



## REVIEW ARTICLE

# Critical neuropsychobiological analysis of panic attack- and anticipatory anxiety-like behaviors in rodents confronted with snakes in polygonal arenas and complex labyrinths: a comparison to the elevated plus- and T-maze behavioral tests

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**Objective:** To compare prey and snake paradigms performed in complex environments to the elevated plus-maze (EPM) and T-maze (ETM) tests for the study of panic attack- and anticipatory anxiety-like behaviors in rodents.

**Methods:** PubMed was reviewed in search of articles focusing on the plus maze test, EPM, and ETM, as well as on defensive behaviors displayed by threatened rodents. In addition, the authors' research with polygonal arenas and complex labyrinth (designed by the first author for confrontation between snakes and small rodents) was examined.

**Results:** The EPM and ETM tests evoke anxiety/fear-related defensive responses that are pharmacologically validated, whereas the confrontation between rodents and snakes in polygonal arenas with or without shelters or in the complex labyrinth offers ethological conditions for studying more complex defensive behaviors and the effects of anxiolytic and panicolytic drugs. Prey vs. predator paradigms also allow discrimination between non-oriented and oriented escape behavior.

**Conclusions:** Both EPM and ETM simple labyrinths are excellent apparatuses for the study of anxiety- and instinctive fear-related responses, respectively. The confrontation between rodents and snakes in polygonal arenas, however, offers a more ethological environment for addressing both unconditioned and conditioned fear-induced behaviors and the effects of anxiolytic and panicolytic drugs.

**Keywords:** Innate fear; panic attacks; prey versus snakes paradigms; polygonal arenas for snakes; elevated plus-maze test; elevated T-maze test

## Introduction

Several studies have focused on the morphological and physiological bases of unconditioned and conditioned fear-related responses resulting from dysfunction of brain systems, anxiety, and panic disorder. There are robust data on the use of simple apparatuses, such as the elevated plus maze (EPM) and T-maze (ETM) tests, for the study of anxiety and other innate fear-related diseases. These devices have been submitted to extensive ethological and pharmacological validation and are considered useful for the study of anxiolytic and panicolytic drugs.<sup>1-10</sup> For example,

the EPM and ETM tests have been used to compare the effects of anxiolytic/panicolytic drugs delivered via systemic injection or microinjection into specific encephalic structures,<sup>11-16</sup> thereby facilitating the study of the neuroanatomical basis of fear- and anxiety-induced reactions<sup>17,18</sup> and the establishment of correlations between behavioral responses and different stress-related models.<sup>19-21</sup>

More recently, prey-versus-predator paradigms have also been used to investigate innate and conditioned fear-related reactions and the effects of drugs that act in the neural substrates of aversive stimulus-induced emotional responses.<sup>22,23</sup> As an example, in our laboratory, different species of rodents were confronted with venomous or constrictor snakes in a complex labyrinth or in polygonal arenas.<sup>24,25</sup> These prey-versus-predator paradigms also seem to be a good experimental tool for studying innate fear-induced behavioral responses elicited in a more ethological situation of threat,<sup>22,23</sup> as well as the effects of new potential panicolytic drugs.<sup>26-28</sup> In this

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sense, the aim of the present article was to discuss the literature regarding the classical EPM and ETM tests, the current knowledge about snake-based prey-versus-predator interaction approaches to anxiety, fear, and panic-related behaviors, and our 10-year experience with snake versus predator interactions.

## Methods

We reviewed PubMed in search of articles focusing on the plus maze test, EPM, and ETM, as well as on defensive behaviors displayed by rodents. We also included in this review selected chapters from classical books about anxiety and defensive behavior in rodents and humans and about anxiety treatment or neuropsychopharmacology of mental diseases.<sup>7,29-33</sup>

For the critical review of the experience of our team, we examined a set of six papers,<sup>22-27</sup> two doctoral theses,<sup>34,35</sup> nine congress abstracts,<sup>28,34,36-43</sup> and one book chapter<sup>44</sup> based on prey-versus-predator paradigms using wild snakes. All these experiments were performed in the Ophidiarium at the Laboratório de Neuroanatomia e Neuropsicobiologia – Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo (LNN-FMRP-USP)/ Instituto de Neurociências e Comportamento (INeC) using polygonal arenas and a complex labyrinth for confrontation between venomous and non-venomous snakes and small rodents, designed by Prof. N.C.Coimbra (first author) and ethologically validated from 2007 to 2013.<sup>24-27</sup> These apparatuses, which are licensed by the Brazilian government (IBAMA Committee; processes 3543.6986/2012-SP and 3543.6984/2012-SP) and by the São Paulo state government (SMA/DeFau 15.335/2012; MEDUSA Project, SISBIO processes 41435-1, 41435-2 and 41435-3), have been used since then in undergraduate medical courses, graduate programs, and in original scientific studies. New data produced during a laboratory demonstration of the model to undergraduate and graduate medical students are also discussed.

## Results

### *Elevated classical labyrinths to record anxiety and fear-related behaviors – EPM and ETM tests*

Elevated labyrinths such as the EPM and the ETM are simple experimental apparatuses with closed and/or open

arms assembled 50 cm above the laboratory floor. They are used to study the natural aversion of rodents to high and open areas.

