# ASSESSING APPROACH-AVOIDANCE CONFLICT IN RESPONSE TO PREDATOR

# THREAT

## By

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## ABSTRACT OF THE THESIS

## Assessing Approach-Avoidance Conflict in Response to Predator Threat

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Approach-avoidance conflict occurs when balancing actions intended to obtain a positive outcome against the known and unknown risks of a negative outcome. Here, we use the PORT, developed by Dent et al., (2014), to evoke a natural approach-avoidance conflict by exploiting predator-prey relationships. Briefly, to obtain a reward, animals must traverse through a chamber containing the innately feared odor, 2-Phenylethylamine (PEA), a synthetic volatile component of cat predator urine. Thus, this task nicely mimics the real-life tension between foraging and predation risk and should recruit natural circuits involved in processing innate threats. Both females and males demonstrate an initial apprehension to enter a chamber scented with predator odor, but not one scented with a novel odorant (MV) or clean bedding (CB). This apprehension was followed by increased time spent within those chambers, indicating similar exploratory behaviors to both a novel and predator odor. This is in direct opposition to previous literature demonstrating avoidance to predator odors. Females and males exposed to MV, exhibited

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similar levels of cFos immunoreactivity within the piriform cortex and when collapsing across all 13 brain regions quantified in this study. However, females had reduced cFos activity within the dorsomedial (DM) hypothalamus compared to males. Unlike MV and CB, behavioral responses between females and males diverged with exposure to PEA. In the presence of the predator cue, all females quickly transited the box to obtain the reward, while males exhibited a highly variable, possibly bi-modal distribution where half the males transited quickly but about half took longer than any female. This differential behavioral response was reflected by a PEA induced enhancement of female cFos immunoreactivity within the piriform cortex, DM hypothalamus, and when collapsing across all brain regions. Interestingly, it was only during PEA exposure, where this behavioral divergence occurred, that females showed greater DM hypothalamic activity than males. In both sexes, PEA exposure evoked heightened cFos activity within the central amygdala which indicates the anxiogenic nature of this stimulus. We also find that regardless of odor condition, females exhibited greater basolateral amygdala activity than males, whereas males demonstrated heightened defensive behaviors such as immobility and time spent close to the chamber walls. Overall, the findings suggest that the behavioral and neural differences between males and females in response to predator threat may reflect sex differences in risk-reward decision-making.

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#### INTRODUCTION

Approach-avoidance conflict occurs when balancing actions intended to obtain a positive outcome against the known and unknown risks of a negative outcome. The ultimate resolution of approach-avoidance conflict into a choice of behavior reflects a decision process that plays a role in everyday choices across a wide range of potential positive and negative outcomes.

Sensory cues that indicate the potential for negative outcomes are regarded as threats and can evoke acute fear or anxiety. Threat cues include both cues whose prediction of potential harm is learned through experience (e.g. through fear conditioning) and a modest number of innately threatening stimuli (Rosen, Asok, & Chakraborty, 2015). These unconditioned threats, here termed "ecologically relevant" threats, include stimuli like predator odors whose meaning is believed to be hard-wired into the neural circuitry of the brain in order to facilitate rapid detection for survival purposes (Ohman, Erixon, & Lofberg, 1975; Ohman, Flykt, & Esteves, 2001). Examining the neural processing of these unconditioned threats and their role in shaping behavior and may provide insight into normal vs. irrational fear and anxiety (Takahashi, 2014) or at least provide more ecologically relevant tests of fear and anxiety than classical conditioning models (Dielenberg, Hunt, & McGregor, 2001; Hendrie, Weiss, & Eilam, 1996; Staples, McGregor, Apfelbach, & Hunt, 2008). This is especially applicable for human phobias, which generally are tied to specific innately threatening stimuli such as heights, blood contamination or infection, and animals (de Jongh, Oosterink, Kieffer, Hoogstraten, & Aartman, 2011; Rosen et al., 2015). This paper focuses on predation threat. Both humans and rodents exhibit a strong innate sensitivity to predatory threats

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which renders these stimuli promising for study in the fields of fear and anxiety (Rosen et al., 2015). Both humans with and without phobias, exhibit enhanced visual detection of predators, while those with animal phobias, (often of spiders and snakes) demonstrate enhanced startle reflex, and greater avoidance of the respective phobic stimuli (Mineka & Ohman, 2002; Ohman et al., 1975). Similarly, predator-related stimuli evoke defensive responses in rodents, such as freezing, enhanced risk-assessment behaviors and avoidance of the threat (Blanchard, Blanchard, Rodgers, & Weiss, 1990; Perrot-Sinal, Heale, Ossenkopp, & Kavaliers, 1996; Shepherd, Flores, Rodgers, Blanchard, & Blanchard, 1992).

