

## RESEARCH ARTICLE

# Aversive memory conditioning induces fluoxetine-dependent anxiety-like states in the crab *Neohelice granulata*

Francisco Javier Maza<sup>1,\*</sup>, Francisco José Urbano<sup>1,2</sup> and Alejandro Delorenzi<sup>1,2,\*</sup>**ABSTRACT**

The interactions between memory processes and emotions are complex. Our previous investigations in the crab *Neohelice* led to an adaptation of the affective extension of sometimes opponent processes (AESOP) model. The model proposes that emotions generate separate emotive memory traces, and that the unfolding of emotional responses is a crucial component of the behavioral expression of reactivated memories. Here, we show that an aversive conditioning, that used changes in an innate escape response to an aversive visual stimulus, induced an emotional behavior that endured beyond the stimuli: the aversive memory training built an anxiety-like state evaluated in a dark/light plus-maze. We found that, after the training session, crabs displayed aversion to maze light areas, and an increased time immobilized in the dark zones of the maze, an anxiety-like behavior induced by stressors or physiological conditions in other crustaceans. The training-dependent anxiety-like behavior was blocked by pretraining administration of fluoxetine, suggesting an underlying serotonin-dependent phenomenon. We hypothesize that this training-induced anxiety-like state generates a separate emotive memory trace that is reinstated and crucial for the modulation of memory expression once the memory is reactivated.

**KEY WORDS:** Memory expression, Emotional state, Crustacea, Consolidation, Fluoxetine, Serotonin

**INTRODUCTION**

The bases of the neurobiological mechanisms and the actions of neuromodulators underlying the impact of emotional experiences on memory appear to be well conserved across evolution. However, the interaction between memory processes and emotions is a complex, multifaceted phenomenon that is not yet fully understood. Arousing experiences have a central role in the various models that have been used to interpret the neurobiological basis of memory modulation. The canonical model proposed that the lability periods of memory provide the opportunity for endogenous stress hormones activated by emotional experiences to modulate the strength of memory (McGaugh, 2000, 2013). Whether and why emotions enhance or impair memory at different stages, the various types of memory, and the difficulty of distinguishing the effects of emotions


from other factors that can influence memory, such as attention and contexts, are still open questions (Lukowiak et al., 2014; Sandi, 2013). Furthermore, there are numerous controversies surrounding the bases of emotion–memory interactions. Findings from reconsolidation studies have highlighted these controversies. Different models have been proposed, but none of them fully address the integration of the different outputs of emotion and memory modulation studies (Agren, 2014; Schroyens et al., 2022; Wolf, 2019). Based on previous models (Gisquet-Verrier and Riccio, 2018; Johansen et al., 2014; Nader, 2009; Osorio-Gómez et al., 2017, 2019; Sierra et al., 2013), and notably on the affective extension of sometimes opponent processes (AESOP) model (Vogel et al., 2019), our current working hypothesis posits that emotions generate separate emotive memory components (Vogel et al., 2019), and that the unfolding of emotional responses is a crucial component of the behavioral expression of reactivated memories (Delorenzi et al., 2014; Larrosa et al., 2017; Maza et al., 2016a, 2022; Sánchez Beisel et al., 2022).

Emotional responses have both behavioral and physiological components that have been studied in various non-human animals, including Arthropoda (Anderson and Adolphs, 2014; Baracchi et al., 2017; Crump et al., 2022; Perry and Baciadonna, 2017). These behaviors outlast the stimuli that elicit them, distinguishing them from simple stimulus–response reflexes (Anderson and Adolphs, 2014). Anxiety is a longer-lasting state of apprehension and heightened vigilance in response to uncertain or unpredictable threats. This state can persist in new contexts even in the absence of the aversive stimuli (Davis et al., 2010; Perrot-Minnot et al., 2017). Complex anxiety behaviors that involve various neuromodulators, such as serotonin, appear to be present across animal phyla. Studies on crayfish have indicated that electric shocks, social stress, exposure to novel environments and molting induce anxiety-like behaviors that can be measured using an aquatic dark/light plus-maze (Bacqué-Cazenave et al., 2017, 2019; Fossat et al., 2014, 2015). In *Neohelice granulata*, a semi-terrestrial crab, various environmental challenges, including water deprivation, can increase expression of the stress protein HSP70 in several brain areas (Frenkel et al., 2008, 2012). Cognitive and physiological mechanisms through which stressors modulate memory processes have been studied in this crab, particularly the role of angiotensin neuropeptides (Barreiro et al., 2013; Caffaro et al., 2012; Delorenzi, 1999; Delorenzi et al., 1995, 2000, 2014; Farhadi et al., 2022; Frenkel et al., 2002, 2005a,b, 2010a, 2012; Gonzalez et al., 2020; Hermitte and Maldonado, 1992; Kaczer et al., 2007, 2011; Klappenbach et al., 2017; Salzet et al., 2001; Santos et al., 2021).

Memory is formed in *N. granulata* through the association of a specific training context with a visual danger stimulus (VDS) resembling an aerial predator. Training comprising 15 trials leads to a decrease in the crab's escape response and an increase in its freezing response. This protocol generates an associative memory that can last up to 5 days and depends on the context in which

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memory was acquired, as well as on the activity of several biochemical pathways involved in long-term memory formation across evolution. Studies at behavioral, anatomical and cellular levels have provided a comprehensive understanding of the various phases of contextual-associative memory in *N. granulata*, including acquisition, consolidation, extinction, retrieval, reconsolidation and memory expression (de la Fuente et al., 2015; Federman et al., 2014; Klappenbach et al., 2017; Maldonado, 2002; Maza et al., 2016b, 2022; Merlo et al., 2020; Ojea Ramos et al., 2021; Tomsic and Romano, 2013). The generation of emotion-like states induced by mnemonic processes remains to be shown in crustaceans (Crump et al., 2022), but the *N. granulata* associative memory paradigm provides the basis for these studies.

