

# Multibehavioral analysis of fear and anxiety before, during, and after experimentally induced predatory stress in *Callithrix penicillata*

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Received 6 January 2004; received in revised form 13 April 2004; accepted 20 April 2004

## Abstract

A detailed behavioral analysis of nine *Callithrix penicillata* was conducted in the Marmoset Predator Confrontation Test (MPCT) during (a) four habituation trials with no “predator,” (b) six confrontation trials with the predator (taxidermized oncilla cat, *Felis tigrina*), and (c) four trials with the predator removed. The marmosets habituated to the test apparatus with significant decreases in locomotion, exploration and long calls. Initial exposure to the predator elicited mainly fear-related behaviors (proximic avoidance, *tsik-tsik* vocalization, swaying/tongue in–out), whereas repeated confrontations attenuated these behaviors, concomitant to an increase in anxiety-associated responses (scratching/grooming/scent marking). The initial behavioral repertoire, observed before confrontations, was fully restored only upon removal of the predator. This easily discernable complex defensive behavioral repertoire is hoped to provide a comprehensive baseline for studying the biological substrates of fear/anxiety parameters in nonhuman primates.

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**Keywords:** Marmosets; Predator confrontation; Defensive behavior; Fear/anxiety; Habituation; Extinction; Contextual conditioning

## 1. Introduction

Mammalian defensive behaviors have markedly similar origins (predators, conspecifics), behavioral outcomes (e.g., flight, freezing, defensive attack), and response-specific neural systems (Blanchard et al., 2003a), probably comprising an evolutionary basis of certain human emotional responses, such as anxiety (e.g., Nesse, 1999). In fact, different natural defense-inducing stressors—in particular, predator-related stimuli—and their consequent behavioral repertoire are being increasingly exploited to investigate the neural mechanisms underlying anxiety disorders with the prospect of developing new anxiolytic drugs. The use of such stimuli to investigate fear and anxiety, in favor of painful stimulation, leads to more natural reactions and a wider range of response patterns that incorporate physio-

logical and phylogenetic aspects (Blanchard et al., 1998; Kavaliers and Choleris, 2001).

Primates, compared to other animal models, can play an important role in clarifying the neural basis of human fear/anxiety due to their closer phylogenetic proximity, resemblance and complexity of the brain and in their physiological and behavioral responses to anxiety-inducing situations (King et al., 1988). Callitrichids, in particular, suffer the highest rates of predation among primates (Cheney and Wrangham, 1987), a feature that has exerted a fundamental selective pressure on their behavioral repertoire (Caine, 1993). In this family of cryptic diurnal neotropical primates, a wide range of complex antipredation strategies are attributed, in part, to this elevated risk of predation. Among these are careful selection of sleeping sites, retirement before sunset, huddled sleeping in a group, arousal only after dawn, mixed-group associations, predator-specific vocalizations, sentinels, and high baseline rates of visual scanning (Dawson, 1979; Ferrari and Lopes Ferrari, 1990; Hardie and Buchanan-Smith, 1997; Heymann, 1995; Peres, 1993; Pook and Pook, 1982; Savage et al., 1996). In fact, these strategies persist even among captive- and captive-born individuals (Barros et al., 2002a; Buchanan-Smith, 1999;

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Caine, 1984, 1998; Koenig, 1998). Furthermore, callitrichids have a low-cost maintenance, adapt rapidly to captive conditions, and have a high reproductive turnover relative to other primates. Taken together, these features make callitrichids a preferred subject for predator-related fear/anxiety studies.

Recently, we developed an ethologically based test to measure anxiety and fear-induced behaviors in these primates—the Marmoset Predator Confrontation Test (MPCT). 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. Validating studies with the benzodiazepine agonist diazepam (Barros et al., 2000) and the serotonergic 5-HT<sub>1A</sub> receptor partial agonist buspirone (Barros et al., 2001) yielded an anxiolytic-like profile, such as reduced avoidance of the predator stimulus and scratching/scent marking, while increasing exploration (e.g., smell/lick the maze, leg stand). The MPCT has been employed to assess the potential anxiolytic action of new agents like the neuropeptide substance P (Barros et al., 2002b) and the selective 5-HT<sub>1A</sub> receptor antagonist WAY 100635 (Barros et al., 2003a).

Given the limited number of predator-encounter studies in primates, the pharmacological validating results of the MPCT, and its usefulness to measure fear/anxiety-related behaviors, this method warrants a more in-depth behavioral evaluation. The present study was designed to investigate marmosets' behavioral repertoire before, during, and after repeated “predator” confrontations. Such analysis was hoped to provide a wider range of dependent variables in future behavioral pharmacological studies employing this test. We conducted a relatively comprehensive behavioral analysis of the black tufted-ear marmoset (*Callithrix penicillata*) in the MPCT setup during different conditions: (a) four maze habituation trials in the absence of the predator, followed by (b) six predator confrontation trials, and (c) four subsequent trials in the absence of the predator to assess possible generalization of the fear/anxiety response to the confrontation context (the maze) and the course of extinction of these responses.

## 2. Materials and methods

### 2.1. Subjects

Subjects were nine experimentally naïve adult male marmosets (*C. penicillata*). They weighed 340–480 g at the beginning of experiments and were housed in separate heterosexual groups in semi-indoor/outdoor cages (2 × 1.3 × 2 m) of the same colony room (female counterparts of each group were not tested). Maintenance and testing of subjects were performed at the Primate Center, University of Brasilia, under natural light, temperature, and

humidity conditions. Except during the brief 30-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-eight maze (Fig. 1), described in detail 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-eight maze. The apparatus, made of 4-mm-thick 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 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. Experimental procedure

For each trial, the subject was captured in its home cage, briefly handled with leather gloves and then placed in a transport cage (35 × 20 × 23 cm). After a 5-min interval, the marmoset was released into the maze's start compartment, thus commencing a 30-min trial. Barriers from this com-

