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Persistent anxiety-like behavior in marmosets following a recent predatory stress condition: Reversal by diazepam

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Abstract

Initial investigations indicated the use of the Marmoset Predator Confrontation Test (MPCT) as an experimental procedure to measure fear/anxiety-related behaviors in non-human primates. However, possible long-term habituation effects and re-use of experimental subjects need to be verified. This study, therefore, compared the behavioral response of experienced versus naïve adult black tufted-ear marmosets (*Callithrix penicillata*) in the MPCT, with/without diazepam administrations. Subjects were tested in the figure-8 maze and confronted with a taxidermized wild-cat predator stimulus. After four initial 20-min maze habituation sessions, each subject was submitted to two randomly-assigned 20-min predator confrontation sessions: vehicle and 2 mg/kg of diazepam. Confrontation with the predator induced significant behavioral changes; i.e., proximic avoidance and *tsik-tsik* alarm call. Diazepam administration, concomitant to predator exposure, reversed the behavioral changes observed. In both the experienced and naïve marmosets a similar behavioral profile and response pattern to diazepam was detected, corroborating the important selective pressure that felines seem to have on marmoset behavioral ecology. Therefore, during a more naturalistic-like regimen — i.e., recurring intermittent predator encounters — the general response pattern remains highly consistent, regardless of prior experience. One may consider the re-use of marmoset subjects in the MPCT, particularly under these specific conditions (i.e. repeated 20-min confrontations, 72-h apart).

Keywords: Marmosets; Predatory stress; Defensive behavior; Fear; Anxiety; Diazepam

1. Introduction

Predator-related stimuli have been increasingly exploited to investigate the neural mechanisms underlying normal and pathological states of anxiety in various animals, as well as to develop novel pharmacological compounds with therapeutic potential. In this sense, we recently developed an ethologically-based test to measure anxiety and fear-induced behaviors in primates — the Marmoset Predator Confrontation Test (MPCT; e.g. Barros et al., 2004). In this method subjects are confronted with a taxidermized predator (wild oncilla cat — *Felis tigrina*) in a previously habituated maze environment, while several easily discernable fear/anxiety-associated responses are measured. This small cryptic diurnal neotropical primate is a unique experimental model for studying various predator-induced defense-related responses, as they suffer one of the highest rates of predation among primates (Cheney and Wrangham, 1987). In fact, marmosets demonstrate a wide-range of complex anti-predation behavioral strategies, many of which seem to persist even among captive and captive-born individuals (Barros et al., 2002a; Buchanan-Smith, 1999; Caine, 1984, 1998; Koenig, 1998). In addition, these monkeys have a low-cost maintenance and high reproductive turnover, relative to other primates, and easily adapt to captive conditions (Barros and Tomaz, 2002).

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Initial pharmacological validating studies pointed towards the potential use the MPCT as an experimental procedure to measure fear/anxiety-related behaviors in non-human primates. Putative anxiolytics, such as the benzodiazepine agonist diazepam (Barros et al., 2000) and the serotonergic 5-HT_{1A} receptor partial agonist buspirone (Barros et al., 2001) reversed the predator-induced proximic avoidance and scratching/scent marking response, while increasing exploration (e.g., smell/lick the maze, leg stand); viewed as anxiolytic-like effects. Similar results were observed with the use of the neuropeptide substance P (Barros et al., 2002b) and the selective 5-HT_{1A} receptor antagonist WAY 100635 (Barros et al., 2003a). In addition, a behavioral validating study (Barros et al., 2004) revealed that acute predator confrontation induced fear-related behaviors, whereas repeated exposures led to full/partial habituation of several reactions. Complete reversal of the behavioral changes was only observed upon immediate removal of the predator. Considering that the order of drug treatments was randomly-assigned to each animal and that marmosets only resumed baseline activity on the absence of the predator, a habituation effect to repeated confrontations does not seem to confound the results observed in the MPCT.