The EPM test is a prototypic anxiety model, and one of the most used ethological tools for measuring and manipulating anxiety in rodents.<sup>7,45</sup> Despite the controversies surrounding the EPM, including the intensity of the anxiogenic stimuli to which the tested animal is submitted,<sup>46</sup> it is a useful tool for verifying the behavioral effects of anxiolytic and anxiogenic drugs.<sup>1,20</sup>

Different species of rodents that vary in size and height can be tested in the EPM, a structure elevated 50 cm from the floor, made of wood or plexiglass and consisting of two open arms placed perpendicularly to two closed high-walled arms of the same size. Some authors claim that replacing the classic opaque walls in the two closed high-walled arms with transparent walls increases the sensitivity of the behavioral effects of anxiolytic drugs.<sup>47-49</sup> In the clear model, each animal is placed in the center of the EPM, generally facing one of the open arms, and is recorded for 5 or 10 min of free exploration. The primary indexes of anxiety-like behavior in the EPM comprise spatiotemporal measures, e.g., the number of entries in the closed arms and the percentage of entries and time spent in the open arms, two significant behavioral parameters related to locomotion and anxiety/fear behaviors in rodents.<sup>1,3,7,50,51</sup> However, as in any other animal model of anxiety, the precision of the EPM depends on many factors, since anti-anxiety effects may have baseline levels of anxiety that are too low or too high to be detected.<sup>7</sup> To avoid these limitations, researchers<sup>1,3,46</sup> have highlighted the necessity of improving the sensitivity, reliability, and ecological validity of the test by focusing on what the animal actually does in the maze, e.g., aspects of defensive behaviors and the location within the maze where these behaviors occur.<sup>1,3,43,52,53</sup> These complementary parameters are sometimes called “risk assessment behaviors” and visually show the type of behavioral strategy the animal actually exhibits in a dangerous situation (Table 1). The biological function of these behaviors is to gather information regarding the potential threat from the environment.<sup>30,54</sup>

However, as there are different parameters of risk assessment behaviors, it is not necessarily clear what each behavior tells us about the elicited anxiety-like behavior in the EPM test. Cruz et al.<sup>3</sup> and Rodgers & Johnson<sup>8</sup> elegantly answer this question by classifying

**Table 1** Description of the complementary behaviors exhibited by rodents in the EPM<sup>55</sup>

Behavioral parameters	Description
Scanning	Looking over the edge of one of the open arms with scanning movement in any direction.
Head dipping	Downward visual screening movement at the edge of the open arm.
End-arm exploration	The animal reaches the end of the open arm and dips its head.
Stretch-attend posture (SAP)	Forward elongation of the body and retreat to the original position when the animal is standing still or moving slowly forward.
Flat-back approach	Forward elongation of the body with frontward movement by slowly pulling the hind body.
Rearing	Bipedal posture supported by the hind paws.
Peeping out	Projection of the head and shoulders from the closed arms toward the central part of the EPM. The four paws are maintained inside the closed arm.
Grooming	Cleaning or scratching of the fur, nose, ears and whiskers using the paws or tongue.
Immobility	Complete stillness of the animal (resembles freezing behavior).

the parameters into factors representing different emotional dimensions. Cruz et al.<sup>3</sup> classified these complementary parameters into four distinct factors: anxiety, motor activity, decision-making, and displacement behavior. The behavioral measures of scanning, head dipping, end of arm exploration, and the classic ethological factors (number of entries into the open and closed arms and time spent inside these arms) are strongly correlated with anxiety; rearing, number of entries into the closed arms, and total number of entries into the arms are correlated with motor activity; time spent in the central square of the maze is correlated with decision-making behavior; and grooming is related to displacement behavior. To this behavioral analysis, Rodgers & Johnson<sup>8</sup> have added three factors: a) stretch-attend posture (SAP), a primary index of learning about a potentially dangerous environment, together with sniffing or investigation; b) exploration, which consists of head-dipping at the edge of the open arm and is considered a primary index of exploration, with high sensitivity for testing anti-anxiety and pro-anxiety drugs<sup>56</sup>; and c) vertical activity, which consists of rearing and grooming (two behaviors that are negatively correlated with each other). It is important to highlight that not only has “factor analysis” been used for identifying the relationship between specific test indexes and factors/dimensions, such as anxiety and locomotor activity, it is also employed to assess whether different animal models can be used to measure the same type of anxiety.<sup>8</sup>

In 1991, Deakin & Graeff<sup>57</sup> proposed a model to separate conditioned fear, which is related to generalized anxiety disorders (GAD), from unconditioned fear, which is related to panic disorders (PD). In that new model, the T-maze test, was adapted from the EPM test by blocking the entrance to one of the closed arms.<sup>58,59</sup> In the ETM, the rat may perform one of two tasks: inhibitory avoidance (conditioned fear) and one-way escape (unconditioned fear).

In the first task, the rat is placed at the end of the enclosed arm so that it can only see the open arms if it positions its head beyond the end of the closed arm. Because the open arm seems to represent an aversive experience for rodents, it evokes inhibitory avoidance, i.e. - if this task is repeated, the latency to leave the closed arm will increase over the trials. Three consecutive trials, with 30-s inter- and intra-trial intervals, are recorded when the animal exits the arm with all four paws during each task.

