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Humans and natural predators induce different fear/anxiety reactions and response pattern to diazepam in marmoset monkeys

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ABSTRACT

The behavioral response of marmoset monkeys in the Human Threat (HT) test of anxiety, and the effects of diazepam (DZP), were compared to those in the Predator Confrontation (PC) procedure. Subjects ($n=13$) were initially submitted to four habituation trials, followed by four random confrontation sessions for each test (DZP 0, 1, 2 and 3 mg/kg). Each trial was divided into three consecutive 5-min intervals: pre-exposure, exposure (human observer, taxidermized oncilla cat) and post-exposure. As DZP induced sedation, marmosets ($n=10$) were re-tested in a second experiment, consisting of two habituation trials and four confrontation sessions per stimulus, with lower DZP doses (0, 0.10, 0.25 and 0.50 mg/kg). Exposure to both stimuli significantly increased direct gazes and alarm calls, being dose-dependently reduced by DZP only in the PC test. In the HT protocol, the significant decrease in aerial scans was not detected with 0.10 mg/kg DZP. Locomotion, proximity, displacement activities and vigilance were not consistently influenced by the stimuli and/or DZP. The results thus suggest that the HT test had a greater impact on the marmosets' behavior, while DZP was more effective on the reactions observed in the PC test, possibly due to the inherent nature of each stimulus, distinct threat levels and/or presentation order.

1. Introduction

Nonhuman primates are an invaluable animal model in behavioral and biomedical research. With a highly developed and complex nervous system, they exhibit behavioral, hormonal and physiological responses to stress and anxiety-related conditions that closely resemble those of humans (e.g., King et al., 1988). These animal models, however, are not always the most feasible choice for several types of studies, considering that they are costly to obtain and upkeep in captivity, in addition to their unique space, sanitary, food and social requirements.

Neotropical monkeys – particularly marmosets – represent an interesting alternative to their Old World counterparts (Abbott et al., 2003; Barros and Tomaz, 2002; Mansfield, 2003; Stellar, 1960). Compared to most simians, these arboreal primates have a simple low-cost maintenance, adapt easily to captivity and possess a high reproductive turnover (for review see Abbott et al., 2003; Barros and Tomaz, 2002). Although they differ from catarrhine primates in a variety of aspects, these same features may allow for unconventional research strategies for several human pathologies (Abbott et al., 2003). Marmosets also exhibit neuroanatomical structures similar to other anthropoids (Reis and Erhart, 1979; Stephan, 1972) and many facilities hold stocks of non-endangered species for different research purposes. Small in size, cryptic in nature and with diurnal activities, they are susceptible to a wide range of potential predators, which in turn seems to have exerted a vital selective pressure on their behavioral ecology (Caine, 1993). Actually, diverse and complex anti-predation strategies are reported for marmosets, ranging from careful selection of sleeping sites, retirement prior to the sunset, huddled-group sleeping and arising after dawn, to the formation of mixed-group associations, use of sentinels and high vigilance rates (e.g. Caine, 1987; Ferrari and Lopes Ferrari, 1990; Hardie and Buchanan-Smith, 1997; Savage et al., 1996). A number of easily discernable fear/anxiety-related behaviors have also been consistently observed in wild and captive populations, including different body postures (e.g., genital display, scratching, scent marking), facial expressions (e.g., slit-stare, lip-smacking, flat-tufted ears) and vocalizations (e.g., *tsik-tsik*, geckering) (Stevenson and Poole, 1976; Stevenson and Rylands, 1988). Taken together, these features make marmosets a prime target for predator-related studies of fear and anxiety.

In fact, two procedures employing such an experimental strategy have been developed for marmosets. The Human Threat (HT) test relies on the presence of a human observer to induce defense attack and anxiety-related behaviors (e.g., Costall et al., 1992), whereas the Predator Confrontation (PC) test explores the response to a natural taxidermized predator (e.g., Barros et al., 2008). Both tests seem to induce a similar response pattern susceptible to several pharmacological treatments (for reviews see: Barros and Tomaz, 2002; Barros et al.,

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2008). Proximic avoidance, defense/aggressive-related postures, displacement activities, vigilance and alarm vocalizations have been reported (see Barros and Tomaz, 2002 for review). As the experimental parameters and drug dose range differ considerably in each test, studies conducted under the same conditions are necessary to determine whether a concurrent validity exists between these experimental strategies with distinct inherent threat sources (i.e., natural vs. non-natural). These increasingly employed models represent a unique opportunity to analyze how fear and anxiety behaviors occur in humans and develop new pharmacological strategies for related human disorders.

Therefore, the fear/anxiety-related response of adult captive marmosets (*Callithrix penicillata*) was assessed before, during and after the confrontation with a human observer and taxidermized predator (oncilla cat; *Leopardus tigrinus*). In the first experiment, the diazepam dose range used corresponded to that of PC tests (Barros et al., 2000, 2007), while in the second experiment doses of this same anxiolytic were given according to previous HT studies (Carey et al., 1992).

