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

Francisco Javier Maza[1,*], Francisco José Urbano[1,2] and Alejandro Delorenzi[1,2,*]

[1]Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-UBA-CONICET), Ciudad Universitaria, Pabellón IFIBYNE, Argentina
[2]Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Argentina

[*]Authors for correspondence:
Francisco Javier Maza , fjmaza@fbmc.fcen.uba.ar
Alejandro Delorenzi , delorenzi@fbmc.fcen.uba.ar

Keywords: memory expression, emotional state, Crustacea, consolidation, fluoxetine, serotonin

Summary statement:

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

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 uses changes in an innate escape response to an aversive visual stimulus, induced an emotional behavior that endured beyond the stimuli: an aversive memory training built an anxiety-like state evaluated in a dark/light plus-maze. We found that, after 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.

Keywords: 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; McGaugh, 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; Osorio-Gómez et al., 2019; Sierra et al., 2013), and remarkable on the AESOP (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 to the behavioral expression of reactivated memories (Delorenzi et al., 2014; Larrosa et al., 2017; Maza et al., 2016a; Maza et al., 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; Bacqué-Cazenave et al., 2019; Fossat et al., 2014; Fossat et al., 2015). In *Neohelice granulata,* a semi-terrestrial crab, various environmental challenges, including water deprivation, can increase expression of stress protein HSP70 in several brain areas (Frenkel et al., 2008; Frenkel et al., 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; Delorenzi et al., 2000; Delorenzi et al., 2014; Farhadi et al., 2022; Frenkel et al., 2002; Frenkel et al., 2005a; Frenkel et al., 2005b; Frenkel et al., 2010a; Frenkel et al., 2012; Gonzalez et al., 2020; Hermitte and Maldonado, 1992; Kaczer et al., 2007; Kaczer et al., 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 freezing response. This protocol generates an associative memory that can last up to five days and depends on the context in which 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; Maza et al., 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 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* measuring between 2.7 and 3.0 cm across the carapace (average weight 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 up to 2 cm depth with brackish water prepared with Coral Pro Salt (Red Sea, Israel) 1% (m/m), pH 7.4-7.6. The holding and experimental rooms were kept at 22–24 °C. Experiments were carried out during the daytime (9 am to 4 pm), between three to 15 days after the crabs had arrived at the laboratory. Between 48 to 24 hours before experiments, crabs were feed during one hour with food sticks (PROCHIN, Molinero, Suaréz 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 U.S.A.

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 (Figure 2a-c). Visual stimulus (termed visual danger stimulus, VDS) consisted of a black opaque rectangle (25 x 7.5 cm) that moved horizontally 12 cm over the actometer in a 90° 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 denominated 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 both 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 solely ("ctx") (Figure 2b above), the VDS trial requires the concomitant ctx to be evaluated ("ctx+vds") (Figure 2b below), thus each VDS trial comprised two movement cycles separated by 2 s at the end of 27 s of active training context.

Trained animals (TR) received 15 VDS trials (i.e., ctx+vds) (interstimulus interval =180 seconds) after a ten-minute acclimation period in the arena. Untrained animals (UN) 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.

Dark/light arms plus-maze

The maze used in this work was adapted from (Fossat et al., 2014). It consists in a cross-shaped clear acrylic container (60x60 cm, arms width 10 cm, wall height 15.5 cm)(Figure 1a). Two arms have their walls covered from outside with black adhesive paper. LEDs were arranged in the outer side of 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 one minute 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 (480x720 px, 30 fps, 3gp format).

Fluoxetine systemic administration

The serotonin reuptake inhibitor fluoxetine (FLX) (Sigma-Aldrich, St. Louis, MO, USA, Cat. #PHR1394) was administered at a final dose of 0.52 microg/crab. Injections were carried out immediately before training sessions. FLX was dissolved in 50 microliters of saline solution as vehicle (450 mM NaCl, 10 mM KCl, 15 mM CaCl2, 10 mM MgCl2) and administered through the right side of the dorsal cephalothoraxic-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 a minimum damage administration of drugs into crab's pericardial sac (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 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 supplemental video S1 examples).

(2) in the dark/light plus-maze, crabs had to explore both light and dark zones (i.e., spend at least a total of 30 seconds in each zone during the 10-minute evaluation period). Table 1 summarizes the final number of included/discarded crabs during this study.