Given that marmosets may demonstrate full/partial behavioral habituation to repeated predator confrontations, the use of a repeated-measure experimental design in the MPCT, and the frequent restricted availably of primate subjects in anxiety-related experiments, further studies are necessary to determine possible long-term habituation effects and re-use of experimental subjects in this test. Accordingly, the present study compared the behavioral response of maze/predator-experienced versus naïve adult black tufted-ear marmosets (*Callithrix penicillata*) in the MPCT. The behavioral effects of the diazepam were also examined in both groups of marmosets.

2. Materials and methods

2.1. Subjects

Twenty-three adult marmoset monkeys (*C. penicillata*: 10 females and 13 males) were used in this study. Animals weighed 333±9 g (mean±SEM; range 255–440 g) at the beginning of the experiment, and were housed in separate heterosexual groups in semi-indoor/outdoor cages (2×1.3×2 m) of the same colony room. Not all members of the housing colony were tested in this experiment. Subjects were divided into two experimental groups, based on previous predator encounters: naïve (n=12) and experienced marmosets (n=11). The later group had been used in an experiment held 6 months before, in which subjects were submitted to three 20-min encounters, 48-h apart, with the same taxidermized predator stimulus in the same test apparatus described below (unpublished results).

Maintenance and testing of subjects were performed at the Primate Center, University of Brasilia, under natural light, temperature and humidity conditions. Except for the brief 20-min test periods, food and water were available ad libitum. All procedures were approved by the Animal Ethics Committee of the Institute of Biology, University of Brasilia, Brazil.

2.2. Apparatus

Testing was conducted in a figure-8 maze (Fig. 1), described in detailed elsewhere (Barros and Tomaz, 2002). Briefly, it consisted of a rectangular field (125×103×35 cm) suspended 1 m from the floor and divided into five arms by two holes and barriers, forming a continuous figure-8 maze. The apparatus, made of 4 mm transparent glass on a metal frame support, was divided into two segments (front and back chambers) by a concrete visual barrier (147×8×218 cm). The back chamber consisted of an arm (125×30×35 cm) with a central guillotine-type door and two removable barriers. The latter formed the start compartment. The front chamber had three parallel arms (40×25×35 cm), 25 cm apart, ending in a common perpendicular arm (125×25×35 cm). Both chambers were interconnected through holes in the visual barrier at each of the three parallel arms. A taxidermized wild oncilla cat (*F. tigrina*), a natural predator of marmosets, was placed outside the maze facing one corner of the outer parallel arms. The visual barrier prevented the subject from viewing the taxidermized cat as it entered the maze, enabling a casual encounter with the stimulus as a result of spontaneous exploration.

2.3. Drugs

Diazepam (Hipolabor, Brazil) was diluted in a solution of 1% Tween 80 (Sigma–Aldrich, USA) and distilled water and administered by oral gavage in the doses of 0 and 2 mg/kg and a volume of 1 ml/kg. Treatments were given 30-min prior to normal morning food placement and behavioral testing commenced 1-h after administrations. The use of diazepam 2 mg/kg was based on a previous behavioral study investigating the anxiolytic effects of this compound in the same experimental protocol (Barros et al., 2000).

2.4. Experimental procedure

Regardless of previous experience, all subjects were first submitted to four 20-min maze habituation trials (MH_1–MH_4), 48-h apart, in the absence of the predator stimulus. Each trial consisted of capturing the subject in its home cage, briefly

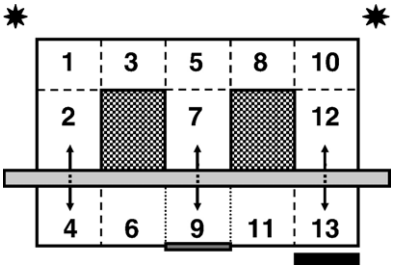

Fig. 1. Schematic top-view illustration of the figure-8 maze employed in the Marmoset Predator Confrontation Test. Stars indicates possible positions of the 'predator' stimulus (see text), arrows show the locations where the two sides of the maze intercommunicate through the concrete visual barrier, dashed lines indicate the 'imaginary' divisions of the maze into 13 sections; dotted lines delimit the start compartment (9), and solid black rectangle represents 25 cm.

handling it with leather gloves and then placing it in a transport cage (35 × 20 × 23 cm). After a 5-min interval, the marmoset was released into the maze's start compartment (Fig. 1), thus commencing a 20-min trial. Barriers from this compartment were then promptly removed, permitting free access to the whole apparatus. At the end of the trial the marmoset was returned to its home cage.