In the second task, the rat is placed at the end of the open arms so it can move towards the closed arm, probably performing an escape response. It is important to highlight that while the latencies to leave the enclosed arm increase over the trials, reflecting habituation to the maze environment, exploratory activity, and learning, the latencies to leave the open arms (escape task) do not change due to a persistent aversive motivation.<sup>4,10,59</sup> This situation is not affected by the administration of diazepam, a classic anxiolytic drug.<sup>58</sup>

The ETM test elicits the activation of different brain areas related to memory formation (hippocampus)<sup>60</sup> and to fear/anxiety responses, as revealed by Fos protein immunolabeling in different brain areas.<sup>60,61</sup> These findings

corroborate behavioral results, and encephalic activation was found to differ during escape and avoidance tasks. Neural activation in the basolateral amygdaloid nucleus and in the dorsal periaqueductal gray matter was observed during escape, whereas enhanced activity in the amygdaloid nucleus, anterior hypothalamic nucleus and the median raphe nucleus was observed in the avoidance task. Both tasks, however, activated common structures, such as the paraventricular nucleus of the thalamus and the dorsomedial hypothalamic nucleus.<sup>61</sup> These structures are related to the elaboration of anxiety and innate fear.<sup>62-67</sup>

Whilst the ETM seems to be a reliable tool for studying the GAD and PD-like behaviors in rats, experiments with mice showed different results. Jardim et al.<sup>68</sup> demonstrated that the time spent to escape from the open arm (escape behavior) was similar to the latency to leave the closed arm (avoidance behavior) in the first trial. Furthermore, the latencies for the mouse leaving the closed arm were high and did not differ significantly between the three consecutive trials. Jardim et al.<sup>68</sup> have asserted that “it does not seem to be possible to separate conditioned and unconditioned fear in the elevated T-maze to mice.”

Nevertheless, Carvalho-Netto & Nunes-de-Souza<sup>2</sup> showed that mice did acquire inhibitory avoidance in the ETM. When the number of exposures to the avoidance trials was increased from three to five, the latency to leave the closed arm increased statistically from baseline. However, even with five trials, the latency to leave the open arm in the escape trial did not change, as verified in rats.<sup>10</sup> Furthermore, they showed that mice in an ETM with transparent walls have a lower avoidance baseline latency and a lower escape latency than those submitted to an ETM with opaque walls, suggesting that the apparatus with transparent walls is more useful for studying avoidance and escape behaviors in mice.

Graeff et al.<sup>4</sup> showed that in rats, anxiolytic and anxiogenic compounds increased and decreased, respectively, the latency for the avoidance task in the ETM. Neuropeptides, psychostimulants, phenylethylamine hallucinogens, and a monoamine oxidase inhibitor A were ineffective, though the avoidance task was impaired by diazepam, buspirone, and ipsapirone, which are three well-known compounds that ameliorate GAD.<sup>31</sup> Furthermore, Carvalho-Netto & Nunes-de-Souza<sup>2</sup> performed the same experiments with similar compounds in mice (five trials). A summary of drugs with pharmacological actions in rodents submitted to the ETM test is shown in Table 2.

#### *Confrontation between rodents and serpents as a model of generalized anxiety and panic attacks*

This may be the first laboratory approach based on snake and rodent interaction in enriched experimental environments, addressing the effect of limbic system activation in a threatening situation that elicits both the predatory and antipredatory ethological repertoires during pharmacological testing of new drugs with potential antiaversive effects.<sup>26,27</sup>

Wild snakes have been used as aversive stimuli to either small non-human primates,<sup>69-74</sup> apes,<sup>75-78</sup> and rodents.<sup>79,80</sup>

**Table 2** Summary of the elevated T-maze test pharmacological validation

Compound	Drug action	Passive avoidance		Escape	
		Rat	Mouse	Rat	Mouse
8-OH-DPAT	5-HT <sub>1A</sub> agonist	?	+	?	0
Buspirone	5-HT <sub>1A</sub> partial agonist	+	+	0	-
Caffeine	Anxiogenic compound (psychomotor stimulant)	0	0	0	+
Clomipramine	5-HT <sub>2A</sub> antagonist; 5-HT uptake blocker	-		0	
D,L-amphetamine	Psychomotor stimulant	0	0	0	0
d-Fenfluramine	5-HT releaser	-	+	+	-
Diazepam	Benzodiazepine agonist	+	+	0	0
DOI	5-HT <sub>2A/2C</sub> agonist	0		0	
FG 7142	Benzodiazepine inverse agonist	-	0	+	0
Flumazenil	5-HT <sub>1A</sub> full agonist		+		0
Haloperidol	Neuroleptic	0	0	0	0
Imipramine	5-HT/NA reuptake blocker	-	0	+	0
Ipsapirone	5-HT <sub>1A</sub> partial agonist	+		0	
mCPP	5-HT <sub>2B/2C</sub> agonist	-	-	+	0
Moclobemide	Monoamine oxidase A inhibitor	0	0	0	0
Ritanserin	5-HT <sub>2A/2C</sub> antagonist	+		0	
SB 200646A	5-HT <sub>2B/2C</sub> antagonist	+	0	0	0
SER 082	5-HT <sub>2B/2C</sub> antagonist	+	0	0	0
SR 46349B	5-HT <sub>2A</sub> antagonist	+		0	
TFPP	5-HT <sub>2B/2C</sub> agonist	-		-	
Yohimbine	Alpha <sub>2</sub> -noradrenergic receptor antagonist	-	0	0	0

5-HT = serotonin.

Recent reports using the apparatuses designed by the first author to contain complex labyrinths and polygonal arenas<sup>24,64</sup> have shown that the rodent versus snake (both venomous and non-venomous) paradigms are useful for conducting ethological and neuropharmacological experiments.<sup>22-27,34,36-39</sup>

#### Simple and complex polygonal arenas for snakes

The polygonal arena consists of a semi-transparent acrylic enclosure (154 × 72 × 64 cm). The inner walls are covered with a reflective film that provides 80% light reflection, and consequently minimal visual contact by the prey and predator with the surrounding experimental area. A green fluorescent line (4.2 mm width; Pritt mark-it) is used to divide the arena into 20 equal rectangles to facilitate analysis of locomotion. The acrylic base of the arena is placed over a rectangular stainless steel platform, and the whole apparatus is placed on a granite surface (2 × 85 × 170 cm) positioned 83 cm above the floor to minimize vibratory stimuli. It is important to highlight that the rodents are habituated to the arena without the snake for seven days before the test.