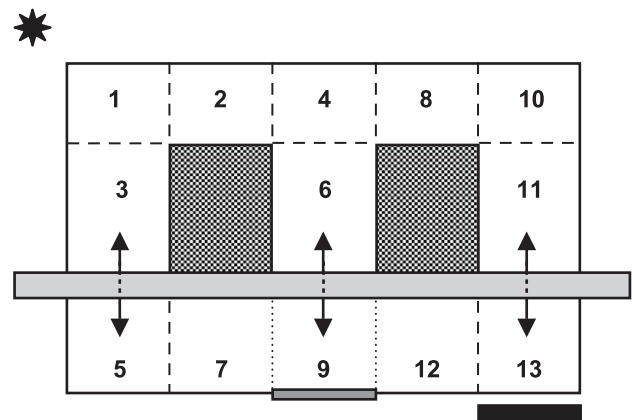


Fig. 1. Schematic top view illustration of the figure-eight maze used in the MPCT. The star indicates the position of the ‘predator’ stimulus (only the left side is represented), arrows show the locations where the two sides of the maze intercommunicated, dashed lines delineate the division of the maze into 13 sections with their respective numbers, dotted lines delimit the start compartment, and the solid black rectangle represents 25 cm.

partment 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. The impact of possible influencing anxiogenic stimuli in the MPCT, such as social isolation and handling stress, has been discussed elsewhere (see Barros and Tomaz, 2002). Order of subjects was randomly assigned for each test day and testing was conducted between 8:00 a.m. and 12:00 p.m. Video cameras were used for online monitoring and all trials recorded for later behavioral analysis. The procedure involved a three-phase design lasting 37 consecutive days.

### 2.3.1. Phase 1: maze habituation

Subjects were first submitted to four 30-min habituation trials (MH<sub>1</sub>–MH<sub>4</sub>), 48 h apart, and in the absence of the predator. These trials are essential to reduce erratic locomotor activity usually observed in captive marmosets exposed to a novel environment. This behavior declines reliably to a baseline level by the fourth trial (e.g., Barros et al., 2000), thus minimizing possible influences of novelty on the fear/anxiety response to the predator stimulus.

### 2.3.2. Phase 2: predator confrontation

Forty-eight hours following MH<sub>4</sub>, each marmoset was introduced into the maze in the presence of the predator for six 30-min confrontation trials (PC<sub>1</sub>–PC<sub>6</sub>) held 72 h apart. During these trials, the stimulus was placed on either the left or the right corner of the maze's front chamber. Half the subjects confronted the predator on the right side of the maze, and the other half, on the left side. Stimulus location remained constant for any given subject over these six trials.

### 2.3.3. Phase 3: predator removal

Seventy-two hours after PC<sub>6</sub>, each marmoset was submitted to four 30-min trials (PR<sub>1</sub>–PR<sub>4</sub>) in the absence of the predator, conducted 72 h apart. These trials aimed to assess the degree of conditioning of the fear/anxiety responses to the confrontation context and the course of their extinction over trials.

## 2.4. Behavioral analysis

For behavioral analysis, the maze was divided into 13 sections (Fig. 1). The following parameters were scored for each 30-min trial by an experienced observer: (1) *locomotor activity*, the total number of maze sections crossed with both forelimbs, indicating total displacement within the maze; (2) *exploratory activity*, the frequency of sniffing/licking any part of the apparatus and/or leg stand (to raise the body into a bipedal position), comprising of nondisplacement explorative-related elements; (3) *proximity to predator*, the frequency and time spent in the maze section (right or left side) closest to the predator location; (4) *long call vocalization*, the frequency of this loud high-pitched contact call; (5) *tsik-tsik vocalization*, the time spent emitting this alarm/mobbing-associated call; (6) the 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) *visual scanning*, the frequency of continuous sweeping movements of the head lasting more than 5 s while the animal remains stationary; (8) the frequency of *swaying* (rapid and repetitive side-to-side movements of the body while sitting) and/or *tongue in-out* (rapid and repetitive movements of the tongue in and out of the mouth); (9) the frequency of *withdrawal* (to press the upper body against the floor with forelimbs extended and lower body raised while stationary and facing stimulus), *stretch approach* (to initially move towards the stimulus elongating the entire ventral part of the body near the floor, then to retract to original position) and/or *flat body* (to elongate the entire ventral part of the body on the floor while stationary and facing the stimulus); (10) the time spent in each maze section.

Locomotor activity, time spent in each maze section, and proximity to predator were scored using a semiautomated behavior analysis program (Chromotrack 4.02, San Diego Instruments), while visual scanning was scored using the PROSTCOM 3.20 program (Conde et al., 2000) and the frequency and/or duration of the remaining behaviors were measured manually by all-occurrences samplings. The behaviors analyzed were based on related studies (Carey et al., 1992; Cilia and Piper, 1997), experiments employing the MPCT (e.g., Barros and Tomaz, 2002), and ethograms (Stevenson and Poole, 1976; Stevenson and Rylands, 1988).

## 2.5. Statistical analysis

Data for each behavioral category were analyzed by one-way analysis of variance (ANOVA) with repeated measures on the trial factor. Subsequent comparisons were performed using the appropriate error variance terms from the ANOVA summary tables with Tukey's test. Analyses between specific trials of the three experimental phases were held with the Bonferroni-corrected paired *t* test ( $P=.05/4$ : MH<sub>4</sub> vs. PC<sub>1</sub>, MH<sub>4</sub> vs. PC<sub>6</sub>, MH<sub>4</sub> vs. PR<sub>1</sub>, PC<sub>6</sub> vs. PR<sub>1</sub>). The correlation between locomotor and exploratory activity of each experimental phase was calculated with the Bonferroni-corrected ( $P=.05/3$ ) Pearson correlation coefficient, using the trend component values of the four habituation trials, six confrontation trials, or four trials with predator removed. For the remaining tests, a *P* value of  $P<.05$  was used for statistical significance.