Each marmoset was then submitted to two 20-min predator confrontation trials in the same maze environment (vehicle+predator and drug+predator). The order of these two sessions varied randomly between subjects. Furthermore, during these confrontation trials, held 72-h apart, the predator was placed on either the left or right corner of the maze's front chamber, such that half the subjects confronted the predator on the right side of the maze and the other half on the left. Predator location remained constant for any given subject during these two trials. Each trial consisted of capturing the subject in its home cage, administering one of the pre-established treatments (0 or 2 mg/kg diazepam; oral gavage 30-min prior to normal morning food placement) and subsequently releasing it back into its home cage. Following a 1-h interval, the subject was submitted to the same procedure described above for the MH trials. Order of subjects was randomly-assigned on each test day and testing conducted between 08:00 and 10:00 a.m. Video cameras were used for online monitoring and all trials recorded for later behavioral analysis.

2.5. Behavioral and statistical analyses

The maze was divided into 13 sections (Fig. 1) for all-occurrences behavioral sampling analysis. The following parameters were scored for each 20-min trial by experienced observers (inter-rater reliability: >95%; ANOVA test): (1) Locomotor activity, the total number of maze sections crossed with both forelimbs, indicating total displacement within the maze; (2) Exploratory activity, frequency of sniffing/licking any part of the apparatus and/or leg stand (to raise the body into a bipedal position); (3) Proximity to predator, frequency and time spent in the maze section (right or left side) closest to the predator's location; (4) Long call vocalization, frequency of this loud high-pitched contact call; (5) *Tsik-tsik* vocalization, time spent emitting this alarm/mobbing-associated call; (6) Displacement activities, frequency of scratching (quick repetitive movements of the hand/foot through the fur), grooming (slow and precise repetitive movements of the hand through the fur) and/or scent marking (to rub the anogenital/circumgenital region on any substratum); (7) Aerial scan, frequency and duration of continuous sweeping movements of the head from the horizontal plane upwards lasting ≥ 5-s while stationary; (8) Terrestrial scan, frequency and duration of continuous sweeping movements of the head below the horizontal plane lasting ≥ 5-s while stationary; (9) Aerial glance, frequency of rapid upward movements of the head lasting < 5-s while stationary; (10) Terrestrial glance, frequency of rapid downward movements of the head lasting < 5-s while stationary. Locomotion and proximity to predator were scored using a semi-automated behavior analysis program (Chromotrack 4.02, San Diego Instruments), while visual scanning was scored using the Prostcom 3.20 program (Conde et al., 2000). Remaining behaviors were measured manually. Behavioral parameters analyzed were based on marmoset ethograms (Stevenson and Poole, 1976; Stevenson and Rylands, 1988), previous investigations employing the MPCT (Barros et al., 2000, 2002b, 2003a,b, 2004) and related studies (Carey et al., 1992; Cilia and Piper, 1997).

The four initial MH trials were compared to determine possible habituation/stable baseline rates of the behaviors analyzed, facilitating interpretation of the subsequent confrontation trials and determining possible baseline differences among the experienced and naïve marmosets. Predator confrontations were compared, together with the fourth habituation trial (as an additional control), to establish possible behavioral effects of the stimulus, of the diazepam treatment and of the previous maze/predator experience. Data for each behavioral parameter were analyzed by means of two-way analysis of variance (ANOVA) with repeated measures on trial and experimental group factors. Subsequent comparisons were performed using the appropriate error variance terms from the ANOVA summary tables with Tukey's test for MH trials or Dunnett's test for MH_4/vehicle/diazepam trials. Data on the behavioral parameters of males and females of each experimental group (naive or experienced) were pooled together as no significant differences in gender were observed. Significance level was set at $p \leq 0.05$.