The confrontation between a rodent and a snake has been proposed as a model of panic attack<sup>26</sup> that has been ethologically, neuropharmacologically, and morphologically validated.<sup>34,38,40-42,81</sup> These experiments were based on confrontations between golden hamsters (*Mesocricetus auratus*) and rattlesnakes (*Crotalus durissus terrificus*), coral snakes (*Micrurus frontalis*), or false coral snakes (*Oxyrhopus guibeii*) in a threatening environment. This approach provides a large experimental environment in which the rodents can exhibit “non-escapable” panic-related reactions in the presence of threatening stimuli.

A burrow (a shelter box with black acrylic walls) can also be positioned inside the arena, allowing rodents to exhibit flight responses to reach the protected area of the

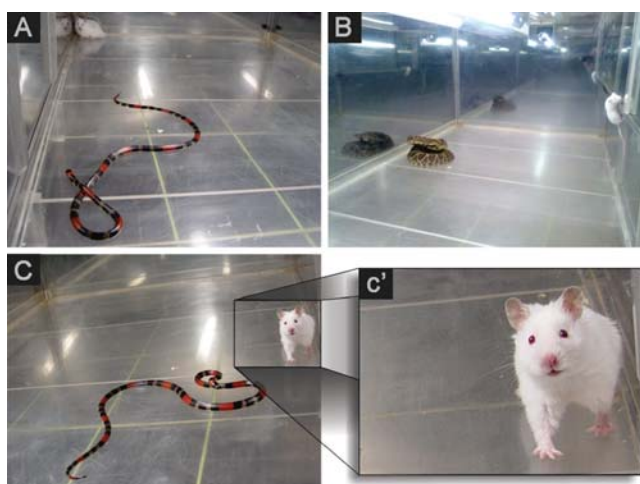
burrow. In our laboratory, two types of burrows have been used: the complex burrow (36 × 26 × 12.5 cm, containing a small, interior, L-shaped labyrinth with two entry/exit apertures) and the simple burrow (26.5 × 17.5 × 12.5 cm, with only one entry/exit aperture). The study of flight/escape behavioral strategy in the presence of burrows has advantageous and disadvantageous points. For example, it can prevent snake attacks, increasing the rodent's chance of survival and permitting the experimenter to study the effects of proximal and distal threatening stimuli on the activation of the rodent's limbic system. Moreover, the rodent can exhibit non-oriented escape behaviors organized by midbrain neurons<sup>22,23,62,64</sup> and oriented escape behaviors in an environment with a shelter organized by diencephalic neurons.<sup>62-64</sup> Nevertheless, the absence of the burrow allows the experimenter to record more uniform and robust panic-like defensive responses, considering that the snake is constantly kept in the rodent's visual field.<sup>26</sup> It is important to highlight that when the burrow is present in the arena, the behaviors may be proportionally recorded in relation to the time spent by each animal inside and outside the burrow through a behavioral index (BI = [100 × number of behavioral responses]/[time in seconds spent outside or inside the burrow]).<sup>26,27</sup> Moreover, the duration of each behavioral response may be expressed as the percentage of a given behavioral response duration displayed outside or inside the burrow, considering as 100% the total time of the behavioral test.<sup>26</sup> In an attempt to compare the defensive behaviors of mice exposed to the polygonal arena without (Figure 1) or with a small burrow present in one of its angular extremities (Figure 2), it was observed that mice confronted with venomous snakes, such as the South American coral snake *Micrurus lemniscatus carvalhoi* (Figure 1A and C), the *Crotalus durissus terrificus* (Figure 1B, Figure 2A, B, and C) or the *Bothrops alternatus* (Figure 2D and E),

displayed expressive anxiety-related defensive responses, such as alertness (Figure 1A, Figure 2A), risk assessment (Figure 2C), and inhibitory avoidance (Figure 2E), panic-related defensive responses, such as freezing (Figure 1C, Figure 2B), and both oriented (Figure 1B) and non-oriented (Figure 2D) escape behavior. Interestingly, even during imminent risk of death, some mice closely interacted with the predator (Figure 2C). In this experiment, the survival rate of mice confronted by snakes was 83.30%, as shown in Table 3. This high rate of survival may have resulted from the presence of the burrow inside the polygonal arena, or may have even been related to the huge size of the simple polygonal arena. However, depending on the genus of the snakes used, the survival rate can be as high as 100% even when the polygonal arena has no burrow (Table 3). This survival rate has been observed in confrontations between golden hamsters and South American coral snakes (the highly venomous *Micrurus frontalis* and the non-venomous *Oxyrhopus guibei*).<sup>34</sup>