## 3. Results

### 3.1. Habituation

Over the course of the four habituation trials (MH<sub>1</sub>–MH<sub>4</sub>), conducted in the absence of the predator stimulus,

the animals displayed a significant decrease in locomotor [ $F(3,24)=5.071, P<.01$ ; Fig. 2A] and exploratory activities [ $F(3,24)=6.496, P<.01$ ; Fig. 2B]. Post hoc analyses revealed that locomotion decreased significantly ( $P<.05$ ) during MH<sub>3</sub> and MH<sub>4</sub>, compared to MH<sub>1</sub>, while exploration was significantly ( $P<.01$ ) lower during MH<sub>2</sub>–MH<sub>4</sub>, relative to MH<sub>1</sub>. Locomotion was not significantly correlated with exploratory activity ( $r=.447, P=.228, \alpha=5/3$ ). The frequency in the maze section closest to where the predator stimulus would be located in future trials (i.e., proximity to predator) also decreased [ $F(3,24)=4.361, P<.05$ ; Fig. 2D], reaching significant values ( $P<.05$ ) during MH<sub>3</sub>–MH<sub>4</sub>, compared to MH<sub>1</sub>. Similarly, the frequency of long calls consistently decreased over sessions [ $F(3,24)=2.969, P=.052$ ; Fig. 3A]. On the other hand, repeated exposure to

the maze environment altered neither the total duration in the maze section where the stimulus would be located in subsequent trials [proximity to predator:  $F(3,24)=1.89, P=.157$ ; Fig. 2C], the frequencies of scratching/grooming/scent marking [ $F(3,24)=0.787, P=.513$ ; Fig. 3C] and visual scanning [ $F(3,24)=0.599, P=.622$ ; Fig. 3D], nor the time spent in each maze section (Table 1). For the latter parameter, however, the time spent in each of the 13 sections differed within each trial; that is, marmosets spent more time in specific sections of the maze, namely, the three parallel arms. *Tsik-tsik* vocalization (Fig. 3B), swaying/tongue in–out, and withdrawal/stretch approach/flat body were not observed during any of the habituation trials. As a whole, the results indicate that marmosets habituated to the maze environment.

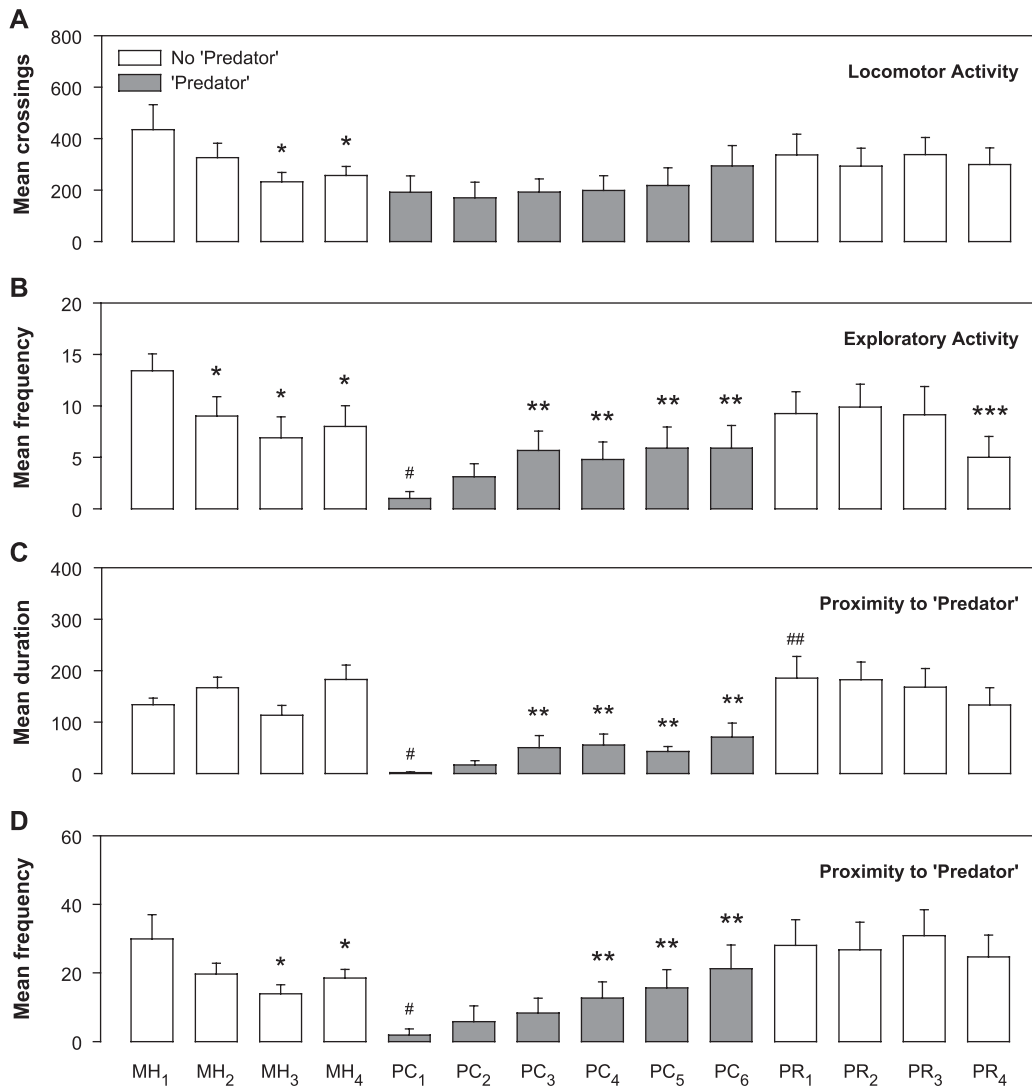


Fig. 2. Mean ( $\pm$  S.E.M.) locomotor activity (A), defined as the number of maze sections crossed; frequency of exploratory activity (smell/lick maze, leg stand) (B), time in seconds (C), and frequency (D) in the maze section closest to the predator stimulus location for each 30-min trial during the three experimental phases: four maze habituations (MH<sub>1</sub>–MH<sub>4</sub>), six ‘predator’ confrontations (PC<sub>1</sub>–PC<sub>6</sub>), and four trials with the ‘predator’ removed (PR<sub>1</sub>–PR<sub>4</sub>). White columns (MH<sub>1</sub>–MH<sub>4</sub>; PR<sub>1</sub>–PR<sub>4</sub>) indicate absence of the predator stimulus, while gray columns show when the ‘predator’ was present (PC<sub>1</sub>–PC<sub>6</sub>). \*  $P<.05$  vs. MH<sub>1</sub>; \*\*  $P<.05$  vs. PC<sub>1</sub>; \*\*\*  $P<.05$  vs. PR<sub>2</sub>; #  $P<.05$  vs. MH<sub>4</sub>; ##  $P<.05$  vs. PC<sub>6</sub>.