Humans exhibit attentional biases towards predator-related stimuli (Flykt, 2005; Ohman et al., 2001; Yorzinski, Penkunas, Platt, & Coss, 2014), which may indicate preferential sensory processing in the brain. Remarkably, these attentional biases are found in infants as young as 5-8 months old (LoBue & DeLoache, 2010; Penkunas & Coss, 2013; Rakison & Derringer, 2008), suggesting that detection of these ecologicallyrelevant threats is still preserved in modern day humans. This may have practical implications for the etiology of anxiety disorders, as noted by De Silva and colleagues (1977), in that people are much more likely to develop phobias to innate threats like heights, snakes, and spiders than to modern-day cultural threats like weapons or motorcycles. Similarly, Cook and Mineka (1989) showed that ecologically-relevant stimuli are more conducive to social fear learning. In their study, lab-reared monkeys watched videos of another monkey acting fearfully to either a snake stimulus (live or toy snake) or a bouquet of flowers. When the observer monkeys were subsequently presented with those same stimuli, they acted fearfully towards the snake stimuli, but not to the bouquet of flowers. This evidence supports the notion of the selective association theory (or preparedness theory) introduced by Seligman (1971) and further built upon by Mineka and Ohman (2002). This theory suggests that certain stimuli are prioritized for rapid fear response learning, even if they do not initially evoke fear directly. Similarly, more rapid, even single trial learning with resistance to extinction can occur when innately fear-related stimuli are paired with an aversive outcome, compared to non-fear related stimuli (Ohman et al., 1975). A study by Chapman and Chapman (1967) powerfully demonstrates the selective association theory. Using an illusory correlation paradigm, participants were shown a series of images (snakes, flowers, mushrooms) in which an aversive shock was administered randomly so that none of the image categories actually predicted an impending shock. However, when asked the probability that each image category resulted in a shock, participants (particularly those with pre-existing fear of snakes) overestimated the probability of snake images resulting in a shock. Finally, recent results from our laboratory have demonstrated that people exhibit enhanced memory for words paired with a snake compared to a non-predator animal (Francesconi, McGann, and Durante, unpublished findings).

Within the animal literature, studies sometimes employ predator and predatorrelated stimuli such as a robotic predator (e.g. Robogator; (Choi & Kim, 2010)) or predator odors to study the neurobiology underlying these responses (Amir, Lee, Headley, Herzallah, & Pare, 2015; Dielenberg & McGregor, 2001; Rosen et al., 2015). In the presence or odor of a cat, rats exhibit increased defensive behaviors such as freezing and risk assessment and decreased non-defensive behaviors such as locomotion, rearing, grooming, drinking, and eating (Blanchard et al., 1990; Perrot-Sinal et al., 1996; Shepherd et al., 1992). Animals avoid predator odors, including single monomolecular components of chemically complex naturally occurring odors like predator urine (Buron et al., 2007; Dewan, Pacifico, Zhan, Rinberg, & Bozza, 2013; Dielenberg et al., 2001; Zhang, Pacifico, Cawley, Feinstein, & Bozza, 2013). The anxiogenic effects of predator odors are observed in anxiety tests such as the Elevated Plus Maze, and trait levels of anxiety are correlated to defensive behaviors in response to these odor cues (Dent et al., 2014; McGregor et al., 2002). In the presence of ferret urine, dominant male mice (defined as high-scent markers) spend less time within the chamber containing the odorant compared to non-dominant males (Roberts et al., 2000). Male rats exposed to natural cat odor exhibit increased escape attempts (jumping), more time with the odor stimulus, and less grooming compared to control odors and 2,5-dihydro-2,4,5-trimethyl thiaszoline (TMT), a synthetic compound isolated from fox feces (Staples et al., 2008). With the exception of Dewan et al. (2013), previous literature has found sex differences in predator defensive behaviors, generally showing that females are more defensive than males (Blanchard et al., 1990; Shepherd et al., 1992). Sometimes these differences were circumstance-dependent, such as females exhibiting fewer risk assessment behaviors than males (e.g. head extensions out of a hide box, stretch-attend posture) but no difference in explicit defensive behaviors (Perrot-Sinal et al. (1996). Buron et al. (2007) saw an avoidance of a chamber containing TMT, and this effect was significantly more pronounced in females.

Researchers have also examined neural activity in various brain regions after exposure to a predator odor. The neuroanatomy of predator odor fear behavior is thought to include regions such as the accessory olfactory bulb (AOB), main olfactory bulb (MOB), the anterior olfactory nucleus (AON), lateral septal nucleus (LSN), dorsomedial hypothalamus (DM), ventromedial hypothalamus (VMH), basolateral amygdala (BLA), Central amygdala (CeA), cortical amygdala (CoA), medial amygdala (MeA), periacqueductal gray (PAG), and the locus coeruleus (LC) (Dielenberg et al., 2001; Staples et al., 2008). Predator-related odorants are suggested to affect the stress response by activation of both the hypothalamic-pituitary-adrenal (HPA) axis and the sympatheticadrenal-medullary (SAM) axis; both being involved in the fight-or-flight response (File, Zangrossi, Sanders, & Mabbutt, 1993; Horii, Nikaido, Nagai, & Nakashima, 2010; Nikaido & Nakashima, 2009).