2. Materials and methods

2.1. Subjects

Adult (2–5 years old) black tufted-ear marmosets (*C. penicillata*), weighing 250–400 g at the beginning of the study, were used as subjects. Both experienced and experimentally-naïve subjects were used, as their general response pattern to similar experimental conditions seems to remain highly consistent, regardless of prior experience (Barros et al., 2007). The former included marmosets that had been previously tested 1 year before in a similar version of the PC test described below, while experimentally-naïve animals had never participated in any type of study.

Animals were pair-housed and tested at the Primate Center of the University of Brasilia, in cages of a same colony room, yet not all member of the colony were used in the present study. Both male and females were tested, some of which had previously produced offspring ($n=4$ pairs). Pairing occurred at least one year prior to the present study, before which housing conditions had consisted of family groups (i.e., parents and their twin).

The colony room consisted of two parallel rows of 12 standard cages (2 m×1.3 m×2 m each), separated by a common wire-mesh enclosed central corridor. Each cage had 2 parallel concrete walls, shared by adjacent cages, a wire-mesh front, rear and top, and a wood-shaving covered floor. Additionally, a solid roof 50–150 cm above the wire-mesh top covered two thirds of all cages. Thus, this colony room formed an outdoor/semi-indoor housing system and animals were housed and tested under natural light, temperature and humidity conditions. Each cage was provided with a suspended nest box, several natural wood perches placed at different heights, a tray for fresh food items and a PVC feeding tube hung from the wire-mesh top containing dry food pellets.

Food was available daily, from 07:30 to 17:30 h, consisting of a mixture of fresh fruits and vegetables. Meal-worms, boiled eggs and/or cooked chicken breast were provided three times a week. Water and dry food pellets were available ad libitum. At any time, natural wildlife can be seen in the vicinity of the Primate Centre, although felines – including oncilla cats – have never been spotted. Also, only husbandry and research personnel are present in the colony room. Housing conditions complied with the regulations of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

2.2. Drugs

In the first experiment (see below), diazepam (DZP; Hipolabor, Brazil) was dissolved in a solution of physiological saline with 1% Tween 80 (Sigma-Aldrich, USA) and administered in the doses of 1, 2 and 3 mg/kg. In the second experiment (see below), DZP was dissolved in the same way, yet administered in the doses of 0.10, 0.25 e 0.50 mg/kg. Saline–Tween solution was used as vehicle in both experiments and the administration volume was always 1 ml/kg. All drug treatments were given intraperitoneally (ip), 20-min prior to the behavioral testing. Dose range for the first (Barros et al., 2000, 2007) and second experiments (Carey et al., 1992) was based on previous behavioral studies with marmosets.

2.3. Experimental procedure

2.3.1. Experiment 1: (higher) diazepam dose range of PC tests

Experienced ($n=7$) and experimentally-naïve subjects ($n=6$) were tested in their own home-cages (total = 13; 6 males, 7 females). Each marmoset, regardless of any previous testing experience, was initially submitted to four 15-min habituation trials (H1–H4), held 48-h apart and in the absence of any threat stimuli. These trials were deemed necessary to habituate subjects to the presence of two observers who would only be scoring the animals' behavioral response. To isolate a specific home-cage, an isolation curtain was placed during each trial around the whole experimental set-up located in the central corridor. Following a 48-h interval, subjects were randomly assigned to one of two experimental groups and confronted with the test stimuli (two-phase cross-over design). Group 1 was initially tested in the Human Threat (HT) protocol and then, 2 weeks later, in the Predator Confrontation (PC) procedure. Group 2, on the other hand, was tested in the PC protocol first and after 2 weeks in the HT procedure. However, four trials were held (48-h apart) for each stimulus tested, consisting of the random administration of one of the following drug treatments: DZP 0, 1, 2 and 3 mg/kg (DZP0, DZP1, DZP2 and DZP3, respectively).

Each confrontation trial, regardless of the stimulus type, consisted of a 15-min observation period divided into three consecutive 5-min intervals. Following an initial pre-exposure baseline observation, the pre-determined stimulus was positioned in the central corridor of the colony room in front of the subjects' home-cage, upon which the exposure interval started. At the end of this interval, the stimulus was removed and the post-exposure interval began. During the exposure interval of the HT protocol, an observer stood motionless 50 cm from the front of the home-cage, with both hands wearing leather gloves held up (beside the head), yet avoiding eye contact with the marmosets. The exposure interval of the PC procedure, on the other hand, consisted of placing a taxidermized oncilla cat (*L. tigrinus*) on a platform located 50 cm from the front of the home-cage and 70 cm above floor level. Subjects from a same home-cage were tested simultaneously, but scored individually. As a result, two observers – one for each focal animal – were positioned in front of the pair's home-cage, 1 m behind the stimuli's location, scoring behaviors on separate laptops. During these trials, the isolation curtain was also placed around the whole experimental set-up in the central corridor in order to isolate a specific home-cage and prevent the stimuli from being seen by other members of the colony room.