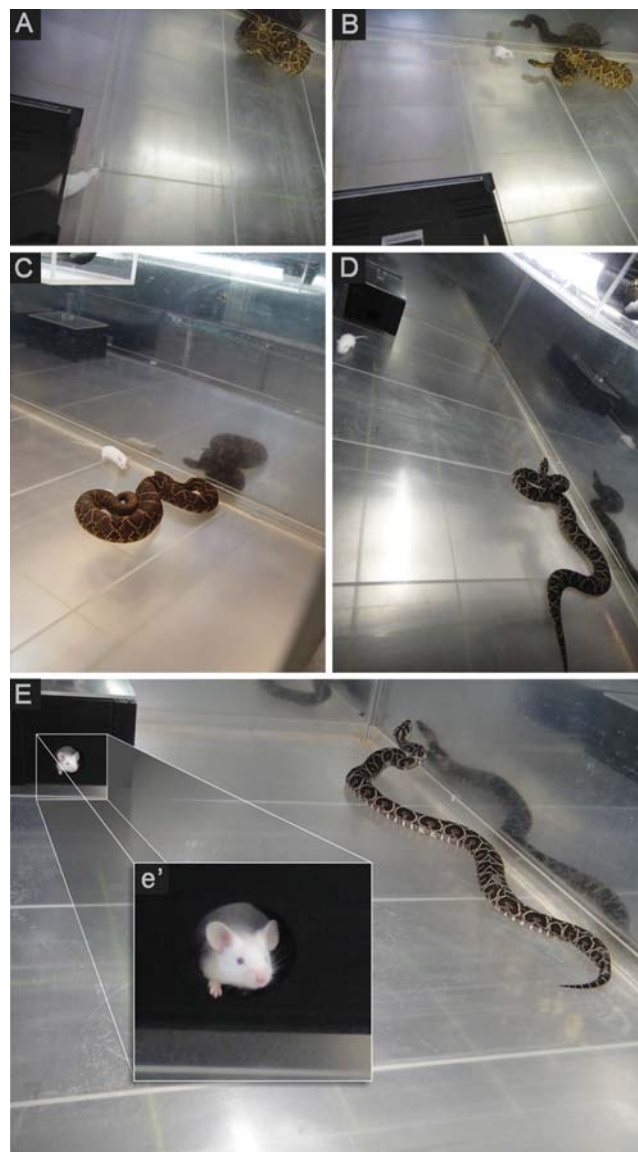
Polygonal arenas with burrows can also potentially be used as a model of post-traumatic stress disorder (PTSD). Experiments focusing on exposure to a live snake and re-exposure to the contextual arena are being conducted with mice and rainbow Boidae constrictor snakes in the LNN-FMRP-USP/INeC Ophiarium to address this possibility in an investigation of comorbidity between PTSD and chronic pain.<sup>28</sup>

#### Complex arenas and labyrinth for snakes

Complex polygonal arenas containing natural rocks and artificial shelters for rodents and natural branches for



**Figure 1** Innate fear-induced defensive responses evoked by *Mesocricetus auratus* confronted with the South American coral snake *Micrurus lemniscatus carvalhoi* (A and C), and the South American rattlesnake *Crotalus durissus terrificus* (B) for 5 min in a polygonal arena without a burrow. Anxiety/fear-related response: alertness (A) elicited in the presence of the coral snake. Panic attack-like responses: oriented escape (B) and freezing (C) elicited in the presence of each venomous snake.



**Figure 2** Instinctive fear-induced defensive responses evoked by *Mus musculus* confronted with the South American Viperidae snakes *Crotalus durissus terrificus* (A, B, C) and *Bothrops alternatus* (D and E) for 5 min in a polygonal arena with a burrow. Anxiety/fear-related response: alertness (A) elicited in the presence of the rattlesnake, and inhibitory avoidance (E, e') displayed in the presence of *Bothrops alternatus*. Panic attack-like responses: freezing (B) displayed by prey threatened by a rattlesnake, and non-oriented escape (D) displayed by prey threatened by *Bothrops alternatus* venomous snake.

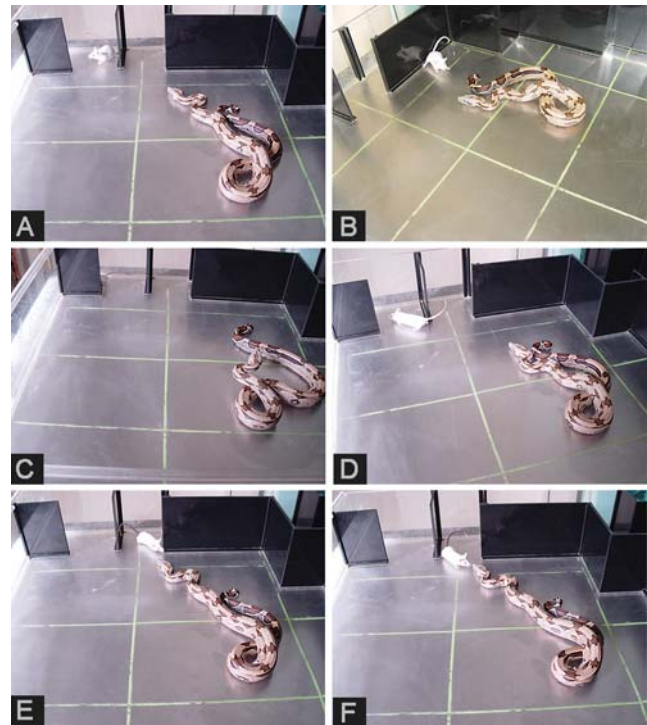
constrictor snakes can be considered as a more ethologically acceptable alternative to study the defensive repertoires of different species of rodents confronted with venomous and Boidae snakes. This approach can be useful to investigate feeding preferences considering the different sizes of laboratory animals. Despite the high frequency of interaction between gerbils and Boidae snakes, no feeding preference was observed when the constrictor snakes *Boa constrictor amarali* and

*Epicrates cenchria crassus* were confronted with *Mus musculus*, *Meriones unguiculatus*, *Cavia porcellus*, or *Rattus norvegicus*.<sup>43</sup>

The complex labyrinth for the confrontation between snakes and rodents, which was designed by the first author in 2000, was ethologically validated by Guimarães-Costa et al.<sup>24</sup> This apparatus consists of a transparent acrylic enclosure containing a small polygonal arena contiguous with a complex maze. The gallery walls are made of black acrylic. The arena measures 38.5 m<sup>2</sup>, and the remaining labyrinth measures 15 cm in height and 6.92 m in length. The whole apparatus containing the complex labyrinth and arena measures 140 × 70 × 15 cm. The top of the labyrinth and the arena contain 84 circular holes (1.5 cm in diameter). The floor of the complex labyrinth is made of a clear crystal acrylic plaque (140 × 70 cm) that is placed on another metallic plaque made of 1-mm-wide stainless steel with the same dimensions. The arena is divided by 0.4 cm green fluorescent lines into 20 equal rectangles (27.7 × 17.2 cm each). It is important to highlight that even wild constrictor snakes as heavy as 2,500 g can invade the galleries during hunting behavior, which increases the panic attack-like behaviors of prey animals.<sup>24</sup> The confrontation between rodents and snakes can occur in both divisions of the complex labyrinth, i.e., within the arena or inside the galleries.