3. Results

Proximity to predator was significantly altered during the course of the four maze habituation trials held in the absence of

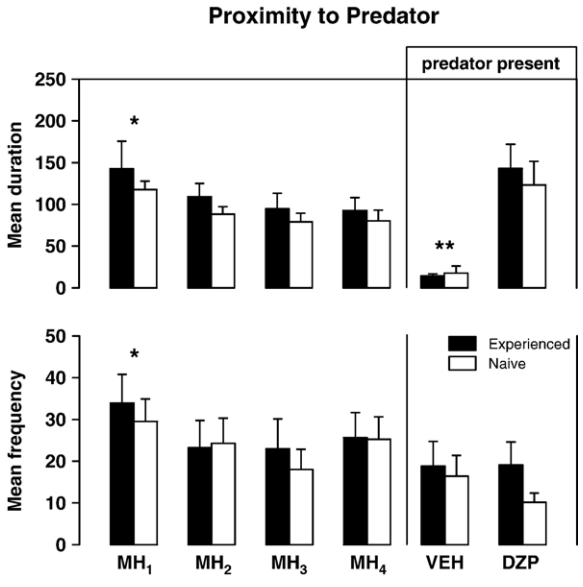

Fig. 2. Mean (+SEM) time spent in seconds (top) and frequency (bottom) in the maze section closest to the predator stimulus location of experienced and naïve-marmosets during each 20-min trial held in the absence (MH_1–MH_4) and presence of the predator stimulus (VEH = vehicle administration and DZP = diazepam 2 mg/kg administration; VEH and DZP trials were counter-balanced between subjects). *$p < 0.05$ vs. MH_3–MH_4 (top: duration) or MH_3 (bottom: frequency); **$p < 0.05$ vs. MH_4 and DZP.

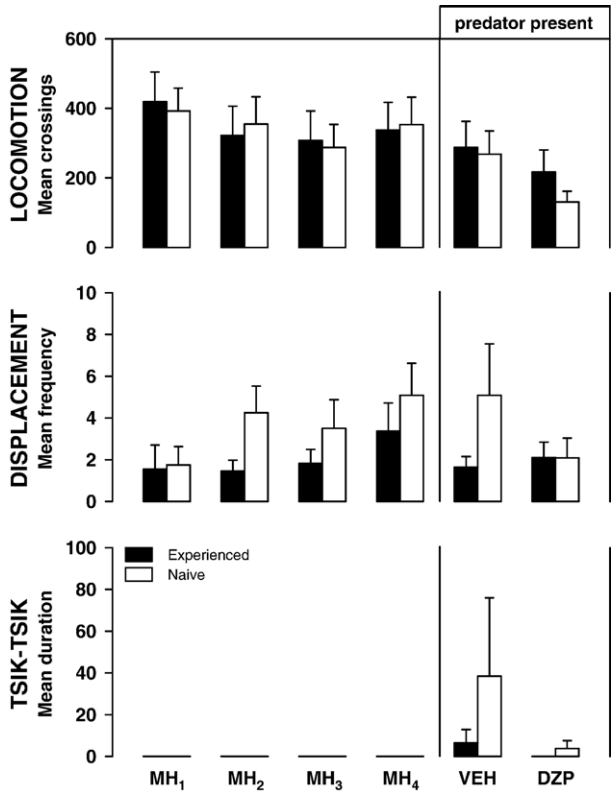

Fig. 3. Mean (+SEM) locomotor activity (top; defined as the number of maze sections crossed), frequency of displacement activities (middle; scratching/grooming/scent marking), and duration in seconds of *tsik-tsik* vocalizations (bottom) of experienced and naïve-marmosets during each 20-min trial held in the absence (MH_1–MH_4) and presence of the predator stimulus (VEH=vehicle administration and DZP=diazepam 2 mg/kg administration; VEH and DZP trials were counter-balanced between subjects).

