opening (Study and Barker, '81), a reduced K_D (greater affinity) would be consistent with an improved $GABA_A$/BZD receptor activity. We observed a reduced K_D in *C. tergolaevigatus* compared to *L. koslowskyi*. Thus, the basal response in terms of GABA-related neurochemical attributes is undoubtedly different between species. Additionally, when we used *z*-scores to specifically analyze the effect of the stress exposure on the CBR affinity between species, we found that the simulated predator condition was the only stressor that altered the affinity of the CBR, and that the direction of the response was opposite in the two species. When compared to their respective control groups, *L. koslowskyi* CBRs showed a decreased receptor affinity and *C. tergolaevigatus* an increased receptor affinity, suggesting that *C. tergolaevigatus* should cope with this particular stressful situation probably faster than *L. koslowskyi*. It should be noted that, from an evolutionary perspective, the Squamate time-tree shows that most major groups diversified in the Jurassic and Cretaceous, 200–66 million years ago; in particular the Teiidae and iguanian lizards have evolved separately for over 150 million years (Vidal and Hedges, 2009). Although the HPA response to stress appears to be conserved in vertebrates, the manner in which it is activated and its actions vary especially in amphibians and reptiles (see review by Moore and Jessop, 2003). Moreover, both shared and novel hormonal targets and mechanisms were documented in the process of investigating the endocrine regulation of life history and reproductive traits in lizards (see review by Lovern, 2011), highlighting the usefulness of this rich taxonomic group for integrative and comparative studies.

Different effects on the CBR/GABA receptors have been reported in different species depending on the type, magnitude, and frequency of the stressor. For example, an immediate increase in B_{max} has been associated with a better adaptation to cope with or to overcome an acute stressful situation (Biggio et al., '90; Kellog et al., '93; Marin and Arce, '95, '96). In turtles, anoxia enhanced FNZ binding in some brain regions (Sakurai et al., '93). While in rats, the exposure to a chronic nonhabituating stress protocol or chronic exposure to stress levels of corticosterone produces changes in the mRNA levels of multiple $GABA_A$ receptor subunits, suggesting that $GABA_A$ receptor subunit composition may be altered at a key regulatory site that may have important implications for studies aimed at understanding GABAergic inhibitory influences upon the HPA axis (Orchinik et al., '95; Cullinan and Wolfe, 2000; Qin et al., 2004). Our results showed that the simulated predator was the only condition where the species studied had clear differences in behavioral responses and also significant differences in the CBR affinity parameter, possibly reflecting changes in expression of GABA subunits due to the chronic stress treatment. Similarly, Labra and Leonard ('99) found that *L. monticola* responded to the predator model by remaining motionless and diminishing the breathing rate.

With regard to the lack of chronic CBR changes induced after 12 days of exposure to high temperature or forced swimming, we offer two potential explanations. First, it is possible that other physiological mechanisms as HPA axis activation are mediating these stress responses (Langkilde and Shine, 2006; French et al., 2008). Second, it is possible that the exposure to high temperature or forced swimming made in this study might not be adequate or it is not strong enough to generate perceptible changes in the GABA/BZD receptors measured. On this topic, a

study on mice showed a stress-induced decrease in BZD binding only after exposure to the predator and not when mice were exposed to an open field test (Chekina et al., 2009). Additionally, a previous study by Langkilde and Shine (2006) evaluating the stress imposed by research practices when measuring corticosterone in *Eulamprus heatwolei* lizards found that the hormone levels in these lizards were not modified under all the situations. For example, the stress imposed by predator scent exposure did not induce significant increases in corticosterone, whereas that imposed by exposure to an unfamiliar enclosure did induce significant increases in corticosterone (Langkilde and Shine, 2006). Finally, it is important to recall that GABA/BZD receptor parameters were not evaluated in a short term after the stressor exposure, but only at the end of the study (24 hr after the end of the 12 days of daily exposure to the stressors). Therefore, the changes expected would involve an up- or downregulation of receptor-binding sites and/or changes in its affinity as well as long-term adaptive changes. Thus, potential acute changes in response to the stressors applied herein should not be ruled out. Obviously, further experimentation is needed to evaluate this hypothesis.

GABA/CBRs have been shown to be genuine stress response mediators in other vertebrates, for example, chickens (Martijena et al., '92) and rodents (Rägo et al., '89). Also, phylogenetic studies have established a high homology among CBRs in mammalian and nonmammalian vertebrates, including lizards (Hebebrand et al., '87; Friedl et al., '98; Schmitz et al., '98). In this regard, our study offers new evidence since we show for the first time that GABA/CBRs have a potential role in the ability of lizards to cope with a repeated exposure to a stressful (predator) condition.

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