According to Guimarães-Costa et al.,<sup>24</sup> Mongolian gerbils display the best exploratory response in this apparatus when compared to Wistar rats and golden hamsters. Gerbils explore the whole arena and the galleries of the complex labyrinth for 5 min, displaying anxiety-related behaviors (such as alertness, flat back approach, and stretch attend posture) and panic-attack-related responses. Similar behavioral reactions can be evoked by mice confronted with constrictor snakes inside the complex labyrinth, as shown in Figures 3 and 4. In this case, the survival rate was 100% (Table 3). The advantages of the complex labyrinth over other types of labyrinths include the possibility of studying aversive memory-related responses and innate fear-related behavior as well as the increased chances of survival of threatened rodents. The disadvantage of this apparatus is the difficulty of interaction between prey and predator, considering the species of rodents and snakes used in each experiment. Some species of rodents, including Mongolian gerbils and mice, can explore the entire apparatus in five minutes, whereas rats and guinea pigs commonly show a delay in finding all gallery exits. To minimize this delay and reduce the species-specific differences in time spent exploring the maze, all rodents are habituated to the complex labyrinth for at least 3 days before the experiments.

Similar patterns of instinctive fear-induced defensive behavior were displayed by mice confronted with three different species of non-venomous rainbow Boidae constrictor snakes: *Boa constrictor constrictor* (Figure 3), *Epicrates cenchria assisi* (Figure 4A, B, C, and D), and *Epicrates cenchria cenchria* (Figure 4E and F). In fact, mice displayed anxiety-related behaviors, such as alertness (Figure 3A, Figure 4B and E), inhibitory avoidance (Figure 3C, Figure 4B), flat back approach and stretch



**Figure 3** Instinctive fear-induced defensive responses evoked by the confrontation between *Mus musculus* and the South American Boidae snake *Boa constrictor constrictor* for 5 min in the polygonal arena of the complex labyrinth. Anxiety/fear-related responses: alertness (A), inhibitory avoidance (C), and stretch attend posture (E and F). Panic attack-like responses: freezing (B) and oriented escape (D) displayed by prey threatened by the rainbow Boidae Amazonian constrictor snake.

attend posture (Figure 3E, Figure 4C), and panic-related behavior, such as freezing (Figure 3B, Figure 4A and F) and escape behavior (Figure 3D). Despite the galleries, prey and predator interacted closely, as shown in Figure 3E and F, and in Figure 4D.

Table 4 summarizes the main characteristics of the EPM, T-maze, and rodent vs. snake models.

#### *Neural substrates involved in threatened prey animals*

Many studies using models of confrontation between rodents and a predator consider explosive escape behavior to be a consequence of periaqueductal gray matter (PAG) neuron activation.<sup>84,85</sup> In fact, the dorsomedial, dorsolateral, and lateral columns of the PAG in Syrian hamsters confronted with coral snakes show several Fos protein-labeled neurons.<sup>40</sup> This finding corroborates previous reports showing that rats exposed to a natural predator (cat) express Fos-labeled neurons in the dorsal and ventral columns of the PAG.<sup>84</sup> These PAG columns are involved in the organization of behavioral and physiological responses that are crucial for the survival of threatened animals based on whether the dangerous situation is distal or proximal.<sup>29,86</sup> In addition, the interaction between the intramesencephalic endogenous opioid peptide-mediated pathways and the GABAergic nigrotectal inputs seems to be critically involved

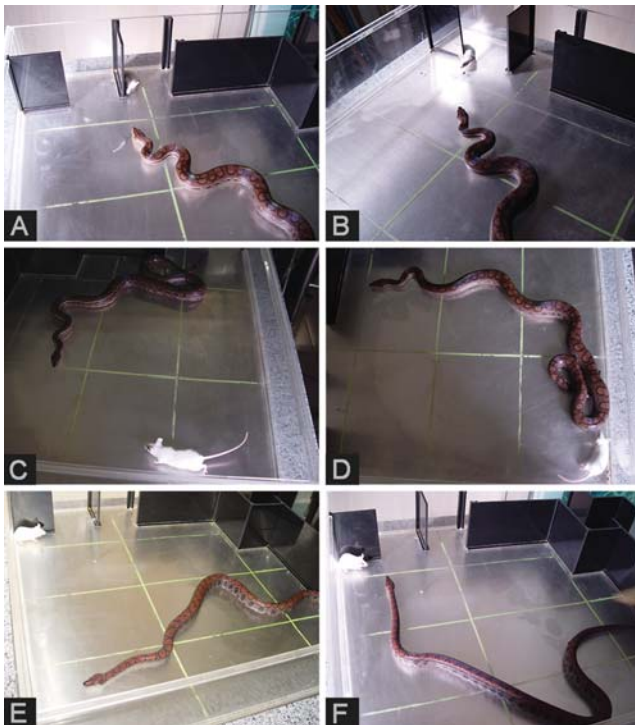
in both the modulation of defensive responses organized by the dorsal midbrain<sup>87-90</sup> and the defensive responses evoked in prey versus wild snakes paradigms.<sup>91</sup>