Here, we used the *N. granulata* aversive memory paradigm to test the induction of anxiety-like emotive states by the acquisition of an associative visual memory. Methods to evaluate these states employed across taxa include the evaluation of how an innate behavior changes when the subject is exposed to unfamiliar aversive places or threats (Anderson and Adolphs, 2014; de Abreu et al., 2020; de Waal and Andrews, 2022; Perry and Baciadonna, 2017). Here, we combined the aquatic dark/light plus-maze approach (Fossat et al., 2014) and the acquisition of an associative visual-aversive memory (Fustiñana et al., 2013; Tomsic and Romano, 2013). We found that in *N. granulata*, aversive training induced an anxiety-like state that is blocked by the serotonin reuptake inhibitor fluoxetine.

## MATERIALS AND METHODS

### Animals

Intermolt adult male crabs of the species *Neohelice granulata* (Dana 1851) measuring between 2.7 and 3.0 cm across the carapace (average mass 17 g) were collected from the narrow coastal inlets of San Clemente del Tuyú, Buenos Aires Province, Argentina. In the laboratory, crabs were kept on a natural light–dark cycle, in collective plastic tanks (20 animals each) filled to 2 cm depth with brackish water prepared with 1% (m/m) Coral Pro Salt (Red Sea, Israel), pH 7.4–7.6. The holding and experimental rooms were kept at 22–24°C. Experiments were carried out during the daytime (09:00 h–16:00 h), between 3 and 15 days after the crabs had arrived at the laboratory. Between 48 and 24 h before experiments, crabs were fed for 1 h with food sticks (PROCHIN, Molinero, Suárez y Cía, S.A, Argentina). All efforts were made to minimize the number of animals. The research was conducted in accordance with the Ethical Reference Frame for Biomedical Investigations of CONICET, equivalent to the standard procedures for animal care and use of the NIH of the USA.

### Dark/light plus-maze

The maze used in this work was adapted from Fossat et al. (2014). It consisted of a cross-shaped clear acrylic container (60×60 cm, arm width 10 cm, wall height 15.5 cm; Fig. 1A). Two of the arms had their walls covered from the outside with black adhesive paper. LEDs were arranged on the outer side of the dark arms. Throughout the experiment, the maze was filled with brackish water to about 0.8 cm depth. Each crab was first placed in the center of the maze and confined for 1 min by covering it with a small opaque chamber. After this delay, the crab was released, and exploratory behavior was video recorded from above with a cellphone (480×720 pixels, 30 frames s<sup>-1</sup>, 3GP format).

### *Neohelice granulata* visual aversive training

The experimental training arena, the actometer (Maldonado, 2002; Tomsic and Romano, 2013), consisted of a bowl-shaped opaque

container with a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter) filled with 50 ml of fresh brackish water where the crab was lodged (Fig. 2A–C). The visual stimulus (VDS) consisted of a black opaque rectangle (25×7.5 cm) that moved horizontally 12 cm over the actometer in a 90 deg clockwise and counterclockwise excursion from the starting position and back (~2.2 s) (Tomsic and Romano, 2013). Each trial was composed of two cycles separated by 2 s. In the training protocol used here, originally termed contextual Pavlovian conditioning, phasic presentation of the training context was achieved by changing background lights in the setup (Fustiñana et al., 2013; Perez-Cuesta and Maldonado, 2009). Training context is considered active when the setup illumination changes by turning off the light below and turning on a light that illuminates from above. This paradigm is interpreted as a contextual conditioning phenomenon; indeed, crabs have been described to recognize the two illumination settings as different contexts (Fustiñana et al., 2013; Perez-Cuesta and Maldonado, 2009). In addition, the above illumination setting does not play a role as a cue with the VDS (Fustiñana et al., 2013).

While the training context can be presented alone (Ctx; Fig. 2B, top), the VDS trial requires the concomitant context to be evaluated (Ctx+VDS) (Fig. 2B, bottom); thus, each VDS trial comprised two movement cycles separated by 2 s at the end of 27 s of active training context.

Trained animals received 15 VDS trials (i.e. Ctx+VDS) (interstimulus interval 180 s) after a 10 min acclimation period in the arena. Untrained animals received the same treatment but without the VDS (i.e. 15 Ctx spaced trials). Activity during each trial was recorded by a camera placed between the VDS and the crab.

### Fluoxetine systemic administration

The serotonin reuptake inhibitor fluoxetine (FLX; cat. no. PHR1394, Sigma-Aldrich, St Louis, MO, USA) was administered at a final dose of 0.52 µg per crab. Injections were carried out immediately before training sessions. Fluoxetine was dissolved in 50 µl of saline solution (450 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> KCl, 15 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 10 mmol l<sup>-1</sup> MgCl<sub>2</sub>) and administered through the right side of the dorsal cephalothoracic-abdominal membrane, using a syringe needle covered with a plastic sleeve to restrict the depth of penetration to a maximum of 4 mm. This injection method has been previously used in *N. granulata* to ensure administration of drugs into crab's pericardial sac with minimum damage (Barreiro et al., 2013; Fustiñana et al., 2013; Gonzalez et al., 2020; Maza et al., 2016a).

### Data analysis

In the training arena, we visually classified the behavior of the animals as escape (when the animals showed an intense response to avoid the VDS), freezing or walking (when animals continuously explored the arena). In the dark/light plus-maze, we tracked the movements of the animals using Kinovea v0.9.5 software (www.kinovea.org). x–y coordinates from tracking were analyzed in Excel (Microsoft) and time spent in the light or dark arms and in the middle of the maze was computed. To be included in the analysis, the crabs had to meet the following criteria: (1) in the training arena, trained crabs had to display an escape response in the first trial and freezing responses in the last trial of training, thus disclosing a VDS training effect; crabs not exposed to the VDS had to display a walking response at the time of the last trial (see Movie 1 for examples); (2) in the dark/light plus-maze, crabs had to explore both light and dark zones (i.e. spend a total of at least 30 s in each zone during the 10 min evaluation period). Table 1 summarizes the final number of included/discarded crabs during this study.