the predator stimulus [duration: treatment — $F(3,30)=3.061$, $p<0.05$; group — $F(1,10)=1.300$, $p=0.281$; interaction — $F(3,87)=2.248$, $p=0.862$; frequency: treatment — $F(3,30)=3.814$, $p<0.05$; group — $F(1,10)=0.001$, $p=0.989$; interaction — $F(3,87)=0.546$, $p=0.602$]; i.e. duration decreased significantly ($p<0.05$) on the third and fourth trial and in frequency on the third trial, compared to their respective first sessions (Fig. 2). The other behavioral parameters analyzed, however, remained constant during these trials, also not differing significantly between experienced and naïve marmosets [Figs. 3, 4 and Table 1; locomotion: treatment — $F(3,30)=1.046$, $p=0.326$; group — $F(1,10)=0.030$, $p=0.865$; interaction — $F(3,87)=0.574$, $p=0.636$; displacement: treatment — $F(3,30)=1.212$, $p=0.062$; group — $F(1,10)=0.130$, $p=0.565$; interaction — $F(3,87)=0.463$, $p=0.584$; *tsik-tsik* vocalization: treatment — $F(3,30)=0.000$, $p=1.000$; group — $F(1,10)=0.000$, $p=1.000$; interaction — $F(3,87)=0.000$, $p=1.000$; terrestrial scan duration: treatment — $F(3,30)=1.534$, $p=0.226$; group — $F(1,10)=0.152$, $p=0.860$; interaction — $F(3,87)=0.986$, $p=0.788$; terrestrial scan frequency: treatment — $F(3,30)=1.346$, $p=0.278$; group — $F(1,10)=1.104$, $p=0.318$; interaction — $F(3,87)=0.692$, $p=0.564$; exploration: treatment — $F(3,30)=2.431$, $p=0.085$; group — $F(1,10)=0.001$, $p=0.987$; interaction — $F(3,87)=2.049$, $p=0.128$; long call vocalization: treatment — $F(3,30)=0.692$, $p=0.564$; group — $F(1,10)=0.047$, $p=0.833$; interaction — $F(3,87)=0.444$, $p=0.723$; aerial scan duration: treatment — $F(3,30)=1.278$, $p=0.300$; group — $F(1,10)=0.497$, $p=0.497$; interaction — $F(3,87)=1.016$, $p=0.399$; aerial scan frequency: treatment — $F(3,30)=1.087$, $p=0.370$; group — $F(1,10)=2.143$, $p=0.174$; interaction — $F(3,87)=0.811$, $p=0.498$; aerial glance: treatment — $F(3,30)=0.816$, $p=0.495$; group — $F(1,10)=0.523$, $p=0.486$; interaction — $F(3,87)=2.857$, $p=0.054$; terrestrial glance: treatment — $F(3,30)=1.142$, $p=0.351$; group — $F(1,10)=0.392$, $p=0.545$; interaction — $F(3,87)=0.877$, $p=0.679$].

Confrontation with the predator stimulus, however, induced a significant change in the frequency and time spent in the maze section closest to the stimulus location (Fig. 2) in both experienced and naïve marmosets [duration: treatment — $F(2,20)=18.594$, $p<0.001$; group — $F(1,10)=2.622$, $p=0.136$; interaction — $F(3,64)=2.320$, $p=0.125$; frequency: treatment — $F(2,20)=7.616$, $p<0.01$; group — $F(1,10)=0.208$, $p=0.658$; interaction — $F(3,64)=1.263$, $p=0.306$; Fig. 3]. Further analysis revealed a significant ($p<0.05$) decrease in the time spent near the predator (vehicle control), which was significantly ($p<0.05$) reversed by the administration of diazepam to both naïve and experimented marmosets. In spite of proximity frequency also being modified, post hoc analysis failed to reveal significant changes relative to vehicle control; i.e. changes are due to other pairwise comparisons (MH_4 vs. DZP). Terrestrial scan rates (Fig. 4) were also significantly influenced during the predator confrontation trials in both experimental groups [duration: treatment — $F(2,20)=9.279$, $p<0.01$; group — $F(1,10)=0.035$, $p=0.856$; interaction — $F(3,64)=0.003$, $p=0.997$; frequency: treatment — $F(2,20)=13.921$,