However, the confrontation between Syrian hamsters and coral snakes showed neuronal activation also in the hamsters' corpora quadrigemina, medial hypothalamus and amygdaloid complex.<sup>39,40</sup> In fact, other encephalic regions are also activated during similar critical situations. For example, the amygdaloid complex is recruited in both unconditioned and conditioned fear-induced responses. Nevertheless, the posterior basomedial amygdaloid nucleus is particularly responsive to cat odor and seems to

be involved in the identification of pheromone cues from predator odors.<sup>92</sup>

The Fos immunoreactivity study showed that the main encephalic structures recruited during a prey versus predator confrontation are situated in the hypothalamus and involve the recruitment of the anterior hypothalamus, the dorsomedial division of ventromedial hypothalamic nucleus, and mainly the dorsal premammillary nucleus.<sup>84,93,94</sup> Previous reports have shown that these nuclei are critically involved in the organization of innate defensive responses; this proposition is based on Fos immunoreactivity in rats exposed to natural predators, such as cats and snakes.<sup>34,35,84,85,93</sup> Other studies using electric or chemical stimulation of the hypothalamic defense system<sup>62,63,95-98</sup> are in agreement with the findings using prey versus predator confrontations, and both these experimental models of panic attacks result in oriented escape reactions.<sup>62,63,95,96</sup>

It is possible that the Fos-labeled neurons in the dorsal midbrain and the hypothalamic nuclei of rodents exposed to a live predator, to the odor of their skin, or to their excrements are indicative of the involvement of these structures in the elaboration of innate fear-induced behaviors, such as defensive alertness, defensive immobility and escape behavior. These behaviors are commonly displayed by rodents confronted with venomous and constrictor snakes and other natural predators.



**Figure 4** Instinctive fear-induced defensive responses in *Mus musculus* confronted with the South American rainbow Boidae snakes *Epicrates cenchria assisi* (A-D) and *Epicrates cenchria cenchria* (E and F) for 5 min in the polygonal arena of the complex labyrinth. Anxiety/fear-related response: alertness (A and E), inhibitory avoidance (B), and flat back approach (C). Panic attack-like responses: freezing (F) displayed by prey threatened by Amazonian rainbow Boidae snake *E.c.cenchria*. (D) Close interaction between prey and *Epicrates cenchria assisi*.

## Discussion

There still are questions regarding how strong EPM-evoked behaviors corroborate anxiety disorders. Studies have shown that exposure to the EPM increases the expression of Fos protein in encephalic areas related to the organization of defensive behaviors, which include the amygdaloid complex, the hippocampal formation, the midbrain periaqueductal gray matter, the hypothalamus, and the prefrontal brain areas.<sup>82,83</sup> Fos protein expression is linked to a general increase in cell metabolism, which is differentially activated in specific brain areas according to the presented stimulus. For example, anxiogenic stimuli such as those present in the EPM open arms can activate neurons from these brain areas related to anxiety or fear responses.<sup>82,83,99</sup> These areas are also activated when the animal is exposed to a predator,<sup>84</sup> the odor of a predator,<sup>92</sup> aversive ultrasonic vocalisations,<sup>100</sup> and the systemic administration of anxiogenic drugs.<sup>101</sup> Corroborating these data,<sup>33</sup> the diverse components of defensive behaviors with distinct brain areas that coordinate the

**Table 3** Offensive/defensive responses of *Crotalus durissus terrificus* and *Bothrops alternatus* snakes in Coimbra polygonal arenas and complex labyrinth during a 5-min exposure to mice

Response	Polygonal arenas		Complex labyrinth	
	<i>C. durissus terrificus</i>	<i>B. alternatus</i>	<i>C. durissus terrificus</i>	<i>B. alternatus</i>
Threatening posture	2	3	3	1
Defensive attack	0	0	0	0
Offensive attack	0	0	0	0
Predation	1	1	0	0
Rodents survival (%)	83.3	83.3	100	100

**Table 4** Comparative scheme of three maze models in the study of anxiety and fear responses in rodents

	Main expected behaviors	Task utility	Fos-labeled brain regions	References
Plus-Maze	Open/closed arm entries Arm latencies Head dippings Defecation/micturition	Anxiety Motor behavior	Amygdaloid complex Hippocampal formation PAG Hypothalamus Prefrontal cortex	Silveira et al. <sup>82</sup> ; Duncan et al. <sup>83</sup>
T-Maze	Avoidance of closed arm Escape from open arm Conditioned aversion (learning) Defecation/micturition	Panic Innate and learned fear/ anxiety Motor behavior	Anterior hypothalamic nucleus (in avoidance task) Median raphe nucleus (in avoidance task) Basolateral amygdaloid nucleus (in escape task) Dorsal PAG (in escape task) Paraventricular thalamic nucleus Dorsomedial hypothalamic nucleus Hippocampal formation Striatum	Silveira et al. <sup>61</sup>
Rodent vs. snake polygonal arenas and complex mazes	Alertness Stretch-attend posture Prey versus predator interactions Freezing Autonomic reactions Oriented escape Non-oriented escape Crossings Rearings	Anxiety Panic-like behavior Innate fear-induced defensive responses Fear-induced antinociception Motor behavior	Dorsomedial, dorsolateral and lateral columns of PAG Corpora quadrigemina Medial hypothalamus Amygdaloid complex Dorsomedial hypothalamus Dorsal premammillary nucleus of the hypothalamus	Weltson et al. <sup>40</sup> ; Paschoalin-Maurin <sup>34</sup> ; Ubiali <sup>35</sup> ; Uribe-Mariño et al. <sup>26</sup> ; Twardowschy et al. <sup>27</sup> ; Almada & Coimbra <sup>22</sup> ; Almada et al. <sup>23</sup>