**Table 1. Experimental groups and final number of crabs after exclusion criteria**

Group	Total no.	Final no.	No. excluded	
			Training arena session	Dark/light plus-maze*
Naive	19	15	–	4
Untrained	18	12	6 freezing at last trial	–
Trained	17	11	1 walking response at last trial 1 escape response at last trial 1 no escape response at first VDS trial	3
Vehicle	13	10	1 freezing at last trial	2
FLX	14	11	1 freezing at last trial	2
Trained-vehicle	21	16	1 no escape response at first VDS trial	4
Trained-FLX	30	15	8 no escape response at first VDS trial	7

Total number of crabs and final number after exclusion criteria are shown. \*Crabs were excluded if they did not explore the light and dark zones in the maze. FLX, fluoxetine.

We computed various measures, including the mean duration per visit in the light and dark arms, latency to first entry into the light and dark arms (in s), time spent in the light as a percentage of total time, total distance traveled (in cm) and time spent immobile in the dark zone (in s), which resulted from the sum of the periods of 15 frames (0.5 s) where the crab's explored distance was less than 1.5 mm.

### Statistics

Statistical comparisons were done in Python using the Pingouin package (Vallat, 2018). Custom scripts can be obtained from the corresponding author upon request. Paired Student's *t*-tests were used for within-group comparisons. Unpaired Student's *t*-tests with Welch's correction were used to compare between two groups. A value of  $P < 0.05$  was considered significant. In addition, effect sizes and confidence intervals (mean differences and 95% confidence intervals, CI) were calculated using bootstrap-coupled estimation statistics (www.estimationstats.com), a statistical method that is less dependent on distribution assumptions (Cumming, 2014; Ho et al., 2019).

Principal component analysis (PCA) was performed using Python's scikit-learn package. The separation between pairs of groups was evaluated by calculating the ratio of the between-group variance to the global variance (Fossat et al., 2014, 2015). We estimated the statistical significance of the ratio for group separation using a permutation test (5000 runs). *P*-value was considered as the

proportion of cases from the permuted groups where the ratio was higher than the actual ratio.