Odorants commonly used in the previous literature include either natural predator odors (complex mixtures of volatile and involatile chemicals naturally emitted by the predator animal), or synthetic volatile components of those odors: 2,5-dihydro-2,4,5trimethyl thiaszoline (TMT) and 2-Phenylethylamine (PEA). Whole predator odors by definition make the experiment more naturalistic but are potentially complicated by variance from source to source (e.g. scent from *which fox?*) and the presence of saliva, dander, fur, etc. (Masini et al., 2010) that can make the experiment irreplicable because the key stimulus is impossible to precisely characterize. However, there is good evidence that single specific odorants within the predator scent drive most of the behavioral response, potentially through dedicated neural circuitry. Remarkably, a single monomolecular amine, 2-Phenylethylamine (PEA), can elicit innate fear in mice and avoidance behavior (Dewan et al., 2013; Zhang et al., 2013), while seemingly activating only a single type of odor receptor. PEA is a biogenic amine found in cat predator urine that selectively activates trace amine-associated receptors (TAARs) expressed in the nasal epithelium (Ferrero et al., 2011; Takahashi, 2014). Rodents have the ability to detect trace quantities of PEA, and this high sensitivity is dependent on the TAAR4 receptor (Dewan et al., 2013; Zhang et al., 2013). It has been speculated that enhanced detection of amines may be the result of evolutionary pressures. That is, the low detection threshold for amines found in predator urine may be an adaptive mechanism to detect the presence of predators even from far distances in order to ensure survival (Dewan et al., 2013).

#### Creating Approach-Avoidance Conflict with the Predator Odor Risk Task

Previous experiments assessing the aversive nature of predator odors have generally utilized paradigms in which animals are passively exposed to the odor without any explicit behavioral goal. While these studies do reveal the innately aversive nature of these predator odorants via behaviors like avoidance, it is currently unknown how these "innately threatening" stimuli could impact conflicting drives to engage in goal-directed approach behavior. This form of decision-making under approach-avoidance conflict may be the key to understanding anxiety disorders (Aupperle et al., 2010, p. 519).

A relatively recent study by Dent and colleagues (2014) demonstrated a "predator odour risk-taking task" (PORT) intended to evoke a natural approach-avoidance conflict by exploiting predator-prey relationships. In this task mice have the opportunity to obtain a desired liquid reward but must traverse a predator odor-scented arena to do so. The task nicely mimics the real-life tension between foraging and predation risk and thus should recruit natural circuits involved in processing innate threats, which is an important step in understanding healthy and aberrant behavioral responses towards innately feared stimuli. The results from Dent et al., (2014) showed that mice exhibited increased latencies to enter and traverse a chamber containing a predator odor compared to an unscented chamber and took longer when the reward was reduced. We adapted this method (see below), with the key additions of a control group exposed to a novel non-predator odor, more detailed analysis of behavior in the chamber, and the assessment of a potential neural correlate of the odor exposure and/or decision making (cFos expression).

### MATERIALS AND METHODS

**Subjects:** A total of 119 C57Bl/6 mice (ages ranging from 8 - 35 weeks) of both sexes (female: n= 59, male: n=60) were used for behavioral analysis. A subset of these mice (n = 58) were used for subsequent cFos quantification. Mice were water restricted to 90% of *ab libitum* weight and maintained on a 12:12 hour light/dark cycle, with behavioral testing occurring during the light cycle. Mice were run in cohorts, with both male and female mice running concurrently.

**Apparatus:** The PORT task apparatus was adapted from Dent, Isles, and Humby (2014). The apparatus was a white, plexiglass arena divided into three equal, distinct chambers (30 x 30 x 40 cm, length x width x height) with doorways between, allowing the middle chamber to be entered from either end chamber. Mice were randomly assigned either the leftmost or rightmost chamber as their "start" chamber, with the other being designated as the "reward" chamber, where 100 uL of 10% sucrose solution was placed.

**Odorants:** 500mL of standard corn cob bedding was mixed with 300uL of either: 2-Phenylethylamine (Sigma-Aldrich) which served as the predator odor, or methyl valerate (Sigma-Aldrich), the novel odor control. 500mL of just standard cage bedding was used for the non-odorized control conditions. All bedding was prepared two days prior to use and stored in sealed Ziploc bags.

## **Procedure:**

*Behavioral Paradigm:* The 6-day behavioral paradigm was adapted from Dent et al., (2014). Mice were single-housed for 6 days prior to task onset with water restriction beginning on the sixth day. Mice began the task on the fourth day of water restriction. On days 1-3 mice underwent sucrose preference testing. During this phase, mice were placed

within standard cages containing clean bedding and two ceramic dishes placed side by side at one end of the cage. One dish contained 3mL of a 10% sucrose solution, while the other contained 3 mL of water. The left/right placement of the dishes was alternated each day. Mice were given 10 minutes to freely sample and consume the liquid from either of the dishes. The total volume of liquid consumed from each of the dishes was recorded each day to quantify the sucrose preference for each animal. On average, animals exhibited a 76% ( $\pm$  1.70) preference for sucrose over water.

On the fourth day, animals were habituated to the testing apparatus in one 20minute session. The middle chamber of the apparatus contained 500mL of clean standard bedding. During this phase no reward was present. On day 5, animals underwent acquisition/training. Animals were placed within the start chamber and were given unlimited time to traverse through the middle chamber (filled with 500mL of clean bedding) to retrieve a reward (100uL of 10% sucrose solution) in the end chamber. After sampling the reward from the dish, animals were removed from the apparatus, and placed in their home cage during the 1 minute intertrial interval in which the apparatus was cleaned first with 1% Tergazyme solution followed by 70% ethanol solution. Animals completed a total of 5 trials during this phase.