Each confrontation trial consisted of capturing both subjects from a same home-cage, administering them the same pre-established drug treatment and subsequently releasing them back into their home-cages. Behavioral testing, as described above, started after a 20-min interval. The procedure followed during the initial four habituation trials consisted only of placing the whole set-up in the colony room's central corridor (including the isolation curtain) and observing the subjects behavior, as described above. No threat stimulus was placed during these trials. The order in which pairs were observed was randomly assigned on each test day for each group. Trials were held between 08:00 and 10:00 h. The procedure described above was approved by the Animal Ethics Committee of the University of Brasilia

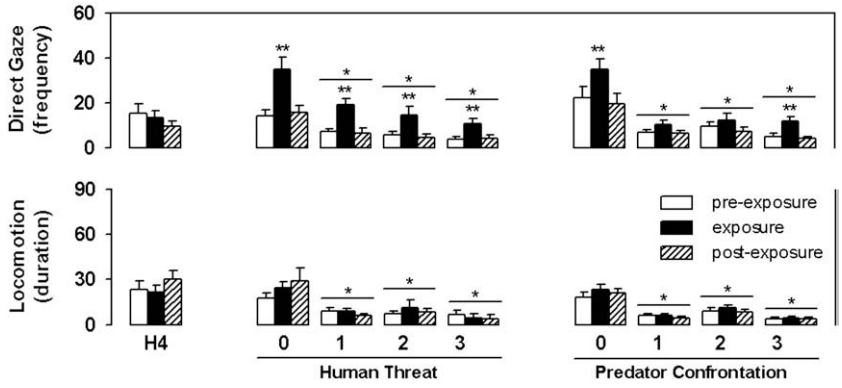

Fig. 1. Mean (+SEM) frequency of direct gazes towards the specific location of where the stimuli would be/was/had been placed (top) and the time spent in locomotion (in s; bottom) during each experimental interval (pre-exposure, exposure, post-exposure) of the last habituation trial (H4), as well as the four confrontation trials with the human observer (Human Threat) and taxidermized oncilla cat (Predator Confrontation). Each confrontation trial was performed with diazepam 1, 2 or 3 mg/kg ip treatment or a vehicle control (0) injection ($n=13$); $^{*}p<0.05$ vs. vehicle control trial, $^{**}p<0.05$ vs. respective pre- and post-exposure intervals.

and complied with the 'Brazilian Principles of Laboratory Animal Use' (COBEA).

2.3.2. Experiment 2: (lower) diazepam dose range of HT tests

Ten subjects (05 males, 05 females) from Experiment 1 were re-tested 6 months later in their own home-cages, with a (lower) diazepam dose range as that of HT tests. Each marmoset was initially submitted to two 15-min habituation trials (H1–H2), held 48-h apart and in the absence of any threat stimuli. After a 48-h period, each subject was submitted to four 15-min HT confrontation trials, held 48-h apart. Each trial consisted of the random administration of one of the following drug treatments: DZP 0, 0.10, 0.25 and 0.50 mg/kg (DZP0, DZP.10, DZP.25 and DZP.50, respectively). Three months after the last HT trial, each subject was again submitted to two 15-min habituation trials, in the same conditions as those described above, and then tested in the PC protocol. The latter consisted of four 15-min cat confrontation trials, held 48-h apart, in which each trial also corresponded to one of the following randomly-assigned drug treatments: DZP0, DZP.10, DZP.25, DZP.50. The procedure followed during the habituation and confrontation trials of this second experiment was as described above for the first.

2.4. Behavioral and statistical analyses

For each trial, the behavioral response was recorded by two experienced observers (one for each focal animal) with a 95% inter-rater reliability and blind to the drug treatment given. The following parameters were scored during Experiment 1 using the focal all-occurrences sampling method: (1) Displacement activities, frequency of scratching (quick repetitive movements of the hand/foot through the fur), scent marking (to rub the anogenital region on any substratum) and/or grooming (slow and precise movements of the hand/mouth through the fur); (2) Direct gaze, frequency of orienting the eyes and head directly at the location of where the stimulus would be/was/had been presented; (3) Aerial scan, duration of scans from the subject's horizontal plane upwards; (4) Terrestrial glance, frequency of quick downward assessments of the surroundings; (5) Locomotion, time spent in motion (>2 s). During Experiment 2, the following behaviors were also scored: (1) Proximity, time spent in contact with the home-cage's front wire-mesh; and (2) Tsik-tsik vocalization, time spent emitting this alarm/mobbing associated call. Based on previous reports by Caine (1984) and Koenig (1998), scan was arbitrarily defined as any long-lasting (≥5 s) sweeping movement of the head directed at the environment. Glance was also arbitrarily defined as a fast (<5 s) deliberate single movement of the head only, directed at the environment (Hardie and Buchanan-Smith, 1997). Scans and glances directed at passing insects were not included. Also, only aerial scans and terrestrial glances were scored as significant quantitative and qualitative differences between these vigilance-associated behaviors have been reported for this marmoset (Barros et al., 2004a, 2008). The remaining behaviors scored were based on ethograms (Stevenson and Poole, 1976; Stevenson and Rylands, 1988) and previous reports (Barros et al., 2002a, 2004a,b, 2007; Carey et al., 1992; Costall et al., 1992). The frequency and duration of the behaviors were recorded, by the observers, on the Etholog 2.2 program (USP, Brazil).