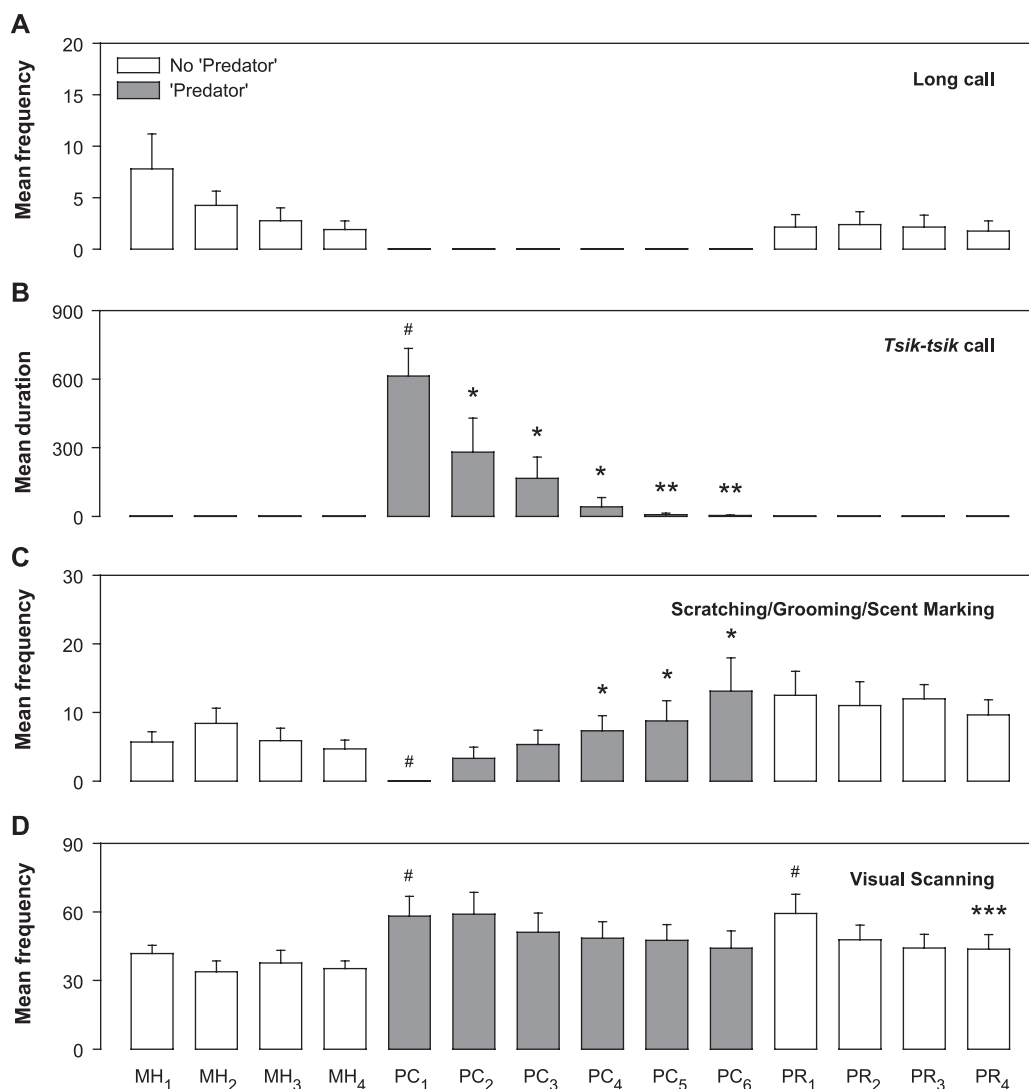


Fig. 3. Mean ( $\pm$  S.E.M.) frequency of long call vocalizations (A); time in seconds spent emitting *tsik-tsik* vocalizations (B); frequency of scratching/grooming/scent marking behavior (C); and frequency of visual scanning (D) for each 30-min session during the three experimental phases: four maze habituations (MH<sub>1</sub>–MH<sub>4</sub>), six ‘predator’ confrontations (PC<sub>1</sub>–PC<sub>6</sub>), and four trials with the ‘predator’ removed (PR<sub>1</sub>–PR<sub>4</sub>). White columns (MH<sub>1</sub>–MH<sub>4</sub>; PR<sub>1</sub>–PR<sub>4</sub>) indicate absence of the ‘predator’ stimulus, while gray columns show when the ‘predator’ was present (PC<sub>1</sub>–PC<sub>6</sub>). \*  $P < .05$  vs. PC<sub>1</sub>; \*\*  $P < .05$  vs. PC<sub>1</sub> and PC<sub>2</sub>; \*\*\*  $P < .05$  vs. PR<sub>1</sub>; #  $P < .05$  vs. MH<sub>4</sub>.

### 3.2. Predator confrontations

Initial (PC<sub>1</sub>) and repeated predator confrontations (PC<sub>2</sub>–PC<sub>6</sub>) significantly changed the marmosets’ behavioral repertoire, compared to the last maze habituation trial (MH<sub>4</sub>) and the first confrontation, respectively. Initial exposure induced a significant decrease in exploratory activity [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = 3.617$ ,  $P < .01$ ,  $\alpha = 5/4$ ; Fig. 2B], which changed over subsequent trials [ $F(5,40) = 3.389$ ,  $P < .05$ ]. Exploration increased significantly ( $P < .01$ ) during PC<sub>3</sub> relative to PC<sub>1</sub>, and stabilized over the following confrontation trials; however, the level remained lower than during MH<sub>4</sub> [PC<sub>6</sub> vs. MH<sub>4</sub>:  $t(8) = 2.589$ ,  $P = .032$ ,  $\alpha = 5/4$ ].

Importantly, duration and frequency in the maze section closest to the predator stimulus (proximity to predator) also

decreased significantly during the first predator exposure [PC<sub>1</sub> vs. MH<sub>4</sub> duration:  $t(8) = 5.947$ ,  $P < .001$ ,  $\alpha = 5/4$ ; frequency:  $t(8) = 6.151$ ,  $P < .001$ ,  $\alpha = 5/4$ ; Fig. 2C and D]. Subsequent exposures significantly influenced this profile [duration:  $F(5,40) = 3.219$ ,  $P < .05$ ; frequency:  $F(5,40) = 4.658$ ,  $P < .01$ ; Fig. 2C and D]. The time spent in proximity to predator significantly increased during PC<sub>3</sub>–PC<sub>6</sub> ( $P < .05$ ), compared to PC<sub>1</sub>. Despite this increase, proximity duration remained stable over trials PC<sub>3</sub>–PC<sub>6</sub> ( $P > .05$ ), and below the rate observed for MH<sub>4</sub>, almost reaching statistical significance [PC<sub>6</sub> vs. MH<sub>4</sub>:  $t(8) = 2.238$ ,  $P = .056$ ,  $\alpha = 5/4$ ]. Post hoc analysis also revealed that proximity frequency steadily increased during PC<sub>4</sub>–PC<sub>6</sub> ( $P < .05$ ), compared to PC<sub>1</sub>, reaching levels comparable to MH<sub>4</sub> [PC<sub>6</sub> vs. MH<sub>4</sub>:  $t(8) = -0.423$ ,  $P = .683$ ,  $\alpha = 5/4$ ].