On the sixth and final day, animals completed a total of 6 trials. Three of these trials occurred in the morning (AM) in which clean bedding was placed within the middle chamber. The last three trials occurred in the afternoon (PM) approximately 3 hours after the initial morning testing. The experimental manipulation of odor cue within the middle chamber occurred in the 3 PM trials. Animals were randomly assigned either clean bedding (CB) control, methyl valerate (MV), or phenlyethtlyamine (PEA) for their PM session. Animals that were randomly assigned to the same odorant condition were run on the same day. This procedure was necessary to prevent the possibility that the PEA odorant (which mice can detect in at picomolar concentrations; (Dewan et al., 2018) contaminated the ambient air for animals running in either MV or CB conditions. To prevent any effects that day of the week might have on behavior, the order in which the groups ran each week varied (e.g. if the PEA group ran Monday, the following week either CB or MV were run Monday). Mice were videorecorded and behavior was analyzed with EthoVision video tracking systems for automation of behavioral experiments (Noldus, Wageningen, the Netherlands).

## Immunohistochemistry:

*Tissue processing*: Forty-five minutes after the PM session in day 6, mice were deeply anesthetized with pentobarbital and transcardially perfused with 40mL of ice cold phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). Extracted brains were postfixed in 4% PFA for 24 hours and subsequently submerged in a 10% sucrose, 0.1% sodium azide solution in PBS until sectioning. 40 micron thick coronal brain sections were cut on a cryostat and submerged and stored in 24- well plates containing 30% sucrose and 0.1% sodium azide solution in PBS until immunohistochemistry processing. *cFos Immunohistochemistry*: Free-floating slices were permeabilized in 0.1% Triton X-100 in 1X PBS for five minutes and subsequently washed with 1X PBS for another five minutes. The slices were then submerged in a citrate buffer for 30 minutes and then blocked with 10% Normal Goat Serum (NGS) and 0.1% Triton X-100 in 1X PBS for one hour. Following the blocking step, slices were incubated overnight at 4 degrees Celsius with primary antibody (cFos-rabbit, CellSignaling), 2% NGS, and 0.3% Triton X-100 in 1X PBS. After at least 18-22 hours, slices were washed in 1X PBS, three times, for five minutes each and then incubated with secondary antibody (goat anti-rabbit IgG Alexa Fluor 488, Invitrogen), 2% NGS and 0.3% Triton X-100 in 1X PBS for two hours at room temperature. Slices were kept in the dark for the remainder of the protocol. After the incubation, the slices were washed with 1X PBS, three times, for five minutes each. The slices were then mounted onto slides and coverslipped with ProLong Gold with DAPI (Invitrogen).

*Imaging:* Slides were imaged with a 10x objective on an Evos FL Auto 2 Imaging System (Invitrogen). UV (4',6-diamidino-2-phylindole (DAPI)-compatible) and blue (AlexaFluor488- compatible) fluorescence channels were utilized for the collection of images. Slides were autofocused on the AlexaFluor488 channel at 50% light intensity, 0.151 second exposure, and 1.0 gain.

*cFos Quantification:* Slices containing specific brain regions such as amygdala, hypothalamus, and olfactory regions were selected and subsequently outlined for their corresponding region of interest (ROI) on the image processing program, ImageJ (Java). Two slices of each region were generally collected per animal. Boundaries of ROIs were determined through the use of a standard mouse brain atlas (Franklin & Paxinos, 2008). Outlines were initially traced onto the DAPI images and then superimposed and saved onto the cFos images for manual cFos quantification. Before counting, delineated cFos images were converted to a 16-bit grayscale and then slightly sharpened with the despeckle and sharpen options. To better visualize the cells, the images' contrast was enhanced to 0.01. Cells within the delineated ROI were manually counted using the multipoint tool. Areas for all sustainable ROIs were measured by ImageJ. Number of

cells counted within each ROI was divided by its corresponding area to attain number of cells per millimeter<sup>2</sup>. The average of all the slices per region per animal was taken to get an overall value.

*Statistical Methods*: Because the behavioral measurement were not (and could not be) normally distributed, a generalized linear model was used for statistical analysis of both behavioral and cFos data, with sex, condition, and the sex\*condition interaction as fixed factors using SPSS (IBM). A gamma probability distribution and log link function were selected, and a robust estimator was used. Pairwise comparisons were adjusted for multiple comparisons with least-square-differences (LSD). Spearman's non-parametric correlation coefficients were used for correlation tests.

#### RESULTS

*Trial 1 Behavior:* Latency to reward served as an overall indicator of trial to trial behavior. As shown in Fig. 1A-C, on the first test trial there were significant differences in the latency to reward across groups [ $\chi^2$  (2) = 15.13, p = 0.00]. Compared to CB, there were increased latencies in both the MV and PEA conditions [ $\chi^2$  (2) = 14.48, p = 0.00; 0.01 (MV, PEA respectively)]. There also was a significant sex\*condition interaction [ $\chi^2$  (2) = 7.87 p = 0.02], such that within the PEA condition females were much quicker than males to obtain the sucrose reward, whereas in the CB or MV conditions they responded similarly to males [ $\chi^2$  (5) = 16.17, p = 0.02; 0.45; 0.95 (PEA, CB, MV, respectively)].