Data for each behavioral category, from a specific test (HT or PC), were analyzed using a two-way analysis of variance (ANOVA) with repeated measures on experimental trial (habituation or confrontation) and interval (pre-exposure, exposure, post-exposure). Whenever significant, subsequent comparisons were performed using the appropriate error variance terms from the respective ANOVA summary tables with Tukey's test for within-trial differences (pre-exposure; exposure;

Table 1
Behavioral response of captive adult marmosets before, during and after being submitted to the Human Threat and Predator Confrontation tests of anxiety in Experiment 1.[a]

Behavior	Human Threat			Predator Confrontation		
	PRE	EXP	POST	PRE	EXP	POST
Displacement activity frequency						
H4	2.0±0.6	2.8±1.0	2.6±0.6	2.0±0.6	2.8±1.0	2.6±0.6
DZP0	2.9±0.9	1.7±0.4	4.2±1.2	2.5±0.9	0.5±0.2	1.8±0.5
DZP1	1.5±0.2	1.0±0.6	1.3±0.2	1.8±0.4	1.1±0.3	1.3±0.5
DZP2	1.0±0.4	0.8±0.5	1.2±0.5	1.1±0.5	0.8±0.2	0.8±0.4
DZP3	0.9±0.8	1.2±0.2	1.3±0.2	1.2±0.7	0.8±0.5	1.3±0.2
Aerial scan duration (s)						
H4	8.9±4.3	9.5±4.5	5.3±2.2	8.9±4.3	9.5±4.5	5.3±2.2
DZP0	6.4±3.4	3.8±2.1	4.8±2.1	3.1±1.8	2.5±2.2	4.4±2.3
DZP1	4.3±1.9	1.5±0.4	1.8±1.4	10.2±5.9	4.7±2.9	12.7±8.0
DZP2	4.1±3.0	1.6±0.8	1.8±0.8	2.7±0.7	2.4±0.4	3.0±1.4
DZP3	6.3±4.3	1.4±1.3	1.9±1.9	2.4±0.8	2.6±1.3	2.2±0.6
Terrestrial glance frequency						
H4	17.5±2.1	16.8±1.4	16.9±1.7	17.5±2.1	16.8±1.4	16.9±1.7
DZP0	20.8±2.5	19.8±2.3	15.8±1.6	19.8±2.9	19.6±2.7	20.6±1.4
DZP1	11.8±1.9[b]	13.4±2.3[b]	11.4±2.0[b]	11.2±1.4[c]	10.7±1.6[c]	8.8±1.7[c]
DZP2	8.1±1.2[b]	10.9±1.8[b]	8.3±1.9[b]	13.2±2.2[c]	10.7±1.8[c]	11.5±2.2[c]
DZP3	5.3±1.3[b]	8.1±1.7[b]	6.8±2.1[b]	6.5±1.3[c]	7.5±1.0[c]	6.5±1.1[c]

[a] Mean±SEM is presented; PRE = pre-exposure interval; EXP = exposure interval; POS = post-exposure interval; H4 = habituation trial 4; DZP0 = saline control trial; DZP1 = diazepam 1 mg/kg; DZP2 = diazepam 2 mg/kg; DZP3 = diazepam 3 mg/kg.
[b] $p<0.05$ vs. DZP0 (PRE+EXP+POST intervals) of the Human Threat test.
[c] $p<0.05$ vs. DZP0 (PRE+EXP+POST intervals) of the Predator Confrontation test.

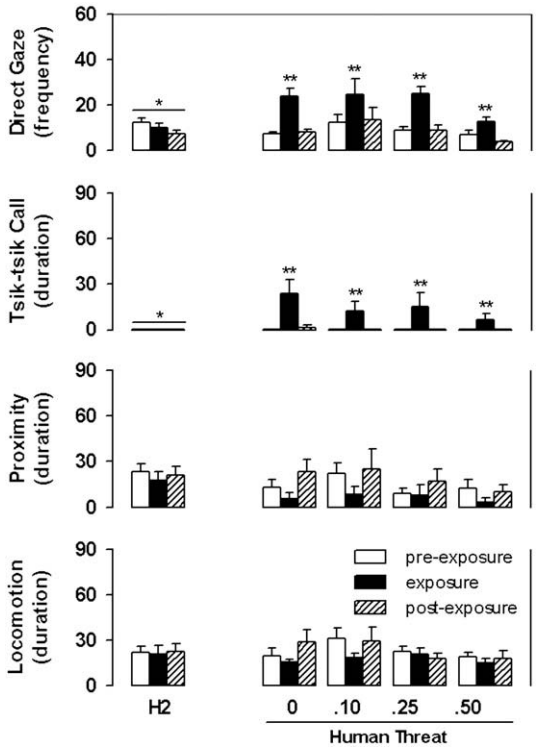

Fig. 2. Mean (+SEM) frequency of direct gazes towards the specific location of where the stimulus would be/was/had been placed (top) and the time spent (in s) emitting tsik-tsik alarm calls (center-top), in proximity with the stimulus (center-bottom) and in locomotion (bottom) during each experimental interval (pre-exposure, exposure, post-exposure) of the second habituation trial (H2), as well as the four confrontation trials with the human observer (Human Threat). Each confrontation trial was performed with diazepam 0.10, 0.25 or 0.50 mg/kg ip treatment or a vehicle control (0) injection ($n=10$); $^{*}p<0.05$ vs. vehicle control trial, $^{**}p<0.05$ vs. respective pre- and post-exposure intervals.