PAG = periaqueductal gray matter.

behavioral strategies when a threat is present have been classified. A potential threat elicits risk assessment and behavioral inhibition, which are related to the activation of the posterior cingulate cortex and septo-hippocampal system and results in anxiety. This same potential threat can also elicit avoidance, activating the anterior cingulate cortex and amygdaloid complex, resulting in anxiety. Complementarily, distal threats elicit freezing behavior via the activation of the ventral columns of periaqueductal gray matter, resulting in instinctive fear. Finally, a proximal threat elicits freezing or fight or flight behavior via the activation of dorsal columns of the periaqueductal gray matter, resulting in a generalized panic reaction.

Interestingly, the EPM test can predict the elicitation of specific behaviors depending on the stress suffered by the animal. Some behavioral parameters evoked in the EPM are related to increased hormonal components of stress response or psychiatric disorders, such as high serum levels of steroid hormones. In patients, anxiety-related symptoms are correlated with an unbalance of steroid secretion within the hypothalamic-pituitary-adrenal axis.<sup>31,33</sup> Corroborating these studies, increased levels of plasmatic corticosterone in rodents have been associated with higher frequencies of anxiety-related SAP in the EPM test.<sup>102-104</sup> Additionally, chronic administration of anabolic androgenic steroids increases the number of rearing response and decreases SAP frequencies without affecting the time spent and frequency of open-arm entries.<sup>105</sup>

Another defensive reaction generally observed in rodents exposed to the EPM is the antinociception induced by instinctive fear, as evaluated by the tail-flick test.<sup>106,107</sup> This reaction has a clear adaptive value because it permits the animal to exhibit other defensive behaviors even though an injury has occurred, consequently increasing its

chances of survival in a dangerous situation, and can be also elicited by electrical and chemical stimulation of diencephalic<sup>95-98,108</sup> and mesencephalic<sup>81,109,110</sup> structures. Nevertheless, this defensive reaction was not observed in mice submitted to the formalin pain test and exposed to the EPM.<sup>13,17,111-113</sup> Therefore, the authors who performed those experiments decided to use the open elevated plus maze, which has the same dimension of the standard EPM but is comprised of four open arms. Using this apparatus, they verified antinociception of high magnitude. The open-arms EPM seemed to be a good model for studying fear-induced antinociception; this modified apparatus has also been behaviorally and pharmacologically validated.<sup>114</sup>

Experimental evidence from the 1970s, 1980s, and early 1990s suggests that serotonin (5-HT) facilitates punished behavior by recruiting the amygdaloid complex activity (resulting in anxiety) and inhibits escape behavior by acting in the dorsal periaqueductal gray matter (the unbalance of which causes panic).<sup>115,116</sup> This led Deakin & Graeff<sup>57</sup> to propose that 5-HT neurons mediate avoidance behaviors while simultaneously inhibiting escape behaviors.<sup>4</sup> Because of its direct implications for anxiety and panic, serotonin and its actions in the amygdaloid complex or the periaqueductal gray matter have been extensively studied in rodent models of anxiety, such as the EPM. These exhaustive investigations have helped establish the EPM test as a reliable experimental tool for studying anxiety in rodents.<sup>65,117-121</sup> Notwithstanding the high reliability of the EPM for measuring anxiety-like behaviors in rodents,<sup>122</sup> this test has been criticized as a mixed model of anxiety that combines an avoidance behavior with an escape behavior: in the avoidance behavior (a conditioned-like behavior or anticipatory anxiety-related response), the animal is in the



closed arms and avoids the open arms, whereas in the escape (panic-like) behavior, the animal is in the open arms and draws back to the closed arms in search of a protected environment. Thus, exposure to the open and closed arms may elicit different types of defensive behaviors. Considering these psychobiological characteristics of the EPM test, this type of mixed model might not be very appropriate for studying the effects of all anxiolytic compounds, or it may need to be used with caution if a unique behavioral task is used to evaluate anxiety.

Prey versus predator paradigms are excellent approaches for studying innate fear-related behaviors, not only because these paradigms allow us to focus on the effects of a discrete intervention in a given structure of the limbic system or a functionally related structure,<sup>22,23</sup> but also because they are useful to study the integrated activation of the limbic system in a threatening situation.<sup>26,27</sup> Although much research now focuses on more invasive methods, such as the use of electrically and chemically restricted brain stimulation, to clarify the involvement of a given brain structure in the organization of defensive behavioral responses,<sup>108-110,123</sup> the prey-versus-predator paradigm is still useful for studying the activity of the brain aversion system activity,<sup>22,84,85,124</sup> for testing new drugs with potential applications in neuropsychiatry, or for assessing the behavioral phenotypes of genetically-modified mice.<sup>25,26</sup>

In conclusion, simple labyrinths such the elevated plus maze and elevated T-maze are excellent apparatuses for the study of anxiety- and instinctive fear-related responses, respectively. Both apparatuses have been sufficiently validated in both behavioral and pharmacological terms. The confrontation between rodents and snakes in polygonal arenas, however, offers a more ethological environment for addressing both unconditioned and conditioned fear-based behaviors and the effects of anxiolytic and panicolytic drugs. More specifically, in the prey-predator confrontation approach, the possibility of testing both anxiety and fear or combined panic-related behaviors allows for a more complete approach to new drug or rodent phenotype testing, considering the diverse aversive stimuli in the rodent ethogram.

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### Disclosure

The authors report no conflicts of interest.

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