Differences in latency to reward could result from either longer latencies from trial start until entering the scented middle chamber or from longer times spent within the middle chamber. We analyzed these separately. As shown in Fig. 1D - I, during the first test trial, there was a significant main effect of odor condition on latency to enter the middle chamber  $[\chi^2 (2) = 11.86, p = 0.00]$ . Mice took significantly longer to enter the middle chamber when it contained PEA than when it contained CB  $[\chi^2 (2) = 8.83, p = 0.00]$ , or when it contained MV (trend level significance:  $\chi^2 (2) = 8.83, p = 0.05$ ). Time to enter the middle chamber did not differ between CB and MV  $[\chi^2 (2) = 8.83, p = 0.26]$ . However, these latencies were short (2-4 sec on average across groups) and thus composed a modest part of the overall latency to reward. We thus also assessed time spent within the middle chamber on test trial 1. There was a significant main effect of odor condition  $[\chi^2 (2) = 8.02, p = 0.02]$ , such that animals spent more time in the middle chamber in both the MV and PEA conditions compared to CB  $[\chi^2 (2) = 8.64, p =$ 

0.01(MV); 0.04(PEA)]. There was also a significant main effect of sex such that males spent more time than females within the middle chamber in trial 1 [ $\chi^2$  (1) = 4.73, p = 0.03]. These durations were on the order of 20-45 sec and thus composed most of the time on each trial.

To further analyze the potential anxiety behaviors of the mice, we analyzed the paths taken and movement speed during the trial, as shown in the heat maps in Fig. 1J. Thigmotaxis was quantified as time spent near the walls of the middle chamber, revealing a main effect of sex such that males spent significantly more time near the walls than females [ $\chi^2$  (1) = 5.23, p = 0.02]. We also quantified immobility and found a significant main effect of sex such that males spent significantly more time (34.12 ± 5.69 sec) immobile than females (18.98 ± 2.37 sec) [ $\chi^2$  (1) = 7.94, p = 0.01].

*Trial 3 Behavior:* We next examined whether animals continued to differ in their response to odorants after multiple trials by repeating the above analyses on the data from test trial 3. As shown in Fig. 2, there were no significant effects of odor condition  $[\chi^2 (2) = 5.48, p = 0.07]$ , sex  $[\chi^2 (1) = 0.00, p = 0.99]$ , or their interaction  $[\chi^2 (2) = 0.46, p = 0.80]$  on overall latency to reward. There were no effects of odor condition  $[\chi^2 (2) = 4.48, p = 0.11]$ , sex  $[\chi^2 (1) = 0.33, p = 0.57]$ , or their interaction  $[\chi^2 (2) = .78, p = 0.68]$  on the latency to enter the middle chamber. Time spent in the middle chamber also did not significantly differ by odor condition,  $[\chi^2 (2) = 2.32, p = 0.31]$ , sex,  $[\chi^2 (1) = 0.57, p = 0.45]$ , or their interaction  $[\chi^2 (2) = 0.37, p = 0.83]$ . Thigmotaxis and immobility were also no different across odor condition or sex (all p values > 0.25). The results indicate that after only two trials, all animals exhibited similar behavioral responses. Animals also

exhibited learning during the task, evidenced by shortened latencies in the third trial, regardless of condition.

*Whole brain average cFos activity:* To assess whether there were neural correlates of the behavioral experiment, we used immunohistochemistry to quantify the number of cells expressing the immediate early gene cFos throughout many individual brain structures detailed below. As shown in Fig. 3, collapsing across all brain regions, there was no significant main effect of odor condition  $[\chi^2 (2) = 2.32, p = 0.31]$ , or sex  $[\chi^2 (1) = 0.01, p = 0.93]$ . However, as in the overall latency to reward metric, there was a significant sex\*condition interaction  $[\chi^2 (2) = 6.36, p = 0.04]$ , such that within the PEA condition females exhibited significantly more cFos expression than males  $[\chi^2 (5) = 9.46, p = 0.04]$ . Note that this is despite the fact that females in this group spent significantly less time exposed to PEA than the males. There were no significant sex differences within the CB or MV conditions  $[\chi^2 (2) = 9.46, p = 0.16$  (CB); p = 0.89 (MV)]. The following sections include further analysis of each individual brain region of interest.

*Olfactory-related* **cFos activity:** The olfactory-related regions used for cFos quantification include: the granule cell layer of the OB, piriform cortex, and AON. As shown in Fig. 4, there were no significant effects of sex, condition, or their interaction on cFos expression within the granule cell layer of the olfactory bulb [ $\chi^2$  (1) = 3.80, p = 0.05 (sex); ( $\chi^2$  (2) = 4.46, p = 0.11 (condition); ( $\chi^2$  (2) = 1.84, p = 0.40 (interaction)]. or the AON [( $\chi^2$  (1) = 0.01, p = 0.92 (sex); ( $\chi^2$  (2) = 0.41, p = 0.81 (condition); ( $\chi^2$  (2) = 0.74, p = 0.69 (interaction)]. However, there was a significant sex\*condition interaction on cFos expression within the piriform cortex ( $\chi^2$  (2) = 13.10, p = 0.00). Within the CB condition,

females had significantly less cFos expression than males ( $\chi^2$  (5) = 18.16, p = 0.01), whereas there was no significant sex effect in the MV condition ( $\chi^2$  (5) = 18.16, p = 0.71). In the PEA condition, however, females had significantly more positive cFos cells than males ( $\chi^2$  (5) = 18.16, p = 0.03) (see Fig. 4D-F).

*Paraventricular Thalamic Nucleus:* Within the PVT, there was a significant sex\*condition interaction  $[\chi^2 (2) = 22.73, p = 0.00]$ . Within the CB condition, females had significantly less cFos expression than males  $[\chi^2 (4) = 41.62, p = 0.01]$ . No significant effects were observed for either the MV or PEA conditions  $[\chi^2 (4) = 41.62, p = 0.33 (MV); p = 0.89 (PEA)]$  (see Fig. 5).