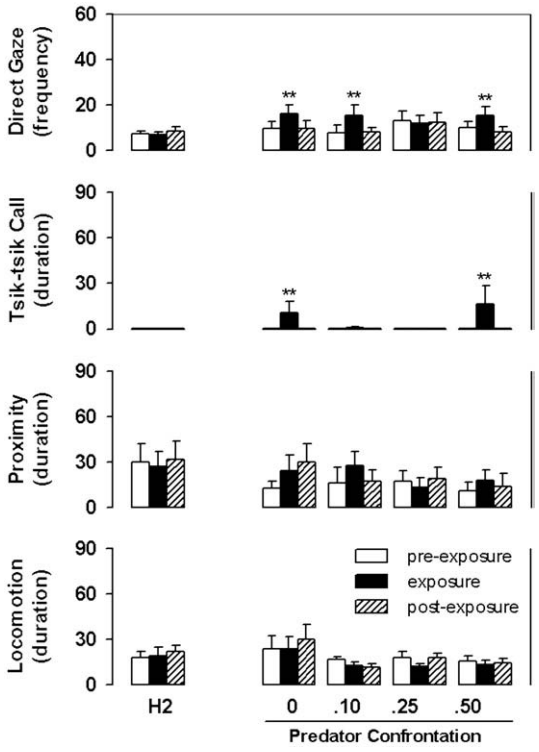

Fig. 3. Mean (+SEM) frequency of direct gazes towards the specific location of where the stimulus would be/was/had been placed (top) and the time spent (in s) emitting tsik-tsik alarm calls (center-top), in proximity with the stimulus (center-bottom) and in locomotion (bottom) during each experimental interval (pre-exposure, exposure, post-exposure) of the second habituation trial (H2), as well as the four confrontation trials with the taxidermized oncilla cat (Predator Confrontation). Each confrontation trial was performed with diazepam 0.10, 0.25 or 0.50 mg/kg ip treatment or a vehicle control (0) injection ($n=10$); $^{*}p<0.05$ vs. vehicle control trial, $^{**}p<0.05$ vs. respective pre- and post-exposure intervals.

post-exposure) or Dunnett's test for between-trial differences (H1–H4; H4/DZP0–3; H2/DZP0/DZP.10/DZP.25/DZP.50). Data from males and females were pooled together, as well as those from experienced and naïve monkeys, as no significant gender and subject-type differences were observed (data not shown). Statistical significance was set at $p\leq 0.05$ and results expressed as the mean ± SEM.

Table 2
Behavioral response of captive adult marmosets before, during and after being submitted to the Human Threat and Predator Confrontation tests of anxiety in Experiment 2.[a]

Behavior	Human Threat			Predator Confrontation		
	PRE	EXP	POST	PRE	EXP	POST
Displacement activity frequency						
H2	2.9 ± 1.4	2.2 ± 0.6	2.7 ± 0.9	1.7 ± 0.4	2.4 ± 0.7	3.3 ± 0.6
DZP0	1.4 ± 0.7	1.5 ± 0.3	2.0 ± 0.6	2.7 ± 0.8	1.5 ± 0.7	2.6 ± 0.9
DZP.10	2.3 ± 0.9	1.3 ± 0.6	1.9 ± 0.8	2.1 ± 0.5	1.4 ± 0.9	2.1 ± 0.9
DZP.25	1.0 ± 0.3	0.3 ± 0.2	1.5 ± 0.5	1.3 ± 0.3	1.1 ± 0.4	2.0 ± 0.7
DZP.50	0.8 ± 0.3	0.4 ± 0.3	0.3 ± 0.1	1.9 ± 0.6	1.3 ± 0.2	1.1 ± 0.2
Aerial scan duration (s)						
H2	5.6 ± 2.1	2.0 ± 1.2	2.0 ± 1.2	11.9 ± 5.9	7.9 ± 3.7	7.7 ± 4.0
DZP0	17.5 ± 5.7	3.1 ± 1.2[b]	7.7 ± 3.6	4.1 ± 2.7	3.1 ± 2.5	14.2 ± 7.6
DZP.10	15.6 ± 6.1	7.8 ± 4.8	8.9 ± 4.7	11.8 ± 5.7	2.6 ± 1.8	7.2 ± 3.0
DZP.25	20.7 ± 5.9	5.8 ± 3.4[b]	8.0 ± 3.4	3.1 ± 1.8	3.8 ± 2.3	3.3 ± 1.8
DZP.50	14.8 ± 4.1	2.9 ± 0.8[b]	4.7 ± 1.6	6.5 ± 2.2	2.9 ± 2.0	8.3 ± 3.3
Terrestrial glance frequency						
H2	20.3 ± 1.8	19.5 ± 2.1	16.0 ± 1.9	24.2 ± 2.6	18.0 ± 2.7	21.7 ± 2.7
DZP0	14.6 ± 2.4	18.7 ± 3.6	18.3 ± 2.9	16.8 ± 2.4	21.4 ± 4.0	21.7 ± 4.1
DZP.10	22.0 ± 2.8	19.6 ± 2.4	21.9 ± 2.8	21.5 ± 3.0	17.5 ± 3.4	17.2 ± 2.4
DZP.25	15.0 ± 1.9	19.0 ± 2.9	17.9 ± 3.2	24.0 ± 2.5	16.7 ± 2.9	19.9 ± 2.7
DZP.50	16.8 ± 4.0	12.6 ± 2.5	12.2 ± 1.9	14.5 ± 1.7	15.9 ± 3.6	17.9 ± 2.1