Table 1

Mean time spent in each of the maze's 13 sections during the first and last 30-min session of each experimental phase

Maze section <sup>a</sup>	Experimental phase <sup>b</sup>					
	Maze habituation		Predator confrontation		Predator removal	
	1	4	1	6	1	4
1	135.92 ± 13.94	171.98 ± 29.20	1.56 ± 1.48	70.83 ± 27.07	185.16 ± 42.09	132.94 ± 33.77
2	78.20 ± 7.28	93.85 ± 26.00	28.47 ± 22.47	84.66 ± 27.71	156.65 ± 42.56	142.79 ± 43.97
3	187.62 ± 28.02	210.87 ± 40.92	6.61 ± 6.44	113.04 ± 37.21	119.35 ± 17.56	138.27 ± 26.72
4	146.85 ± 18.01	143.06 ± 23.06	12.03 ± 9.32	120.03 ± 30.93	280.77 ± 59.19	325.54 ± 137.83
5	240.47 ± 25.38	225.97 ± 40.57	60.71 ± 36.38	163.26 ± 55.32	164.68 ± 30.01	183.87 ± 43.23
6	152.36 ± 11.26	95.56 ± 14.63	38.32 ± 22.10	117.25 ± 24.68	133.90 ± 40.09	85.35 ± 21.01
7	64.50 ± 7.16	41.31 ± 7.53	23.57 ± 11.68	65.35 ± 24.78	57.41 ± 12.19	77.65 ± 34.22
8	81.02 ± 7.92	119.62 ± 42.23	53.73 ± 30.79	96.32 ± 46.46	144.41 ± 49.81	176.09 ± 46.65
9	126.18 ± 19.91	112.88 ± 18.40	79.97 ± 22.66	102.80 ± 28.38	116.98 ± 21.73	92.73 ± 24.11
10	138.49 ± 18.16	131.44 ± 26.76	310.70 ± 151.78	134.26 ± 37.83	184.26 ± 50.73	138.77 ± 34.00
11	145.08 ± 17.86	145.78 ± 17.06	451.23 ± 94.70	497.75 ± 223.90	90.91 ± 30.93	95.67 ± 17.95
12	81.37 ± 16.83	57.86 ± 16.96	132.70 ± 20.84	46.31 ± 6.53	48.31 ± 11.05	51.93 ± 12.98
13	213.04 ± 22.51	244.96 ± 78.61	596.73 ± 133.36	183.64 ± 27.18	111.24 ± 27.74	153.76 ± 39.75

<sup>a</sup> Maze section numbers are equivalent to those schematically illustrated in Fig. 1, with Section 1 being that closest to the predator and successively larger numbers being farther away from the stimulus.

<sup>b</sup> Data are expressed as mean ± S.E.M (seconds).

Analysis of the time that marmosets spent in each maze section (Table 1) indicated that on the first confrontation, subjects spent on average 65% of their time in the three maze sections opposite the predator, compared to 2% in the three sections closest to it. From these locations, the predator stimulus could not easily be seen. Subsequent exposures altered this pattern, inasmuch as during PC<sub>6</sub> the mean time spent in the three sections opposite the stimulus decreased, while in the closest three it increased.

Furthermore, the frequency of scratching/grooming/scent marking significantly decreased upon the marmosets' first exposure to the predator [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = 3.255$ ,  $P < .01$ ,  $\alpha = 5/4$ ; Fig. 3C]. This initial profile changed due to further confrontations [ $F(5,40) = 6.628$ ,  $P < .01$ ; Fig. 3C], in that these behaviors increased significantly ( $P < .05$ ) over PC<sub>3</sub>–PC<sub>6</sub>, compared to PC<sub>1</sub>. In fact, the frequency of scratching/grooming/scent marking was higher at PC<sub>6</sub> than MH<sub>4</sub>, although not significantly [ $t(8) = -2.786$ ,  $P = .024$ ,  $\alpha = 5/4$ ]. *Tsik-tsik* vocalization [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -5.100$ ,  $P < .001$ ,  $\alpha = 5/4$ ; Fig. 3B], swaying/tongue in-out [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -4.601$ ,  $P < .01$ ,  $\alpha = 5/4$ ; Fig. 4A], and withdrawal/stretch approach/flat body [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -2.715$ ,  $P < .01$ ,  $\alpha = 5/4$ ; Fig. 4B], which were not observed over the four maze habituation trials, occurred during the first predator confrontation. The initial high rate of these behaviors was influenced by subsequent exposures [*tsik-tsik* vocalization:  $F(5,40) = 12.606$ ,  $P < .05$ ; Fig. 3B; swaying/tongue in-out:  $F(5,40) = 10.948$ ,  $P < .001$ ; Fig. 4A; withdrawal/stretch approach/flat body:  $F(5,40) = 4.503$ ,  $P < .01$ ; Fig. 4B]. The subjects rapidly and significantly decreased the time spent emitting *tsik-tsik* calls [ $P < .001$ ] and the frequencies of swaying/tongue in-out [ $P < .001$ ] and withdrawal/stretch approach/flat body [ $P < .05$ ] compared to PC<sub>1</sub>, reaching low levels by the last trial.

The frequency of visual scanning (Fig. 3D) also increased significantly upon first exposure to the predator [PC<sub>1</sub> vs.