*Hypothalamic Regions:* There were no significant effects of sex  $[\chi^2 (1) = 1.28, p = 0.26]$ , condition  $[\chi^2 (2) = 2.00, p = 0.37]$ , or their interaction  $[\chi^2 (2) = 4.73, p = 0.09]$  on cFos expression within the VM hypothalamus (see Fig. 6D-E). However, there was a significant sex\*condition interaction on cFos expression in the DM hypothalamus  $[\chi^2 (2) = 17.58, p = 0.00]$ . As shown in Fig. 6A-C, females had significantly fewer positive cFos cells than males in both the CB and MV conditions  $[\chi^2 (5) = 16.09, p = 0.01(CB); p = 0.05 (MV)]$ . However, a reversal was observed where females exhibited significantly more cFos expression than males in the PEA condition  $[\chi^2 (5) = 16.09, p = 0.04]$ .

*Amygdala Nuclei:* There were no significant effects of condition,  $[\chi^2 (2) = 4.18, p = 0.12]$ , or their interaction  $[\chi^2 (2) = 0.56, p = 0.76]$ , on cFos expression within the cortical amygdala (see Fig. 7A-C). Although there was a marginal main effect of sex  $[\chi^2 (1) = 3,72, p = 0.05]$ , with pairwise comparisons revealing females showing higher numbers of

cFos positive cells than males [ $\chi^2(1) = 3.27$ , p = 0.08]. Within the medial amygdala, there was a main effect of condition  $[\chi^2(2) = 8.63, p = 0.01]$ . Pairwise comparisons revealed that there were significantly more cFos positive cells for animals in the PEA condition compared to both CB and MV [ $\chi^2$  (2) = 7.90, p = 0.01 (CB); p = 0.04 (MV] (see Fig. 7G-I). There was no significant difference in cFos expression between MV and CB conditions  $[\chi^2(2) = 7.90, p = 0.70]$ . There was a main effect of condition on cFos expression within the BMA [ $\chi^2$  (2) = 6.92, p = 0.03]. Compared to the CB condition, there was greater cFos for animals within the PEA, but not MV condition [ $\chi^2$  (2) = 6.14, p = 0.01(PEA); p = 0.31 (MV)]. However, the number of cFos positive cells did not differ between PEA and MV conditions [ $\chi^2(2) = 6.14$ , p = 0.12] (see Fig. 7D-F). As shown in Fig. 8A-C, there were no significant effects of sex [ $\chi^2$  (1) = 1.07, p = 0.30], condition,  $[\chi^2 (2) = 0.51, p = 0.77]$ , or their interaction  $[\chi^2 (2) = .43, p = 0.81]$  on cFos expression within the lateral amygdala. There was a main effect of condition on cFos expression within the central amygdala [ $\chi^2(2) = 8.68$ , p = 0.01]. There were significantly more cFos positive cells for animals in the PEA condition compared to both CB and MV conditions  $[\chi^2 (2) = 4.67, p = 0.04 (CB); p = 0.03 (MV)$  (see Fig. 8D-F). There was no significant difference in number of cFos positive cells between CB and MV [ $\chi^2$  (2) = 4.67, p = 0.81]. As shown in Fig. 8G-I, within the BLA, there was a significant main effect of sex [ $\chi^2$  (1) = 14.63, p = 0.00], where females had more cFos positive cells than males, regardless of condition.

*Lateral Septal Nuclei:* There were no significant effects of sex  $[\chi^2 (1) = 0.15, p = 0.70]$ , condition  $[\chi^2 (2) = 2.85, p = 0.24]$ , or their interaction  $[\chi^2 (2) = 0.60, p = 0.74]$  on cFos expression within the lateral septal nuclei (see Fig. 9).

*Correlating Individual Behavior to cFos Activity:* Next, we examined if individual differences in behavior would correlate to cFos positive cells within the brain regions quantified. First, collapsing across condition, there were modest but significant positive correlations between latency to reward in trial one, and cFos counts in AON, BLA, and LSN (r = 0.35, p = 0.05; r = 0.36, p = 0.02; r = 0.33, p = 0.04) (see Fig 10A-C). However, there were no significant correlations of latency to reward and cFos counts within individual conditions, perhaps because of the smaller number of subjects. Collapsing across condition, there were significant positive correlations between individual animals' latencies to enter the middle chamber (LTM) and cFos counts in piriform and PVT (r = 0.27, p = 0.05, r = 0.31, p = 0.03) (see Fig. 10E,F). Within the CB condition there was a significant negative correlation between latency to the enter the middle chamber and cFos counts within the OB (r = -0.79, p = 0.04) (see Fig. 10D). Within the MV condition, there was a significant positive correlation between LTM and cFos in the piriform cortex (r = 0.54, p = 0.04) (see Fig. 10E). Collapsed across condition, there were no significant correlations between time spent in the middle chamber and cFos counts in any region. Within the CB condition there was a significant negative correlation between TSM and lateral amygdala (r = -0.55, p = 0.04) (see Fig. 10G). Collapsing across condition, there were no significant correlations between time spent in the periphery of the middle chamber (TSP) and cFos counts in any region. There were no significant correlations of TSP by condition.