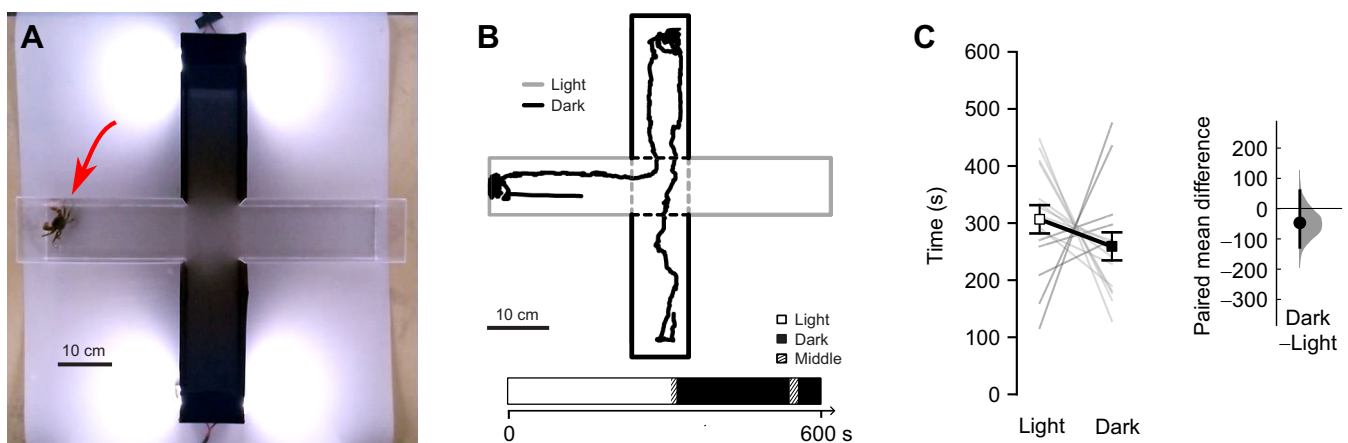
## RESULTS

### Crab exploration in a dark/light plus-maze

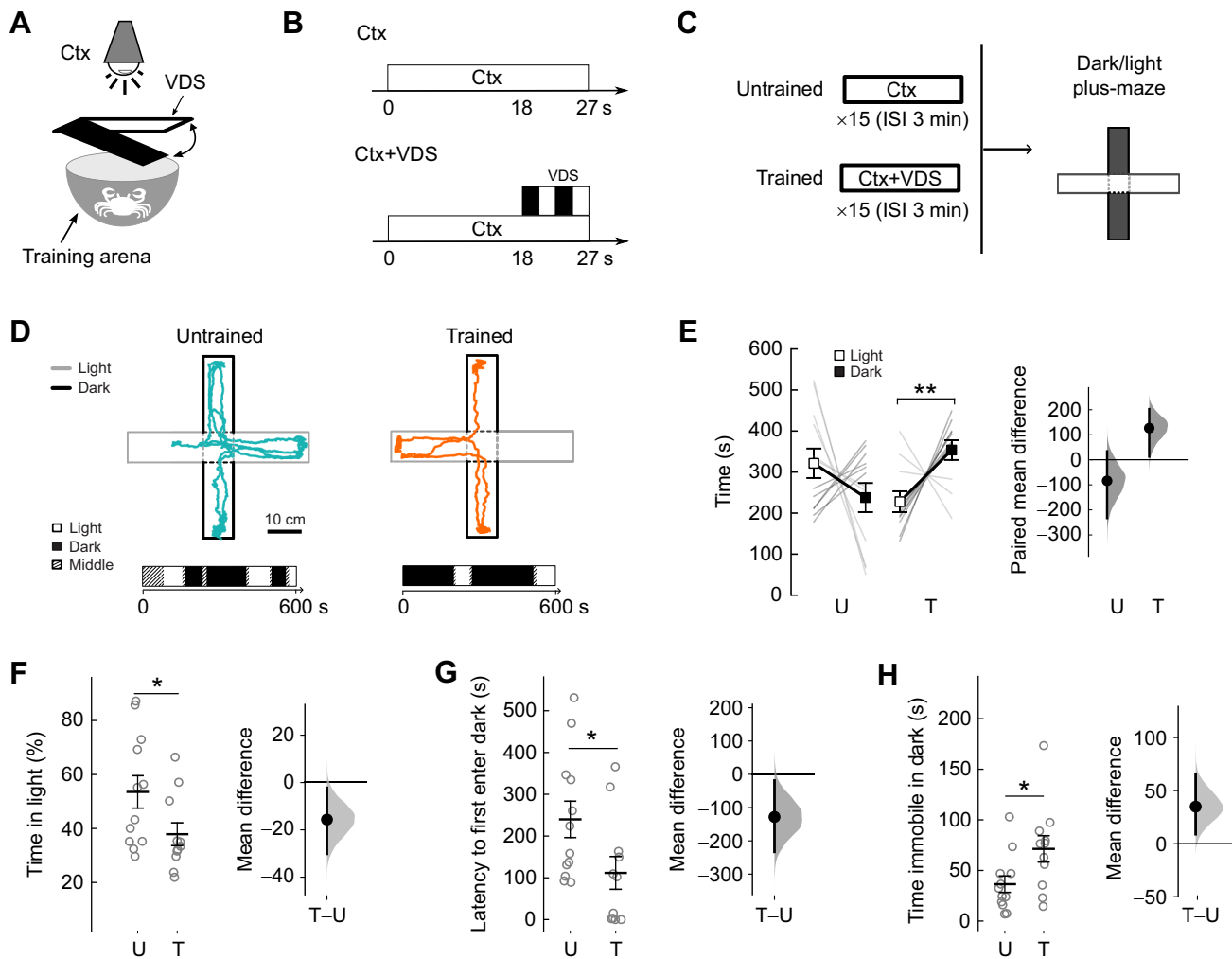
To evaluate the basal behavior of crabs in the dark/light plus-maze and test for possible preferences for dark or light zones, we commenced evaluating the maze exploration of naive crabs taken directly from collective tanks (Fig. 1). After 10 min of isolation in a tank, crabs were placed in the maze (Fig. 1A). Fig. 1B shows an example of a naive crab's tracked path during the 10 min evaluation. We found no preference for the time spent in the dark or light arms of the plus-maze (two-tailed paired *t*-test,  $t_{14} = 0.98$ ,  $P = 0.34$ ; Fig. 1C, left). The paired mean difference (Fig. 1C, right) calculated using bootstrap-coupled estimation statistics between time spent in the light and time spent in the dark was  $-47.4$  (95.0% CI:  $-126, 57.3$ ).

### Aversive training and its effects during the exploration of the dark/light plus-maze

We next evaluated whether visual aversive training with a training known to elicit short- and long-term memory has effects on the crab's behavior in the dark/light plus-maze tested immediately after training, indicative of a change in the crab's emotional state. Two groups of crabs were evaluated (Fig. 2): trained crabs that received 15 VDS trials (interstimulus interval, ISI 3 min) and untrained crabs



**Fig. 1. Dark/light plus-maze.** (A) Overview of the maze with a crab in it (arrow). (B) Example of a crab's tracked path. Below is a summary of maze exploration during the 10 min (600 s) evaluation. (C) Left, time spent in the light and dark zones for naive crabs ( $n = 15$ ). Each gray line corresponds to a crab, the black line to the mean and error bars to the s.e.m. Two-tailed paired *t*-test,  $P > 0.05$ . Right, effect size (mean difference between dark and light zone exploration time) calculated using bootstrap-coupled estimation statistics. Curve shows the resampled distribution of mean differences and error bars show 95% CI.



**Fig. 2. Visual danger stimulus training and anxiety-like state in the dark/light plus-maze.** (A) Schematic diagram of the training arena and the visual danger stimulus (VDS). A crab is inside the actometer, which is illuminated from below. Phasic presentation of the training context is done by a change in setup illumination from above (Ctx). The VDS comprises a black rectangular panel moving above the horizon of the crab. (B) Ctx and VDS trials scheme. Ctx presentation involves a phasic change in setup illumination over 27 s. The VDS is presented during the last 9 s of the Ctx presentation, moving back and forth twice in each trial (black bars). (C) Experimental groups. Crabs were evaluated in the dark/light plus-maze immediately after taking them from the training arena. ISI, interstimulus interval. (D) Examples of crabs' tracked paths (untrained, Ctx only; trained, Ctx+VDS). Below is a summary of maze exploration during the 10 min (600 s) evaluation. (E) Left, time spent in light and dark zones by untrained (U) and trained (T) crabs. Each gray line corresponds to a crab, the black line to the mean and error bars to the s.e.m. One-tailed paired *t*-test, \*\* $P < 0.01$ . Right, effect size (mean difference between dark and light zone exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars show 95% confidence interval (CI). (F–H) Time spent in the light (F), latency to first enter the dark (G) and time spent immobile in the dark (H). Untrained, trained:  $n = 12, 11$ . Error bars over dot plots represent means  $\pm$  s.e.m. One-tailed Welch *t*-test, \* $P < 0.05$ .