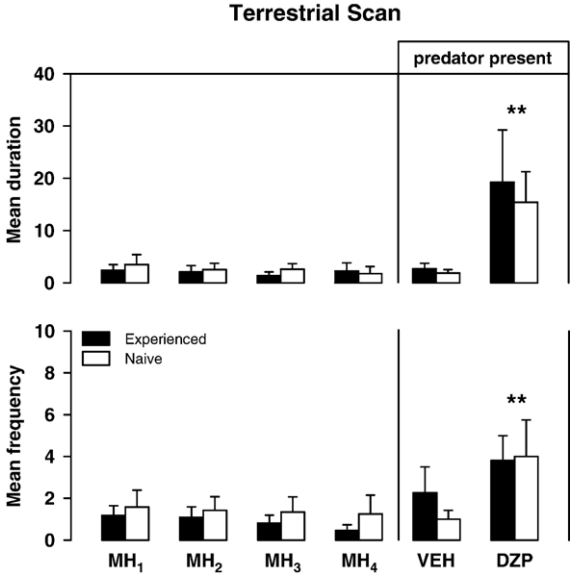

Fig. 4. Mean (+SEM) time spent in seconds (top) and frequency (bottom) of terrestrial scans of experienced and naïve-marmosets during each 20-min trial held in the absence (MH_1–MH_4) and presence of the predator stimulus (VEH=vehicle administration and DZP=diazepam 2 mg/kg administration; VEH and DZP trials were counter-balanced between subjects). **$p<0.05$ vs. MH_4 and DZP.

Table 1
Behavioral pattern observed in MPCT experienced and naïve-marmosets during each 20-min trial [a]

Behavioral parameter	Experimental trial			
	MH_1	MH_4	VEH	DZP
Experienced-marmosets				
Exploration [b]	15.00±4.09	14.64±2.99	9.64±2.23	12.00±1.90
Long call [b]	6.27±3.37	9.45±6.86	8.36±4.94	6.20±4.94
Aerial scan duration [c]	295.56±91.02	347.19±87.47	407.33±99.38	421.51±66.32
Aerial scan frequency [b]	33.09±4.00	39.09±4.46	37.64±5.65	40.00±3.06
Aerial glance [b]	27.36±8.19	32.73±12.27	32.45±12.74	13.50±3.88 [d]
Terrestrial glance [b]	59.18±8.78	63.82±10.30	56.09±9.95	52.50±9.89
Naïve-marmosets				
Exploration [b]	14.75±2.52	9.50±2.50	7.33±1.83	8.75±1.73
Long call [b]	10.33±7.38	9.83±5.48	5.58±4.15	12.33±7.75
Aerial scan duration [c]	211.91±48.67	360.45±80.17	397.96±71.23	512.99±80.22
Aerial scan frequency [b]	46.33±6.72	47.25±6.74	51.33±5.98	40.33±4.18
Aerial glance [b]	45.42±8.80	32.17±8.99	35.75±9.30	12.25±2.34 [d]
Terrestrial glance [b]	67.25±8.04	67.08±10.85	61.58±8.21	37.83±5.23

[a] Each marmoset was initially submitted to four maze habituation trials (MH_1–MH_4), followed by two confrontation trials (VEH = vehicle administration in presence of the predator; DZP = diazepam 2 mg/kg administration in the presence of the predator; data were analyzed using repeated measure ANOVAs; see text for further details).
[b] Mean frequency ± SEM.
[c] Mean duration in seconds ± SEM.
[d] $p<0.05$ vs. respective MH_4 and VEH.