[a] Mean ± SEM is presented; PRE = pre-exposure interval; EXP = exposure interval; POS = post-exposure interval; H2 = habituation trial 2; DZP0 = saline control trial; DZP.10 = diazepam 0.10 mg/kg; DZP.25 = diazepam 0.25 mg/kg; DZP.50 = diazepam 0.50 mg/kg.
[b] $p<0.05$ vs. respective PRE interval of the Human Threat test.

3. Results

3.1. Experiment 1: (higher) diazepam dose range of PC tests

Direct gazes towards the location of where the stimuli would be placed in subsequent trials changed over the course of the four habituation trials. In fact, the rate of this parameter decreased significantly during the second and third trials, compared to the first, with no significant interval-effects and trial × interval interactions [trial-effect: $F(3,12)=3.90$, $p<0.05$; interval-effect: $F(2,12)=2.74$, $p=0.09$; interaction: $F(6,72)=1.10$, $p=0.37$; data not shown]. The remaining behavioral parameters analyzed remained constant during these initial four trials [Displacement Activities — interval-effect: $F(2,12)=0.35$, $p=0.71$; trial-effect: $F(3,12)=2.78$, $p=0.07$; interaction: $F(6,72)=1.13$, $p=0.36$; Aerial Scan — interval-effect: $F(2,12)=0.78$, $p=0.47$; trial-effect: $F(3,12)=0.97$, $p=0.42$; interaction: $F(6,72)=1.47$, $p=0.20$; Terrestrial Glance — interval-effect: $F(2,12)=0.56$, $p=0.58$; trial-effect: $F(3,12)=0.79$, $p=0.51$; interaction: $F(6,72)=1.24$, $p=0.30$; Locomotion — interval-effect: $F(2,12)=0.59$, $p=0.56$; trial-effect: $F(3,12)=0.92$, $p=0.44$; interaction: $F(6,72)=1.35$, $p=0.25$; data not shown].

On the other hand, marmosets detected and responded to the presence of both stimuli-types, as the rate of direct gazes towards the specific threat location increased significantly during the exposure interval of the vehicle control trial (DZP0), compared to its pre- and post-exposure intervals [HT test: $F(2,12)=37.51$, $p<0.001$; PC test:

$F(2,12)=14.13$, $p<0.001$; Fig. 1]. For the remaining behaviors analyzed, within-trial effects were not observed in both tests [Displacement Activities — HT test: $F(2,12)=2.04$, $p=0.10$; PC test: $F(2,12)=1.68$, $p=0.21$; Aerial Scan — HT test: $F(2,12)=0.23$, $p=0.79$; PC test: $F(2,12)=2.43$, $p=0.11$; Terrestrial Glance — HT test: $F(2,12)=1.78$, $p=0.19$; PC test: $F(2,12)=0.23$, $p=0.79$; Locomotion — HT test: $F(2,12)=1.14$, $p=0.34$; PC test: $F(2,12)=0.61$, $p=0.55$; Table 1].

Administration of all three doses of DZP significantly decreased the rates of direct gaze and terrestrial glance, compared to their respective DZP0 trial [Direct Gaze — HT test: $F(4,12)=5.70$, $p<0.001$; PC test: $F(4,12)=10.13$, $p<0.001$; Terrestrial Glance — HT test: $F(4,12)=11.77$, $p<0.001$; PC test: $F(4,12)=13.06$, $p<0.001$; Fig. 1 and Table 1]. However, all three doses of DZP also induced a significant decrease in the marmosets' locomotion during the three experimental intervals of both stimuli-types, compared to the DZP0 trial [HT test: $F(4,12)=8.47$, $p<0.001$; PC test: $F(4,12)=14.77$, $p<0.001$; Fig. 1]. For the remaining behavioral analyzed, however, significant between-trial effects were not detected in both tests [Displacement Activities — HT test: $F(4,12)=1.14$, $p=0.32$; PC test: $F(4,12)=1.85$, $p=0.28$; Aerial Scan — HT test: $F(4,12)=1.24$, $p=0.31$; PC test: $F(4,12)=1.69$, $p=0.17$; Table 1]. In terms of trial×interval interactions, significant effects were only seen for direct gazes [HT test: $F(6,72)=3.89$; $p<0.001$; PC test: $F(6,72)=2.03$; $p<0.05$; non-significant data not shown].