that were exposed to the same 15 trials of training context presentation but without the VDS. Immediately after the experimental manipulation, we evaluated their behavior in the dark/light plus-maze. We hypothesized that the aversive nature of the VDS training would promote avoidance of the light zones, reflecting an anxiety-like state similar to that observed in crayfish (Fossat et al., 2014, 2015). The data showed that trained crabs spent less time in the light zone than in the dark zone (Fig. 2E; paired *t*-test light less than dark, trained  $t_{10} = -2.55$ ,  $P = 0.014$ ; paired mean difference 126.45 s, 95% CI: 16.30, 202.00). Untrained crabs did not spend less time in the light than in the dark zones (paired *t*-test light less than dark, untrained  $t_{11} = 1.18$ ,  $P = 0.869$ ; paired mean difference  $-84.02$  s, 95% CI:  $-229.81, 33.24$ ). Trained crabs spent a lower percentage of time in the light than untrained crabs (one-tailed Welch *t*-test,  $t_{19.35} = -2.13$ ,  $P = 0.023$ ; unpaired mean

difference  $-15.66$  s, 95% CI:  $-30.12, -2.25$ ; Fig. 2F). Light avoidance was also reflected in a shorter time to enter a dark arm for the trained versus untrained crabs ( $t_{20.93} = -2.19$ ,  $P = 0.02$ ; unpaired mean difference  $-128.21$  s, 95% CI:  $-232.98, -18.76$ ; Fig. 2G). Trained crabs also spent more time immobilized in the dark ( $t_{16.97} = 2.27$ ,  $P = 0.018$ ; unpaired mean difference 34.88 s, 95% CI: 8.90, 65.86; Fig. 2H).

#### Effect of acute fluoxetine in the anxiety-like state induced by training

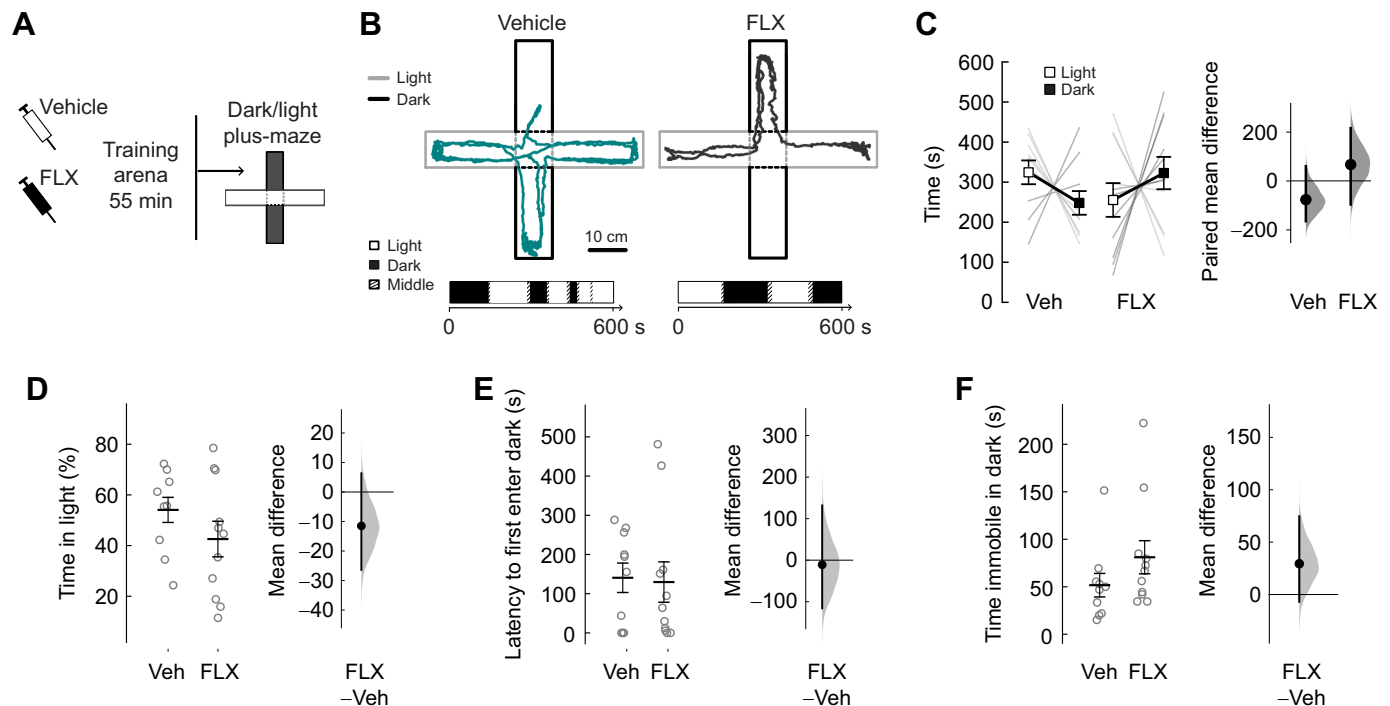
We next wanted to test whether fluoxetine, a selective serotonin reuptake inhibitor (SSRI) that is commonly used in the treatment of depression and anxiety disorders in humans, affected the anxiety-like state induced by the training session. Acute fluoxetine treatment has been shown to have anxiolytic-like effects in the crab

*Pachygrapsus crassipes* (Hamilton et al., 2016), and to both induce an increase in glucose levels (Santos et al., 2001) and interfere with long-term memory in *N. granulata* crabs (Pedetta and Maldonado, 2008, 2009). To test whether fluoxetine altered the crab's activity on the dark/light plus-maze per se, crabs were injected with either vehicle or fluoxetine (0.52 µg per crab) and then isolated in the actometer with light above the bowl for 55 min, i.e. continuously exposed to the same illumination conditions as for the training context (this fluoxetine dose has been shown to increase glucose levels in *N. granulata*; Santos et al., 2001). They were then immediately tested in the dark/light plus-maze (Fig. 3). Crabs injected with fluoxetine showed no difference in the time spent in dark/light zones (Fig. 3C; two-tailed paired *t*-test, vehicle  $t_9=1.29$ ,  $P=0.23$ , paired mean difference 76.34 s, 95% CI: -164.66, 60.42; FLX  $t_{10}=-0.82$ ,  $P=0.43$ , paired mean difference 67.21 s, 95% CI: -96.15, 216.09). They also did not show differences from vehicle-treated crabs in the percentage of time spent in the light (Fig. 3D; two-tailed Welch *t*-test,  $t_{17.58}=-1.34$ ,  $P=0.197$ , unpaired mean difference -11.50%, 95% CI: -26.41, 6.33), the latency to enter the dark arms (Fig. 3E;  $t_{17.85}=-0.17$ ,  $P=0.86$ , unpaired mean difference -10.92 s, 95% CI: -115.88, 131.68) or the time spent immobilized in the dark (Fig. 3F;  $t_{17.65}=1.37$ ,  $P=0.186$ , unpaired mean difference 29.40 s, 95% CI: -6.88, 74.62).