### DISCUSSION

In the current study, we demonstrate that in an approach-avoidance paradigm, females and males exhibit differential behavioral and neural responses to a predatorrelated odorant, 2-Phenylethylamine (PEA). During initial exposure, animals exhibited increased latencies to cross the threshold into the PEA-scented chamber compared to when the chamber was scented with a neutral novel odorant (MV) or clean bedding. Surprisingly, the time spent within the scented chamber before exiting to obtain the liquid reward was comparably extended for both odors compared to the clean bedding, despite previous reports of PEA avoidance (Buron et al., 2007; Dewan et al., 2013; Dielenberg et al., 2001; Zhang et al., 2013). Interestingly, females and males differed in their contact with the odor. In the presence of the predator cue, all females quickly transited the box to obtain the reward, while males exhibited a highly variable, possibly bi-modal distribution where half the males transited quickly but about half took longer than any female. cFos expression exhibited an analogous pattern in the olfactory cortex and when collapsing across all brain regions, where females in the PEA group (and only the PEA group) exhibited significantly higher cFos expression than the males despite their shorter PEA exposure times. Some other brain regions exhibited PEA-specific effects, such as significantly elevated expression of cFos in the fear-related central nucleus of the amygdala (regardless of sex).

The PORT task was designed to explicitly place the subject in conflict between the desire to obtain the reward and the desire to avoid the predator odor (Dent et al., 2014). The present study, which used more than four times as many subjects, reports a more complex dataset that challenges this interpretation. First, the Dent et al. study found no significant effect of test trial number (p=0.10, mice from two very different strains pooled) and thus averaged all their data across trials. We observed diverse behavior on test trial 1, but by trial 3 almost all mice quickly transited the middle chamber for a reward and all effects of odor type or sex had vanished. We conclude that the task includes an implicit learning element as the animal habituates to the novel odor and/or learns not to be afraid. Second, like the Dent et al. study, we observed that mice took significantly longer to enter the PEA-scented middle chamber compared to the MVscented or unscented chamber on trial 1. However, this accounted for only a few seconds before the mice entered the middle arena, where mice spent most of their time. For both the PEA and MV groups, mice spent more time in the middle chamber than in the clean bedding condition. This seemingly contradicts the expected result that animals would seek to avoid PEA by minimizing their exposure to it, even though the elevated central and amygdalar cFos expression suggests that PEA exposure was more anxiogenic than MV or clean bedding. It may be that in this experimental context PEA evokes exploratory behavior as a form of threat assessment (Blanchard et al., 1990) that is similar to the novel odor investigation presumably evoked by MV.

Behavior on trial 1 exhibited a robust sex difference in the PEA group, where all females transited the PEA-scented bedding quickly but many males spent long periods of time in the middle chamber (Fig. 1B,E,H). This contrasts with the response to MV, where females and males both sometimes exhibited long exploration times. This may parallel the findings in the dorsomedial hypothalamus, where PEA evoked a large increase in cFos expression in females that was absent (or opposite) in males and did not occur with MV (Fig. 6B. The Dent et al. study only used males, but previous work by Perrot-Sinal et al. (1996) suggests that female rodents may engage in fewer threat assessment behaviors. This difference in response to predator threat could thus reflect sex differences in riskreward decision-making. Moreover, male mice were more likely to exhibit thigmotaxis and immobility in the middle chamber than female mice were, regardless of odor condition, which suggests a potential interaction of PEA with an underlying difference in anxiety-related behaviors between the sexes.

We observed significant sex by condition interactions for cFos expression within piriform cortex, which is the primary olfactory cortex, receiving projections from the olfactory bulb (Schoenbaum & Eichenbaum, 1995). The piriform cortex has also been found to be active in response to stress (Abraham & Kovacs, 2000). We observed an interesting effect where females displayed lower levels of cFos expression even in the CB condition. However, when exposed to the novel MV odor, both females and males had similar numbers of cFos positive cells in the piriform. Exposure to PEA resulted in greater cFos activity within piriform in females compared to males. This was the only olfactory-related region that displayed disparities between males and females in the PEA condition, though it should be noted that our exposures were notably brief (typically under two minutes exposure time) compared to the 30-60 minutes used in a typical odordriven cFos study (Salcedo, Zhang, Kronberg, & Restrepo, 2005). It may be important that this study used PEA, a monomolecular odorant that selectively activates the TAAR4 receptor, rather than whole predator urine that would activate many receptors at once. Given our previous report of differences in peripheral sensory responses to odors in male and female mice (Kass, Guang, Moberly, & McGann, 2016), it is conceivable that males

and females selectively differ in their sensory sensitivity to this odorant, though this has not been observed in previous studies (Dewan et al., 2018) and is not supported by the latency to enter the middle data. Nonetheless, given the larger cFos response to PEA in piriform cortex, it will be important to confirm that females are equally sensitive to PEA.

As noted above, we also found that females showed elevated levels of cFos in the DM hypothalamus in the PEA condition compared to the CB or MV conditions, while males exhibited a decrease in cFos positive cells compared to the CB and MV conditions. This result is quite interesting because DM hypothalamus is involved in regulating emotional responses such as arousal, attention, motivation, and the innate defensive response (Freitas, Uribe-Marino, Castiblanco-Urbina, Elias-Filho, & Coimbra, 2009; Staubli, Schottler, & Nejat-Bina, 1987) as well as the representation of thirst and fear (Sewards & Sewards, 2003). The dramatic differential activation of DM hypothalamus in females and males in the PEA group could be considered a correlate of the animal's decision to "ignore the threat and go for water."