MH<sub>4</sub>:  $t(8) = -2.824$ ,  $P < .01$ ,  $\alpha = 5/4$ ]. Further confrontations altered this response pattern, almost attaining statistical significance [ $F(5,40) = 2.117$ ,  $P = .083$ ], such that during PC<sub>6</sub> its frequency was comparable to MH<sub>4</sub> [ $t(8) = -1.287$ ,  $P = .234$ ,  $\alpha = 5/4$ ]. Conversely, long call vocalization was not detected during any of the confrontations (Fig. 3A). Locomotor activity (Fig. 2A) was influenced neither by the initial presence of the predator [PC<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = 1.234$ ,  $P = .252$ ,  $\alpha = 5/4$ ], nor by further confrontations [ $F(5,40) = 2.089$ ,

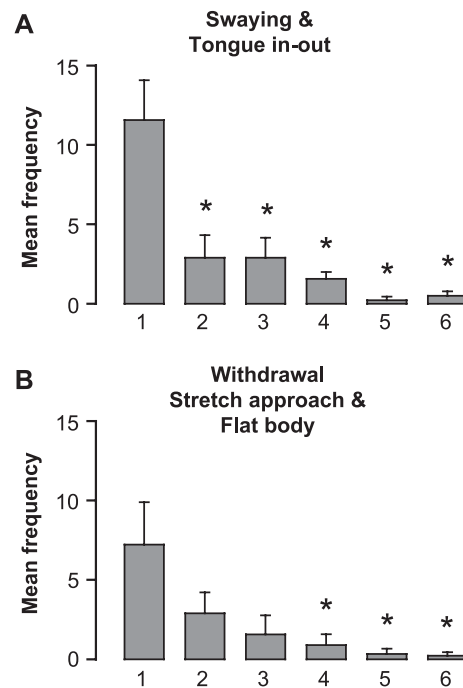


Fig. 4. Mean ( $\pm$  S.E.M.) frequency of swaying/rapid tongue in-out (A), and withdrawal/stretch approach/flat body (B) behaviors for each 30-min session during the six predator confrontations when the 'predator' stimulus was present. \* $P < .05$  vs. Trial 1.

$P=.087$ ]. In fact, similar locomotion rates were also observed between PC<sub>6</sub> and MH<sub>4</sub> [ $t(8) = -0.530$ ,  $P=.611$ ,  $\alpha = 5/4$ ], and this parameter was not significantly correlated with exploratory activity ( $r=.565$ ,  $P=.113$ ,  $\alpha = 5/3$ ).

### 3.3. Predator removal

Over the four trials in the absence of the predator (PR<sub>1</sub>–PR<sub>4</sub>), exploratory activity (Fig. 2B) increased during the first trial, although not significantly [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = -1.986$ ,  $P=.082$ ,  $\alpha = 5/4$ ]. In fact, exploration was observed at levels similar to those observed before confrontations [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -0.499$ ,  $P=.632$ ,  $\alpha = 5/4$ ]. Subsequent trials, on the other hand, significantly influenced this profile [ $F(3,24) = 3.495$ ,  $P < .05$ ], in that exploration during PR<sub>4</sub> was significantly lower ( $P < .05$ ) than during PR<sub>2</sub>. The time marmosets spent in the maze section closest to where the stimulus had been previously encountered increased significantly on the first trial with the predator removed [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = 3.789$ ,  $P < .01$ ,  $\alpha = 5/4$ ; Fig. 2C], thus returning to initial baseline levels [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -0.263$ ,  $P=.799$ ,  $\alpha = 5/4$ ]. Proximity duration did not change significantly during the following three trials [ $F(3,24) = 0.922$ ,  $P=.445$ ]. Similarly, the frequency in the maze section closest to where the predator had been encountered initially increased, although not significantly [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = -1.170$ ,  $P=.276$ ,  $\alpha = 5/4$ ; Fig. 2D]. This parameter remained constant over subsequent trials [ $F(3,24) = 0.757$ ,  $P=.529$ ], and at rates comparable to MH<sub>4</sub> [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -1.608$ ,  $P=.146$ ,  $\alpha = 5/4$ ]. Furthermore, the time spent in the three sections closest to where the stimulus had been presented increased, while in the opposite three sections it decreased (Table 1). This profile was similar to that observed during MH<sub>1</sub> and MH<sub>4</sub>, remaining stable over the next three trials. Immediate removal of the predator [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = 0.841$ ,  $P=.425$ ,  $\alpha = 5/4$ ] and subsequent trials [ $F(3,24) = 0.466$ ,  $P=.709$ ] did not significantly alter the frequency of scratching/grooming/scent marking (Fig. 3C), although it seemed at a higher frequency than during MH<sub>4</sub> [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -2.432$ ,  $P=.041$ ,  $\alpha = 5/4$ ]. Furthermore, *tsik-tsik* vocalization (Fig. 3B), swaying/tongue in–out (Fig. 4A), and withdrawal/stretch approach/flat body (Fig. 4B) were not observed in the absence of the predator stimulus, whereas long calls (Fig. 3A) were emitted at a low stable level during the trials with the predator removed [ $F(3,24) = 0.114$ ,  $P=.951$ ]. Visual scanning (Fig. 3D) increased upon removal of the stimulus, although not significantly [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = -2.080$ ,  $P=.071$ ,  $\alpha = 5/4$ ]. It occurred at a significantly higher frequency than during baseline [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -2.880$ ,  $P < .01$ ,  $\alpha = 5/4$ ]. Over subsequent trials this parameter decreased significantly [ $F(3,24) = 8.849$ ,  $P < .01$ ]. Post hoc analysis revealed that only PR<sub>4</sub> differed significantly [ $P < .05$ ] from PR<sub>1</sub>. Lastly, immediate removal of the predator [PR<sub>1</sub> vs. PC<sub>6</sub>:  $t(8) = 0.641$ ,  $P=.539$ ,  $\alpha = 5/4$ ], as well as the further three trials [ $F(3,24) = 0.761$ ,  $P=.527$ ] did not

significantly alter the level of locomotor activity (Fig. 2A), which remained similar to baseline [PR<sub>1</sub> vs. MH<sub>4</sub>:  $t(8) = -1.153$ ,  $P=.282$ ,  $\alpha = 5/4$ ]. Locomotion and exploration were also not significantly correlated during these trials ( $r = -.055$ ,  $P=.898$ ,  $\alpha = 5/3$ ).