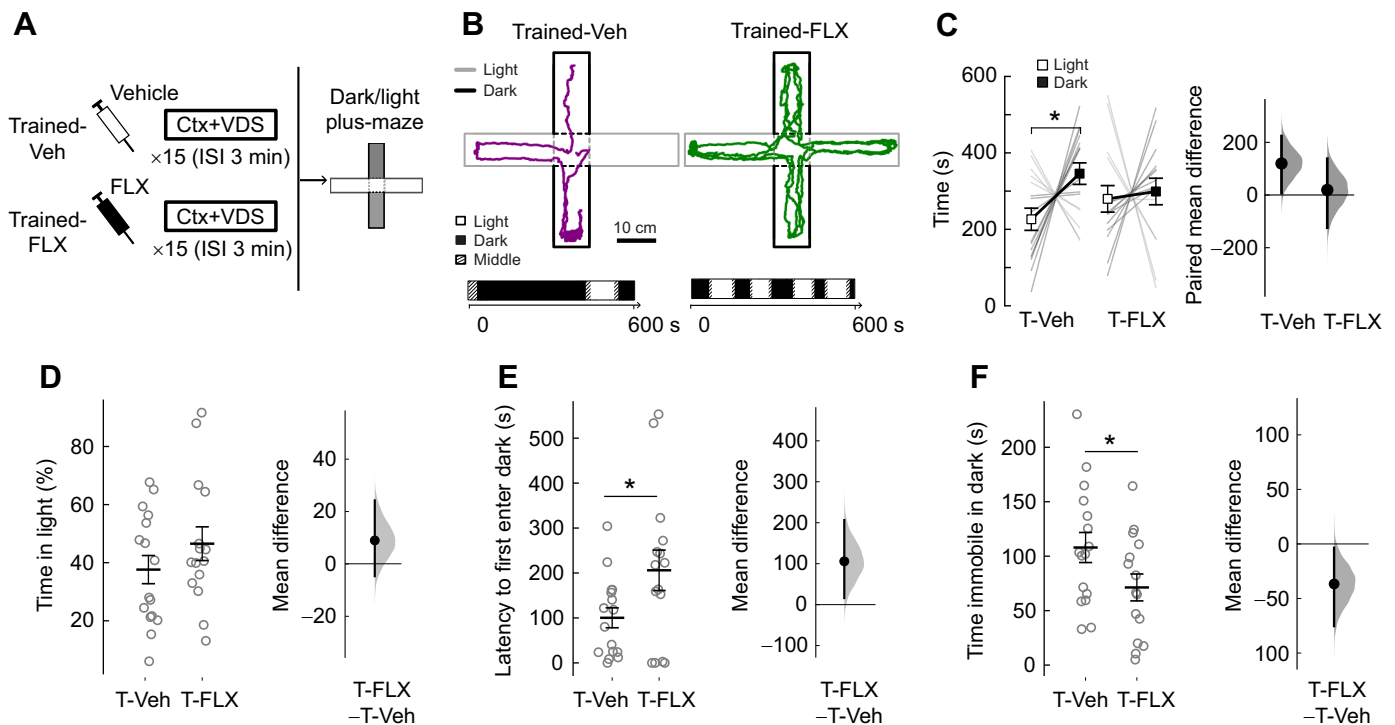
When applied concomitantly with crab training, acute fluoxetine blocked the anxiety-like state induced by VDS training (Fig. 4). Trained crabs that were treated with vehicle immediately pre-training spent less time in the light zone than in the dark zone (paired *t*-test, light less than dark, trained-vehicle  $t_{15}=-2.09$ ,  $P=0.027$ , paired mean difference 119.74 s, 95% CI: 7.68,

223.97). However, when crabs were treated with fluoxetine, no difference was found between time spent in the dark and the light zones (trained-FLX  $t_{14}=-0.28$ ,  $P=0.392$ , paired mean difference 19.32 s, 95% CI: -123.95, 138.16; Fig. 4C). Although not statistically significant, trained-vehicle crabs displayed a lower percentage of time in the light than did trained-FLX crabs (one-tailed Welch *t*-test,  $t_{27.74}=1.18$ ,  $P=0.124$ , unpaired mean difference 8.93%, 95% CI: -4.72, 24.20; Fig. 4D). Light avoidance was significantly reduced for the trained-FLX crabs as they showed a longer time to enter a dark arm compared with the trained-vehicle crabs ( $t_{20.49}=-2.11$ ,  $P=0.023$ , unpaired mean difference 105.72 s, 95% CI: 16.08, 206.43; Fig. 4E). Trained-FLX crabs also spent less time immobilized in the dark ( $t_{28.83}=-1.98$ ,  $P=0.028$ , unpaired mean difference -36.67 s, 95% CI: -75.29, -3.44; Fig. 4F).