$p<0.001$; group — $F(1,10)=0.004$, $p=0.951$; interaction — $F(3,64)=0.529$, $p=0.598$], such that diazepam administration significantly ($p<0.05$) increased its duration and frequency compared to vehicle control and MH_4 trial. Aerial glance (Table 1) was also influenced by the confrontation trials (treatment — $F(2,20)=7.824$, $p<0.01$; group — $F(1,10)=0.004$, $p=0.948$; interaction — $F(3,64)=0.123$, $p=0.885$); i.e. the frequency decreased significantly ($p<0.05$) following diazepam administration compared to vehicle control and MH_4 trial. Furthermore, *tsik-tsik* vocalization — which was not observed during the maze habituation trials — was detected during confrontation with the predator (i.e. vehicle), particularly in naïve marmosets, and reversed by diazepam administration, albeit not significantly (treatment — $F(2,20)=1.068$, $p=0.362$; group — $F(1,10)=0.473$, $p=0.507$; interaction — $F(3,64)=0.778$, $p=0.474$; Fig. 3). On the other hand, locomotion (Fig. 3) and terrestrial glance (Table 1) were significantly altered during the confrontation trials (locomotion: treatment — $F(2,20)=8.441$, $p<0.01$; group — $F(1,10)=0.042$, $p=0.842$; interaction — $F(3,64)=0.986$, $p=0.391$; terrestrial glance: treatment — $F(2,20)=6.912$, $p<0.01$; group — $F(1,10)=0.017$, $p=0.898$; interaction — $F(3,64)=2.937$, $p=0.077$). Further post hoc analysis failed to reveal significant changes relative to vehicle control; i.e. changes are due to other pairwise comparisons (MH_4 vs. DZP). The remaining behavioral parameters were not significantly influenced by the presence of the predator, diazepam administration, nor prior experience [Fig. 3 and Table 1; displacement activity: treatment — $F(2,20)=2.791$, $p=0.084$; group — $F(1,10)=2.539$, $p=0.142$; interaction — $F(3,64)=1.117$, $p=0.348$; exploration: treatment — $F(2,20)=3.257$, $p=0.058$; group — $F(1,10)=1.401$, $p=0.264$; interaction — $F(3,64)=0.401$, $p=0.675$; long call: treatment — $F(2,20)=0.468$, $p=0.633$; group — $F(1,10)=0.107$, $p=0.750$; interaction — $F(3,64)=0.556$, $p=0.583$; aerial scan duration: treatment — $F(2,20)=1.944$, $p=0.168$; group — $F(1,10)=0.056$, $p=0.818$; interaction — $F(3,64)=1.042$, $p=0.372$; aerial scan frequency: treatment — $F(2,20)=0.777$, $p=0.473$; group — $F(1,10)=1.097$, $p=0.319$; interaction — $F(3,64)=0.958$, $p=0.401$].

4. Discussion

The present study further indicates that the Marmoset Predator Confrontation Test (MPCT) is capable of inducing significant typical fear and anxiety-related behaviors. Importantly, these changes were reversed by the administration of the putative anxiolytic diazepam (2 mg/kg) in both experienced and naïve marmosets. It is also important to emphasize that, among the marmosets tested, the vehicle + predator session was counter-balanced with the drug + predator trial, thus minimizing possible confounding habituation and drug effects on the results observed.

During the initial maze habituation trials, locomotor (Fig. 3) and explorative activities (Table 1) remained constant, unlike previous investigations where these behaviors decreased significantly (Barros et al., 2000, 2002b, 2003a,b, 2004). Factors such as previous experience in the maze environment (experienced subjects) or recent transfer to the Primate Center from other locations (<6-months; naïve subjects), may have contributed to this result. Nonetheless, subsequent confrontation with the wild-cat significantly induced a typical proximic avoidance behavior (Fig. 2), as well as an alarm/mobbing *tsik-tsik* call (Fig. 3); behaviors which were reversed by the administration of diazepam 2 mg/kg. A possible sedative effect does not seem to confound these results, since this same dose did not significantly alter locomotion, exploration and proximity frequencies, as observed in a previous study (Barros et al., 2000). The predator also had an interesting effect upon vigilance-