3.2. Experiment 2: (lower) diazepam dose range of HT tests

Of the two habituation trials initially held for each test, only the second session was included in the present analyses, as subjects had been recently tested (6 months before) in both protocols during the first experiment.

In the HT test, the human's presence induced a significant increase in the marmosets' direct gaze and tsik-tsik alarm call rates during the exposure interval, compared to their respective pre- and post-exposure intervals [Direct Gaze: $F(2,9)=37.13$, $p<0.001$; Tsik-tsik Call: $F(2,9)=8.72$, $p<0.01$; Fig. 2]. Although the same profile was observed in spite of DZP treatments, this response was not seen during the preceding habituation trial, with a significant trial×interval interaction [Direct Gaze — trial-effect: $F(4,9)=2.66$, $p<0.05$; interaction: $F(8,72)=4.66$, $p<0.001$; Tsik-tsik Call — trial-effect: $F(4,9)=1.72$, $p<0.05$; interaction: $F(8,72)=1.99$; $p<0.05$; Fig. 2]. Furthermore, when in the presence of the human observer (DZP0), marmosets spent significantly less time aerial scanning, relative only to the pre-exposure interval [$F(2,9)=12.07$, $p<0.001$; Table 2]. Such response pattern was observed with the DZP treatments, except DZP.10, although a significant trial-effect [$F(4,9)=1.25$, $p=0.31$] and trial×interval interaction were not detected [$F(8,72)=0.34$, $p=0.57$; Table 2]. Proximity, locomotion, displacement activities and terrestrial scans remained constant within and between trials [Proximity — interval-effect: $F(2,9)=1.58$, $p=0.23$; trial-effect: $F(4,9)=1.32$, $p=0.21$; Locomotion — interval-effect: $F(2,9)=2.22$, $p=0.14$; trial-effect: $F(4,9)=0.20$, $p=0.46$; Displacement Activities — interval-effect: $F(2,9)=2.00$, $p=0.17$; trial-effect: $F(4,9)=1.34$, $p=0.22$; Terrestrial Scan — interval-effect: $F(2,9)=0.18$, $p=0.84$; trial-effect: $F(4,9)=1.64$, $p=0.18$; Fig. 2 and Table 2].

When marmosets were confronted with the taxidermized oncilla cat (DZP0), direct gazes and tsik-tsik calls also increased significantly, compared to their respective pre- and post-exposure intervals [Direct Gaze: $F(2,9)=9.45$, $p<0.01$; Tsik-tsik Call: $F(2,9)=3.46$, $p<0.05$; Fig. 3]. When DZP was administered, this behavioral pattern was detected for direct gazes — except for DZP.25, with a significant trial×interval interaction [trial-effect: $F(4,9)=0.45$, $p=0.77$; interaction: $F(8,72)=2.83$, $p<0.01$; Fig. 3]. The oncilla cat's presence also induced tsik-tsik calls during the DZP.50 treatment, yet not for DZP.10 and DZP.25, with a significant trial×interval interaction [trial-effect: $F(4,9)=1.58$, $p=0.20$; interaction: $F(8,72)=2.58$; $p<0.05$]. The other behaviors analyzed remained constant within each trial, as well as between trials [Proximity — interval-effect: $F(2,9)=0.45$, $p=0.65$; trial-effect: $F(4,9)=1.35$, $p=0.27$; Displacement Activities — interval-effect: $F(2,9)=2.73$, $p=0.09$; trial-effect: $F(4,9)=2.33$, $p=0.08$; Aerial Scan — interval-effect: $F(2,9)=1.46$, $p=0.26$; trial-effect: $F(4,9)=0.71$, $p=0.59$; Terrestrial Glance — interval-effect: $F(2,9)=3.24$, $p=0.07$; trial-effect: $F(4,9)=0.80$, $p=0.54$; Locomotion — interval-effect: $F(2,9)=3.32$, $p=0.07$; trial-effect: $F(4,9)=1.54$, $p=0.21$; Fig. 3 and Table 2].

4. Discussion

In Experiment 1, marmosets detected the presence of both stimuli, with the number of gazes made towards their specific location increasing significantly during the control trial (DZP0). This behavioral pattern was not seen during the preceding habituation sessions, similar to previous reports (Caine, 1998; Hankerson and Caine, 2004; Hayes and Snowdon, 1990; Searcy and Caine, 2003). However, exposing these monkeys to the cat and human stimuli did not alter the levels of displacement activities, aerial scans and terrestrial glances. In the PC test, both similar (Barros et al., 2002b, 2004b, 2007) and contrary response patterns have been seen (Barros et al., 2000, 2001), while in the HT test these behaviors have not been analyzed. Also, all DZP-treatments (1, 2 and 3 mg/kg) significantly decreased locomotion. Benzodiazepines, such as DZP, typically induce ataxia and sedation, particularly at higher doses (e.g. Argyropoulos et al., 2000). Such an effect was not presently expected, considering that the dose range used had been shown effective in previous PC studies (Barros et al., 2000, 2007). This sedative effect thus precludes the correct interpretation of other behavioral and DZP results.