To analyze the effects of the aversive training using the seven variables measured in all groups, a PCA was performed. This type of analysis had been useful to reveal components related to anxiety-like behavior and to compare different levels of anxiety in crayfish (Bacqué-Cazenave et al., 2017, 2019; Fossat et al., 2014, 2015). The first two components of the PCA accounted for 79% of the variance (PC1: 51%, PC2: 28%). Time in the light, latency to first enter the dark and time spent immobile in the dark were the variables that had more contribution to variance in the first component (PC1), which can be interpreted as the component related to 'anxiety', with anxiety increasing to positive values (Fig. 5A). The second component mainly described the crab's locomotion, as its major contribution came from the variable distance (Fig. 5A). Trained animals were the groups most separated to the right in the 'anxiety' component, indicating higher levels of anxiety than the other groups



**Fig. 3. Acute fluoxetine treatment.** (A) Crabs injected with fluoxetine (FLX, 0.52 µg per crab) or vehicle were isolated in the training arena for 55 min. Immediately after, we evaluated their behavior in the dark/light plus-maze. (B) Examples of crabs' tracked paths. Below is a summary of maze exploration during the 10 min (600 s) evaluation. (C) Left, time spent in the light and dark zones. Each gray line corresponds to a crab, the black line to the mean and error bars to the s.e.m. One-tailed paired *t*-test,  $P>0.05$ . Right, effect size (mean difference between dark and light zone exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars show 95% CI. (D–F) Time spent in the light (D), latency to first enter the dark (E) and time spent immobile in the dark (F). Vehicle, FLX:  $n=10$ , 11. Error bars over dot plots represent means  $\pm$  s.e.m. One-tailed Welch *t*-tests,  $P>0.05$ .



**Fig. 4. Acute fluoxetine treatment and anxiety-like behavior.** (A) Crabs injected with fluoxetine (FLX, 0.52  $\mu\text{g}$  per crab) or vehicle were trained with the VDS. Immediately after, we evaluated their behavior in the dark/light plus-maze. (B) Examples of crabs' tracked paths. Below is a summary of maze exploration during the 10 min (600 s) evaluation. (C) Left, time spent in the light and dark zones. Each gray line corresponds to a crab, the black line to the mean and error bars to the s.e.m. One-tailed paired  $t$ -test,  $*P < 0.05$ . Right, effect size (mean difference between dark and light zone exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars show 95% confidence intervals. (D–F) Time spent in the light (D), latency to first enter the dark (E) and time spent immobile in the dark (F). Trained-vehicle, trained-FLX:  $n = 16, 15$ . Error bars over dot plots represent means  $\pm$  s.e.m. One-tailed Welch  $t$ -test,  $*P < 0.05$ .

(Fig. 5B). When evaluating group separation, trained and trained-vehicle crabs were the only groups separated from the untrained group (ratio of the between-group variance to the global variance, permutation tests untrained versus trained and untrained versus trained-vehicle:  $P < 0.05$ ; untrained versus all other groups  $P > 0.05$ ).

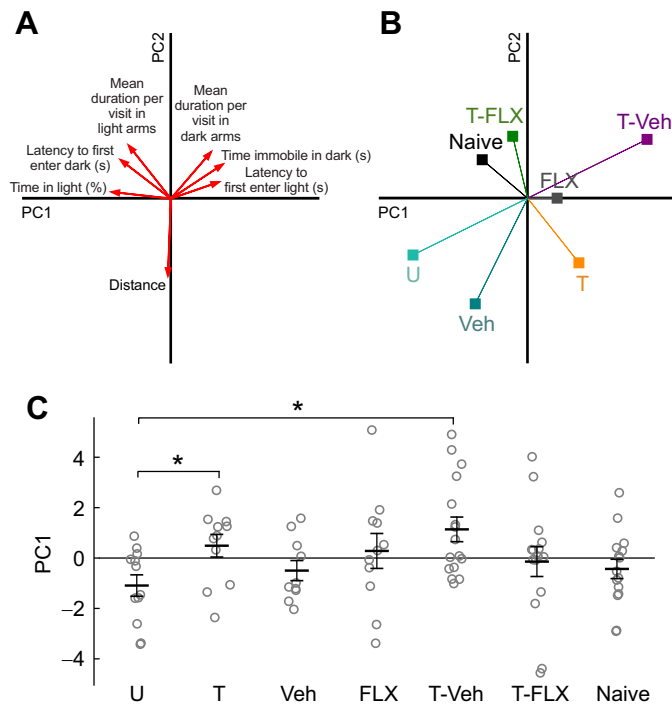
## DISCUSSION

We conducted behavioral and pharmacological assays in the Brachyura crustacean *Neohelice granulata* to investigate the emotional responses generated by a training protocol known to induce an associative long-term memory. Using anxiety-like behaviors as a measure, we employed an aquatic dark/light plus-maze to examine the induction of anxiety by a contextual Pavlovian conditioning training session. Our results suggest that the training session induced an anxiety-like state, as indicated by a decrease in time spent in the light arms, a shorter latency to enter the dark arms, and an increased time spent immobilized in the dark zones (Fig. 2). The serotonergic agent fluoxetine appears to block these behavioral responses (Fig. 4), which is difficult to explain by unspecific effects as no significant differences were observed between vehicle- and fluoxetine-treated animals continuously exposed to the same illumination conditions as the training context for 55 min (Fig. 3). PCA revealed that time in the light, latency to first enter the dark arms, and immobilization in the dark were the variables that contributed the most to the variance (Fig. 5).

The paradigm used to examine anxiety-like behaviors in this study is based on a minor adaptation of the one originally developed by Fossat and colleagues (Bacqué-Cazenave et al., 2019; Fossat et al., 2014) and confirms that the variables used to describe

anxiety-like emotions in crayfish can also be applied to Brachyura crustaceans. The relevance of these variables was also confirmed by PCA, as previously proposed (Fossat et al., 2014). The role of serotonergic neuromodulatory actions during induction of anxiety-like states has been proposed and shown to be central to the similarities between crustaceans and vertebrates (Curran and Chalasani, 2012; de Abreu et al., 2020; Fossat et al., 2015; Hamilton et al., 2016; Kravitz, 1988). Consistent with an anxiety-like state being a serotonin-dependent phenomenon, acute administration of a single dose of fluoxetine before training reduced the training-induced anxiety in the light/dark plus-maze. This result is also in line with the anxiolytic effect of acute fluoxetine previously described in other crab species, such as *Pachygrapsus crassipes*, where crabs acutely treated with fluoxetine reduced their avoidance of the light zone of a light/dark aquarium after an aggression test (Hamilton et al., 2016). In *N. granulata*, it has been shown that both serotonin and fluoxetine, at the dose used here, have a potent effect on increasing blood glucose levels (Santos et al., 2001), a suggested stress biomarker in crustaceans (Hall and van Ham, 1998; Soares et al., 2022). Additionally, fluoxetine has also been reported to impair memory consolidation in *N. granulata* in both aversive and appetitive paradigms, suggesting that serotonin may be involved in memory processes regardless of the learning paradigm (Pedetta and Maldonado, 2008, 2009).