associated behaviors (Fig. 4, Table 1). Marmosets commonly demonstrate constant high rates of aerial scan and terrestrial glance, whereas terrestrial scan and aerial glance occur less frequently (Barros et al., 2003b). This pattern — also seen during the present maze habituation trials — is thought to ensure effective and continuous monitoring of the surroundings (Caine, 1984), according to the differential aerial vs. terrestrial threat to which marmosets are exposed (Ferrari and Lopes Ferrari, 1990; Heymann, 1990; Peres, 1993). In addition, vigilance increases in response to a potential threat, facilitating the early detection and avoidance of predators (Caine, 1984, 1998; Ferrari and Lopes Ferrari, 1990; Hardie and Buchanan-Smith, 1997; Koenig, 1998). When confronted with the wild-cat, however, general vigilance rates remained constant. Interesting though was the significant increase in terrestrial scan and decrease in aerial glance — the less frequent behaviors — when 2 mg/kg of diazepam was administered. As this is the first study to report the effects of an anxiolytic agent on vigilance-associated behaviors in these primates, further studies are required to better elucidate the possible: 1) influence of aerial vs. terrestrial aversive stimuli, 2) association of vigilance responses with the 'behavioral inhibition' system observed during risk-assessment in rodents (e.g., Blanchard and Blanchard, 1989), and 3) partial sedative effect of diazepam on the complex vigilance response.

Of particular interest was the similar behavioral response of both experienced and naïve marmosets to the predatory stress procedure. Such result suggests that felines may have had an important selective pressure upon this species behavioral ecology and that a more naturalistic-like situation — i.e., recurring intermittent predator encounters — induces a highly consistent general response pattern, regardless of prior experience. Only *tsik-tsik* call and displacement activity were greater in the naïve than the experienced marmosets during confrontations (i.e. vehicle control; Fig. 3), albeit not significantly possibly due to inter-individual variations — particularly for *tsik-tsik* call. Previous studies have detected higher rates of this fear-associated call (Barros et al., 2004). Such discrepancy may come to minimize the usefulness of this vocalization in the present procedure, also having been found to vary in feral marmosets according to age, gender and social rank (e.g. Stevenson and Rylands, 1988). Nonetheless, increases in these behaviors have been described as fear/anxiety-related responses in the MPCT (e.g., Barros et al., 2004), the latter being significantly reversed by different putative anxiolytics (e.g., Barros et al., 2000, 2001).

In fact, when considering the response to diazepam administration, again a similar profile was observed between the two experimental groups. In the MPCT, a recent (i.e., <72-h) predator and diazepam exposure also did not significantly alter the marmosets' response, as a repeated-exposure paradigm is usually adopted in this test (Barros et al., 2000). To our knowledge, similar studies in other primate tests of anxiety have not been conducted. In rodents, (cat) predator exposure induced significant and persistent (20-day) behavioral and hormonal effects (Blanchard et al., 1998). However, a recent prior test experience has repeatedly resulted in a distinct behavioral profile and response pattern to benzodiazepines (e.g., Holmes et al., 2001; Rodgers and Shepherd, 1993). Studies on the long-term effects, such as a 6-month interval, are scarce. Thus, comparative studies may help elucidate possible distinct long-term behavioral and pharmacological responses in different animal models of anxiety.

Taken together, the present results suggest that a long-term habituation effect (>6-months) of the behavioral profile and the response to diazepam was generally not observed (experienced vs. naïve subjects). During few and frequent brief encounters, a rapid habituation effect has been observed (three 3-min confrontations 3–4 days apart; Dacier et al., 2006), whereas several frequent long-lasting exposures lead to a full/partial habituation (six 30-min confrontations 3 days apart; Barros et al., 2004). Therefore, the marmosets' response to repeated predator encounters seems highly dependent on its duration, number of confrontations and exposure frequency. Under the present experimental conditions (i.e. repeated 20-min confrontations, 72-h apart), one may consider the re-use of these primates.

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