## 4. Discussion

### 4.1. Habituation to novel environment

In marmosets, exposure to novel environments leads to fear, anxiety, and behavioral/physiological stress-related responses (Dettling et al., 2002; Smith et al., 1998). These reactions may be due, in part, to isolation from group members, unfamiliarity with the surroundings, and nonestablishment of escape routes and/or protective shelters. The initial behavioral response, characterized primarily by high levels of locomotion and distress/contact vocalizations (Dettling et al., 2002; Smith et al., 1998), has been shown to rapidly decrease to a stable baseline level following repeated exposures (e.g., Barros and Tomaz, 2002). Consistent with this profile, the marmosets employed in the present study habituated to the novel maze environment in the absence of the predator stimulus, as locomotor and exploratory activity decreased and stabilized. These behaviors are often perceived as a good measure of approach towards novelty in such stress-related conditions (Prut and Belzung, 2003). Importantly, locomotion and exploration seem to reflect independent variables, not being significantly correlated. The frequency of long calls, a common, social contact call in marmosets (Stevenson and Poole, 1976; Stevenson and Rylands, 1988), also decreased following repeated maze exposures. On the other hand, the high frequency of visual scanning remained stable over the repeated maze exposures. Constant high rates of this vigilance-associated response have been reported in marmosets (Barros et al., 2003b; Caine, 1998) and other callitrichids (e.g., Caine, 1984; Hardie and Buchanan-Smith, 1997), playing a vital role in their antipredation strategy. Finally, repeated exposure to the maze did not influence the time the marmosets spent in its different sections, having a similar between-trial profile. Of interest were the consistent within-trial differences, i.e., a preference for specific locations that was maintained over the course of the habituation trials. Taken together, the results indicate that the marmosets habituated to the novel maze environment over the four trials; the preexposure data provide a baseline for comparing possible behavioral changes induced by the predatory stress condition.

### 4.2. Predator confrontations

Initial exposure to the predator stimulus elicited diverse changes in the subjects' behavioral repertoire compared to the preceding maze habituation trials, whereas further con-

frontations altered this response pattern. Locomotor activity did not seem to confound these changes, as this parameter differed significantly from neither the last maze habituation trial, nor between the six confrontations.

The animals demonstrated a significant avoidance of the predator, in both duration and frequency. This pattern is commonly observed in marmosets confronted with a human observer (e.g., Carey et al., 1992) and in rodents exposed to a live predator (e.g., Blanchard et al., 2003b) or its cues (fur/feces odors) (e.g., Blanchard et al., 2001). In the MPCT, proximal avoidance is reversed by the administration of anxiolytic compounds (Barros et al., 2000, 2001). With repeated confrontations, the initial proximal avoidance response significantly decreased; that is, duration and frequency in the maze section closest to the predator increased. However, the specific pattern differed between these two measures. Whereas duration stabilized below initial baseline levels, frequency steadily returned to baseline, indicative of approach–avoidance conflict. Concomitantly, the time spent in the three maze sections opposite the stimulus increased during initial confrontation, summing 65% of the total time, opposed to the 2% spent in the closest three. From these locations, the marmosets could not easily visualize the predator stimulus. Although subsequent confrontations altered this profile, it did not return to baseline values. Lasting proximal avoidance, together with the high percentage of time spent in the extreme opposite side of the maze, suggests that the other behavioral reactions measured occurred essentially as far away from the stimulus as possible, as previously described in rodents exposed to predator odors (Blanchard et al., 2001).

In addition, a significant decrease in exploratory activity (smell/lick the apparatus, leg stand) was observed upon first exposure to the predator. This decline has been found in the MPCT to be reversed by the administration of anxiolytics (Barros et al., 2000, 2001). Further confrontations significantly increased exploration frequency, although it stabilized by the third trial. Commonly measured in some rodent tests (e.g., Prut and Belzung, 2003), exploration has only recently been increasingly addressed in nonhuman primates as a behavioral indicator of anxiety. As it was not significantly correlated to locomotor activity, exploration and locomotion during confrontations with a taxidermized predator may constitute independent, although possibly complementary, variables of the marmosets' repertoire.

A significant decrease in scratching/grooming/scent marking was also observed during initial confrontation. Following subsequent trials, its frequency significantly and steadily increased above original baseline rates. These behaviors are frequently classified as “displacement activity,” involving mainly routine body care activities often exhibited out of context or may be exacerbated within a given normal situation (Bradshaw, 1993). In primates, in general, displacement activity characteristically occurs in situations of frustration, tension, uncertainty, and/or conflict (Bradshaw, 1993; Diezinger and Anderson, 1986; Maestri-

pieri et al., 1992), and thus is commonly employed as a behavioral indicator of anxiety. As the levels of scratching/grooming/scent marking increased, while other defense/anxiety indicators decreased following repeated confrontation, this behavioral category may be more congruent with the “behavioral inhibition” system induced by aversive stimuli (Gray, 1996). Studies employing the MPCT, however, have detected an increase in response to anxiogenic stimuli and a decrease following the administration of diazepam and buspirone (Barros et al., 2000, 2001). Accordingly, further studies would be necessary to better clarify this issue.

In contrast, long call vocalization seems to have been inhibited by the initial and repeated exposure to the stimulus. Other behavioral responses, which did not appear during exposure to the maze environment alone, were elicited during the first confrontation, namely, *tsik-tsik* vocalization, swaying/tongue in–out, and withdrawal/stretch approach/flat body. However, these behaviors rapidly declined over subsequent trials, inasmuch as fast within- (data not shown) and between-trial decreases were observed. In addition, treatment with different compounds (i.e., diazepam, buspirone, WAY 100635, substance P) did not modulate these responses (unpublished data). Swaying/tongue in–out is commonly observed during dangerous or alarming situations (Stevenson and Rylands, 1988), while *tsik-tsik* vocalization is described as an alarm/mobbing-associated call in callitrichids (Stevenson and Rylands, 1988; Stevenson and Poole, 1976). Withdrawal/stretch approach/flat body, albeit normally associated with “risk assessment” behaviors in rodents, has not been taken note of in primates. In rodents, these behaviors are related to assessing the potentiality of a threat, including danger orientation, investigative approach, and sensory (olfactory, auditory, visual) scanning (e.g., Blanchard et al., 2003b).

A significant transient increase in visual scanning occurred during initial predator confrontation, which gradually decreased with repeated exposures. In callitrichids, this behavior facilitates the detection of objects in the environment (Caine, 1984; Hardie and Buchanan-Smith, 1997), increases only after the presentation of a potential threat (Caine, 1984, 1998; Ferrari and Lopes Ferrari, 1990; Hardie and Buchanan-Smith, 1997; Koenig, 1998), and enables the early detection and avoidance of predators, thus being viewed as an effective and reliable measure of vigilance (Caine, 1984; Koenig, 1998). In fact, vigilance behaviors have recently been suggested as a possible correspondent in animals such as birds and primates, of the risk assessment behavior described in rodents (Blanchard et al., 2003b). Although the significant increase in withdrawal/stretch approach/flat body and visual scanning in response to the presence of the predator concurs with this suggestion, further studies would be necessary before safely classifying them as risk assessment behaviors in primates. The withdrawal/stretch approach/flat body profile in the MPCT may be more related to fear-related behaviors in marmosets (i.e.,



*tsik-tsik* call/swaying/tongue in–out) than to classical anxiety-associated responses (i.e., displacement activities).