Our present results open a path to incorporate a cognitive approach in the study of the neurobiology of emotional behaviors in crustaceans (Anderson and Adolphs, 2014; Perry and Baciadonna, 2017). Studies have shown that anxiety-like states are present across animal evolution (Perrot-Minnot et al., 2017). In arthropods,



**Fig. 5. Principal component analysis with all experimental series.**

(A) Contribution of each variable to the variance in the first and second components of the principal component analysis (PCA). Time spent in the light, latency to first enter the dark and time spent immobile in the dark were the variables that had more contribution to variance in the first component (PC1), which can be interpreted as the component related to 'anxiety'. Variance in the second component (PC2) was dominated by the walked distance, which can be interpreted as the component related with 'locomotion'. (B) Location of each group mean in the plane defined by the first and second components of the PCA. Note that trained animals (T and T-Veh) are on the right side of the PCA plane, corresponding to a higher anxiety. (C) First principal component, 'anxiety', for all experimental series groups. Each circle represents a crab. \* $P < 0.05$ , permutation tests against untrained (U) (ratio of the between-group variance to the global variance). Mean and s.e.m. are shown for each group.

cognitive studies of fear or aversive conditioning that operate with the innate fear-like response to aversive stimuli have been used to evaluate behavioral and physiological components of emotional responses in insects. Aversive stimuli, electric shocks, social stress and molting have been applied to show the induction of anxiety-like behaviors in crayfish, amphipods and crabs (Appel and Elwood, 2009; Bacqué-Cazenave et al., 2017, 2019; Fossat et al., 2014, 2015; Hamilton et al., 2016; Perrot-Minnot et al., 2017). The associative memory paradigm studied here is based on an association between the context presentation and the visual danger stimulus. During 15-trial training, a crab's escape response decreases in intensity and is progressively replaced by a freezing response, and this preference for freezing is present in testing sessions (Pereyra et al., 2000). It was assumed, as shown here in Movie 1, that crabs under contextual-Pavlovian conditioning exhibit similar changes after training (Fustiñana et al., 2013; Gonzalez et al., 2020; Merlo et al., 2020). The emotional behavior response studied here showed that the change in the innate escape response to freezing outlasts the training session, differentiating it from simple stimulus–response reflexes as it persists in the absence of the visual danger stimulus and in the presence of the new context of the light/dark plus-maze (Anderson and Adolphs, 2014; Baracchi et al., 2017; Crump et al., 2022; Perry and Baciadonna, 2017).

In *N. granulata*, weak training protocols generate consolidated memories that, although they can be reactivated in the long term and become labile, do not result in observable behavioral expression (Barreiro et al., 2013; Caffaro et al., 2012; Delorenzi et al., 2014; Frenkel et al., 2010b; Klappenbach et al., 2017; Maza et al., 2016a); with increments in the salience of the VDS, five trials (instead of 15) are sufficient to generate an associative memory (Gonzalez et al., 2020). Additional experiments investigating the relationship between the anxiety-like behavioral changes observed here and the different strengths of training sessions are needed to support the view that changes in the responses in the light/dark plus-maze evaluate behavioral components of emotional responses in *N. granulata*. As a model system, *N. granulata* provides an opportunity to investigate the neural mechanisms underlying emotional behavior in invertebrates. Future studies exploring whether mushroom body activity (Maza et al., 2016a,b, 2022) is part of the neural circuits involved in the processing of contextual information embedded in an emotional state could shed light on the neurobiological basis of these behaviors. Understanding the neural mechanisms underlying emotional behaviors in *N. granulata* may also provide new insights into the evolution of emotional processing in animals.

Our current working hypothesis posits that the emotional internal states induced during training sessions play a crucial role in determining the behavioral expression of reactivated memories during testing sessions. The results from our study show that training that induces an associative long-term aversive memory leads to a change in the crab's emotional behavior (de Waal and Andrews, 2022). The hypothesis proposes that during consolidation and reconsolidation, this internal state links with the memory trace and, at retrieval, the unfolding of this internal state will modulate the behavioral expression of the memory (Delorenzi et al., 2014; Maza et al., 2022; Sánchez Beisel et al., 2022). The results presented here open the possibility of using an experimental approach in crustaceans to further investigate the regulation of anxiety states by cognitive processes, as well as to understand how changes in internal states triggered by the reactivation of memory traces can influence the behavioral expression of reactivated aversive memories.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: F.J.M., A.D.; Methodology: F.J.M., A.D.; Formal analysis: F.J.M., A.D.; Investigation: F.J.M.; Data curation: F.J.M.; Writing - original draft: F.J.M., F.J.U., A.D.; Writing - review & editing: F.J.M., F.J.U., A.D.; Supervision: A.D.; Project administration: A.D.; Funding acquisition: F.J.U., A.D.

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#### Data availability

Data for this paper can be found within the article and its [supplementary information](#).

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**Summary:** Crab associative aversive conditioning leads to the formation of enduring emotional behavior, revealed through dark/light plus-maze evaluation, involving serotonin-dependent processes.

## Funding details

S.No.	Funder name	Funder ID	Grant ID
1	Agencia Nacional de Promoción Científica y Técnica de Argentina		PICT 2016-1875 PICT 2019-00284
2	Universidad de Buenos Aires		UBA-UBACYT 2018 20020170100119BA