Our results show that females exhibit less cFos positive cells than males in the PVT only in the CB condition. The PVT has been found to be activated by both unconditioned and conditioned fearful stimuli (Sewards & Sewards, 2003) and projects to both DM hypothalamus and piriform cortex. It is uncertain why females and males would differ in the CB condition, but not when odors are present. Females had significantly more BLA activity than males regardless of condition. The BLA is suggested to be involved in emotional processing, and is implicated in both the fear response and appetitive processing which make it difficult to interpret its particular role in the current

paradigm where both a predator cue and reinforcer are presented (Parkinson, Robbins, & Everitt, 2000).

It is tempting to speculate that females' reduced latency to reward may be indicative of a more motivated state for the sucrose. However, females show increased cFos immunoreactivity in the BLA, DM hypothalamus, and piriform cortex, all regions that are involved in the stress and fear response. Therefore, the most parsimonious explanation is that females exhibit a heightened level of fear that may result in a different defensive strategy than males.

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## FIGURE LEGENDS

Figure 1. Trial 1 Behavioral Analysis. **A-C**. Trial 1 latencies to attain reward during the PM session by condition and by condition by sex, respectively, (C) displays individual results **D-F**. Trial 1 latencies during the PM session to enter middle chamber by condition and by condition by sex, respectively, (F) displays individual results **G-I**. Time spent in the periphery of the middle chamber during trial 1 of the PM session by condition and by condition by sex, respectively (I) displays individual results **J**. Heatmap representation of mouse route through apparatus to attain reward. Figure shows configuration for mice traversing left to right. Conditions from top to bottom: CB, MV, PEA; males on the left, females on the right.

Figure 2. Trial 3 Behavioral Analysis. **A-C**. Trial 3 latencies to attain reward during the PM session by condition and by condition by sex, respectively, (C) displays individual results **D-F**. Trial 3 latencies during the PM session to enter middle chamber by condition and by condition by sex, respectively, (F) displays individual results **G-I**. Time spent in the periphery of the middle chamber during trial 3 of the PM session by condition and by condition by sex, respectively (I) displays individual results **J**. Heatmap representation of mouse route through apparatus to attain reward. Figure shows configuration for mice traversing left to right. Conditions from top to bottom: CB, MV, PEA; males on the left, females on the right. The animals corresponding with these maps are the same animals that were used for **Figure #J**.

Figure 3. Average cFos positive cells collapsed across brain regions by condition and sex. This graph represents the average total number of cFos positive cells collapsed across all brain regions quantified.

Figure 4. cFos positive cells in olfactory-related regions. **A-C.** cFos counts for OB, first collapsed across sex (A), then by condition and sex (B) and individual results (C). **D-F.** cFos counts for piriform cortex, first collapsed across sex (D), then by condition and sex (E) and individual results (F). **G-I.** cFos counts for AON, first collapsed across sex (G), then by condition and sex (H) and individual results (I).

Figure 5. cFos positive cells in PVT. **A-C.** cFos counts for PVT, first collapsed across sex (A), then by condition and sex (B) and individual results (C).

Figure 6. cFos positive cells in hypothalamic nuclei. **A-C.** cFos counts for dorsomedial hypothalamus, first collapsed across sex (A), then by condition and sex (B) and individual results (C). **D-F.** cFos counts for ventromedial hypothalamus, first collapsed across sex (A), then by condition and sex (B) and individual results

Figure 7. cFos positive cells in olfactory-related amygdala. **A-C.** cFos counts for CoA, first collapsed across sex (A), then by condition and sex (B) and individual results (C). **D-F.** cFos counts for BMA, first collapsed across sex (D), then by condition and sex (E) and individual results (F). **G-I.** cFos counts for MeA, first collapsed across sex (G), then by condition and sex (H) and individual results (I).

Figure 8. cFos positive cells in fear-related amygdala. **A-C.** cFos counts for LAT, first collapsed across sex (A), then by condition and sex (B) and individual results (C). **D-F.** cFos counts for CeA, first collapsed across sex (D), then by condition and sex (E) and individual results (F). **G-I.** cFos counts for BLA, first collapsed across sex (G), then by condition and sex (H) and individual results (I).

Figure 9. cFos positive cells in LSN. **A-C.** cFos counts for LSN, first collapsed across sex (A), then by condition and sex (B) and individual results (C).

Figure 10. Correlations of behavior with cFos positive cells. A-C. Correlations of AON
(A), LSN (B), and BLA (C) to latency to reward. D-G. Correlations of OB (D), piriform
(E), PVT (F), and LSN (G) to latency to middle chamber. Colors and shapes denote different experimental conditions.



Figure 1. Trial 1 behavioral analysis.



Figure 2. Trial three behavioral analysis.



Figure 3. Average cFos positive cells collapsed across brain regions by condition and sex.



Figure 4. cFos positive cells in olfactory-related regions.



Figure 5. cFos positive cells in paraventricular thalamic nucleus.



# Figure 6. cFos positive cells in dorsomedial and ventromedial hypothalamus.



Figure 7. cFos positive cells in olfactory-related amygdalar nuclei.



Figure 8. cFos positive cells in fear-related amygdalar nuclei.



Figure 9. cFos positive cells in lateral septal nuclei.