Therefore, a group of subjects was re-tested 6 months later (Experiment 2) with a (lower) DZP dose range reported to be effective in the HT test (0.10, 0.25 and 0.50 mg/kg; Carey et al., 1992). Here, sedation was not observed, as the time spent in motion remained constant within and between trials, with and without DZP treatment. Thus, the remaining behavioral changes observed were likely due to the stimuli's presence.

In this sense, during the DZP0 control trial, the number of gazes made towards the threat location increased significantly. Also, tsik-tsik mobbing calls recorded in this second experiment were emitted exclusively in the stimuli's presence. Although data on the vocal response of marmosets towards humans is scarce, Newman and Farley (1995) recorded a higher incidence of alarm calls in rhesus monkeys, while both feral (Bartecki and Heymann, 1987; Bezerra et al., 2009; Buchanan-Smith, 1990; Corrêa and Coutinho, 1997; Ferrari and Lopes Ferrari, 1990; Heymann, 1987; Passamani, 1995; Tello et al., 2002) and captive callitrichids consistently vocalize towards natural predators (Barros et al., 2002a, 2004b, 2007; Epple, 1968).

DZP treatment – depending on the test – reversed the threat-induced behavioral changes. In the PC test, DZP.10 and DZP.25 significantly reduced tsik-tsik call duration, while only DZP.25 effectively decreased the number of direct gazes; effects not previously reported for this procedure. In the HT protocol, the significant decrease in aerial scans was not observed with DZP.10. This is the first report on scan behavior in the HT test, as other vigilance-related behaviors have only been recently assessed in non-human primates. In fact, Barros et al. (2007) using the PC test in the Figure-8 maze, reported changes in different vigilance-related parameters with a 2 mg/kg DZP dose. Lastly, fear-induced proximic avoidance and high levels of displacement activities were not presently observed, contrary to earlier studies using both tests (e.g., Barros et al., 2000; Carey et al., 1992).

Therefore, compared to previous reports in marmosets, several differences in the behavioral response towards the threat stimuli and the effects of DZP were observed. In fact, in order to readily compare both tests, modified versions of the original protocols were used in the present study, which in turn may have played a significant role in

the discrepancies observed. For instance, exposure duration and/or frequency may influence the response pattern seen over time, leading to a possible habituation effect of specific behaviors (Barros et al., 2004a,b, Dacier et al., 2006). Similarly, the use of experienced and naïve subjects may induce variable reactions, although significant differences were not presently observed between these two types of subjects (data not shown), as indicated in the general low between-subject variability. Recently, Barros et al. (2007) also found persistent anxiety-like behaviors and response pattern to DZP in marmosets after a recent predator stress condition. Familiarity with the environment (home-cage vs. novel environment), social context (pair vs. isolation-testing) and use of captive and feral-born subjects with different exposure histories to the stimuli used are likely additional influencing factors, particularly for the PC test. Lastly, when interpreting the results, one must consider that the presence of the observers, as well as the drug administrations, may have become conditioned stimuli – influencing pre-exposure levels – as a repeated-exposure procedure was used.

Both tests, however, may still be viewed as invaluable non-human primate models for studying how fear and anxiety occur in humans and screening new potential pharmacological strategies for related human disorders, considering the consistency of several of the reactions observed, in spite of significant methodological changes. In humans, as in the marmosets presently tested, several fear and anxiety-related behaviors are described, depending on the nature and distance of the threat source, with considerable inter- and intra-individual variability in response type and intensity (for review see Sandford et al., 2000). Behavioral indicators include facial expressions, body postures and vocal responses (e.g. Barros et al., 2008). Furthermore, both humans (e.g. Argyropoulos et al., 2000) and marmosets (Barros et al., 2000, 2007) demonstrate an anxiolytic-profile following diazepam treatments, as well as sedation and ataxia with high dosing.

Nonetheless, the HT test seemed to have had a greater impact on the marmosets' behavior, whereas DZP was more effective on the reactions observed in the PC test. This pattern could correspond to the distinct inherent nature of the stimuli, where constant negative interactions with humans persistently reinforce fear/defensive aggression, while innate/socially-learned fear responses to natural predators habituate in the absence of appropriate attack reactions. Then again, reactions induced by low-level threats – such as the ambiguous taxidermized cat stimulus – are more susceptible to anxiolytics than an unmistakable high-level threat, like a human observer (e.g. Blanchard et al., 2003). Accordingly, each test may be evaluating distinct aspects of the marmosets' fear and anxiety response. Alternatively, the order in which the stimuli were tested may have contributed to the distinct profile detected and should be better evaluated in the future. Thus, a concurrent validity between these non-human primate tests of anxiety remains unclear and merits further investigation.

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