Taken together, the results indicate that acute predatory stress, employing a taxidermized natural predator, elicits mainly innate fear-related responses (i.e., *tsik-tsik* vocalization, swaying/tongue in–out, proximic avoidance) that decrease, to varying degrees, following recurring confrontations; that is, some behaviors rapidly and fully habituated (e.g., *tsik-tsik* vocalizations, swaying/tongue in–out, withdrawal/stretch approach/flat body), others gradually decreased/increased (e.g., visual scanning, exploration), while a few failed to return to baseline (e.g., proximic avoidance duration). The latter indicates that even after six 30-min confrontations with the taxidermized predator, this stimulus still retained threat elements. Others have reported a general lack of habituation to live/cued predators (for a review, see Dielenberg and McGregor, 2001), a response-specific habituation effect (Blanchard and Blanchard, 1989; Blanchard et al., 2001), and a persistent avoidance behavior of the predator stimuli (Blanchard et al., 2001, Mineka, 1978). In callitrichids, reduction of antipredation costs is achieved by rapidly resuming previous activities once the potentiality of a threat has been accurately established (Caine, 1998). In fact, learning to differentiate threatening from harmless objects/situations is essential not only for survival, but also to maximize energy allocation towards defensive versus nondefensive behaviors (Kavaliers and Choleris, 2001). This seems to be the case even among captive- and captive-born callitrichids (Barros et al., 2002a; Buchanan-Smith, 1999, Caine, 1998; Koenig, 1998), further indicating the persistence and importance of antipredation behaviors to these primates.

A striking result was the rapid decrease in fear-related behaviors concomitant to the gradual increase in anxiety-associated responses (e.g., scratching/grooming/scent marking), as a result of repeated predator confrontations. A cost-effective defense strategy is especially important to these small simians, as they suffer the highest rates of predation among primates (Cheney and Wrangham, 1987). For example, rapid evaluation and habituation to a potential threat has been shown to minimize highly costly and disruptive fear-related defensive behaviors (Caine, 1998). As the potentiality of the threat decreases with exposure, anxiety-associated responses recede and appear intermittently with other nondefensive behaviors (Blanchard et al., 2003b). In addition, the increase in anxiety-related behaviors over exposure trials suggests that the predator stimulus in the MPCT represents more a potential and/or ambiguous threat, rather than a “real” threat. This may be particularly true following a second or more exposures, since habituation or extinction to predators/predatory cues may decrease the intensity and certainty of a threat source, while increasing ambiguity (e.g., Blanchard et al., 2003b). Additionally, in spite of being clearly visible and in proximity to the maze/subject, ambiguity of the threat may also be achieved in the MPCT situation by the presence of glass panels that physically

separate the predator from the marmoset, and by the lack of typical odors, vocalization, and animated movements associated with the stimulus.

#### 4.3. Contextual conditioning and extinction

Conditioning of the fear/anxiety responses to the context in which the confrontation took place (i.e., maze) was evaluated over four trials upon removal of the stimulus. In general, the marmosets' immediate responses to the absence of the predator and subsequent “predator-removal” trials were dependent on prior degree of habituation observed during the preceding confrontation trials; that is, rapidly habituated behaviors were not observed (i.e., *tsik-tsik* vocalization, swaying/tongue in–out, withdrawal/stretch approach/flat body), responses that returned to baseline values remained unaltered (i.e., exploratory activity, frequency of proximity), and nonfully habituated behaviors immediately returned to baseline (i.e., proximity duration; time spent in maze sections). Therefore, these results provide meager evidence for contextual conditioning of fear/anxiety behavior in marmosets. Interestingly, visual scanning increased significantly when the predator was removed, only to rapidly return to baseline over further trials. This behavior may possibly be employed as an information-gathering strategy upon immediate changes in their environment. Furthermore, long calls, which had not been observed during the previous phase, were emitted at a constant baseline level.

Trial duration (30 min) and repeated confrontations led to habituation of various responses to the predator stimulus, which, in turn, very likely influenced the profile observed during the contextual conditioning test trials. Studies adopting a no-predator control group or single short-term exposure followed by contextual conditioning might provide a different profile. Both feral and captive marmosets avoid locations where predators were previously spotted (e.g., Hankerson et al., 2001) or where attempted/successful predation episodes have recently taken place (e.g., Tello et al., 2002). In rodents, contextual conditioning to predator stimuli has been demonstrated, even if dependent on specific environmental and stimulus properties (for discussion, see Blanchard et al., 2003a). In these animals, habituation to the context alone (maze) and during successive predator confrontations has been shown to decrease the incidence of contextual conditioning (Dielenberg et al., 2001; Blanchard et al., 2001, Radulovic et al., 1998).

#### 4.4. Concluding remarks

An easily discernible diverse behavioral repertoire can be induced in the MPCT, comprising bodily reactions and specific threat vocalizations. Whereas acute confrontation with the predator induced fear-related behaviors, repeated predator exposures led to full/partial habituation of these reactions concomitant to an increase in anxiety-associated

responses. Reversal of the proximal avoidance was only observed upon immediate removal of the stimulus. Thus, the MPCT reveals a complex defense strategy, which can be decomposed by differential behavioral analysis. Such a component analysis holds promise for a more differentiated pharmacological investigation of the behavioral manifestations of fear/anxiety, including the search for novel anxiolytic drugs.

## Acknowledgements

This work was supported by CAPES/DAAD/PROBAL (137/02 to CT and JPH) and the Deutsche Forschungsgemeinschaft (HU 306/23-1 to JPH and MADSS). MB was a recipient of a postdoctoral fellowship from CAPES, and CT a recipient of a CNPq researcher fellowship (no. 300364/1986-5). Thanks to Dr. R. de Oliveira for animal care and maintenance, and R.S. Maior, E.L. Mello, Jr., C. Alencar, and G. Camargo for assistance in running the tests.

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