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 (Latency to first in light(dark) (s)), time spent in the light as a percentage of total time (Time Light (%)), total distance traveled in cm (Distance), and time spent immobile in the dark zone (Immob dark (s)), which resulted from the sum of the periods of 15 frames (about 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). Paired Student's *t*-test were used for within group comparisons. Unpaired Student's *t*-test with Welch's correction were used to compare between two groups. A value of $p<0.05$ was considered significant (*P<0.05, **P<0.01). In addition, effect sizes and confidence intervals (mean differences and 95% confidence intervals) were calculated using bootstrap-coupled estimation statistics (https://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; Fossat et al., 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

Crabs' exploration in a dark/light arms plus-maze

To evaluate the basal behavior of crabs in the dark/light arms plus maze and test for possible preferences for dark or light zones we commenced evaluating the maze exploration of naïve crabs taken directly from collective tanks (Fig. 1). After 10 minutes of isolation in a tank, crabs were placed in the maze (Fig.1A). Fig. 1B shows an example of a naïve crab's tracked path during 10-minute 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. 1 C, left). The paired mean difference (Fig. 1 C, right) calculated using bootstrap-coupled estimation statistics between Time Light (s) and Time Dark (s) was -47.4 [95.0%CI: -126, 57.3].

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

We next evaluated whether visual aversive training with a training known to elicit short and long-term memory have effects on the crab's behavior in the dark/light arms maze tested immediately after training, indicative of a change in the crab emotional state. Two groups of crabs were evaluated (Fig. 2): trained crabs (TR) that received 15 VDS trials (ISI 3 min) and untrained crabs (UN) that were exposed to the same 15 trials of training context presentation as the TR 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 to the light zones, reflecting an anxiety-like state similar to that observed in crayfish (Fossat et al., 2014; Fossat et al., 2015). Data showed that TR crabs spent less time in the light zone than in the dark zone (Fig. 2E; paired t- test light less than dark, TR t(10)=-2.55, p=0.014; paired mean difference 126.45 s [95% CI:16.30, 202.00]). UN crabs did not spend less time in the light than in the dark zones (paired t- test light less than dark t(11)=1.18, p=0.869; paired mean difference -84.02 s [95% CI: -229.81, 33.24]). TR displayed less percentage of time in the light than UN (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 is also reflected in less time to enter to a dark arm for the TR crabs vs UN (t(20.93)=-2.19, p=0.02; unpaired mean difference -128.21 % [95% CI: -232.98, -18.76])(Fig. 2G). TR 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 treatments have been shown to have anxiolytic-like effects in the crab *Pachygrapsus crassipes* (Hamilton et al., 2016), and both to induce an increase in glucose levels (Santos et al., 2001) and to interfere with long term memory in *N. granulata* crabs (Pedetta and Maldonado, 2008; Pedetta and Maldonado, 2009). To test whether fluoxetine altered the crab's activity on the dark/light maze *per se*, crabs were injected with either vehicle or fluoxetine (0.52 microg/crab) and then isolated in the actometer with light above the bowl for 55 minutes, i.e., continuously exposed to the same illumination conditions of the training context (this fluoxetine dose increase glucose levels in *N. granulata* (Santos et al., 2001)). They were then immediately tested in the dark/light maze (Fig. 3). Crabs injected with fluoxetine showed no differences in the time spent in dark/light zones (Fig. 3C; two-tailed paired t-test VEH 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 neither showed differences with 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]) nor in 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 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]).

Concomitantly with crabs training, acute fluoxetine blocked 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, TR-VEH 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 (TR-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, TR-VEH displayed less percentage of time in the light than TR-FLX (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 is significantly reduced for the TR-FLX as they showed a higher time to enter to a dark arm compared with the TR-VEH (t(20.49)=-2.11, p=0.023, unpaired mean

difference 105.72 s [95% CI: 16.08, 206.43])(Fig. 4E). TR-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 principal component analysis (PCA) was performed. This type of analysis had been useful to reveal components related with the anxiety-like behavior and to compare different levels of anxiety in crayfish (Bacqué-Cazenave et al., 2017; Bacqué-Cazenave et al., 2019; Fossat et al., 2014; Fossat et al., 2015). The first two components of the PCA accounted for 79 % of the variance (PC1: 51 %, PC2: 28 %). Time in light (%), Latency to first in dark (s), and Immobilization in dark (s) were the variables that had more contribution to variance in the first component (PC1), which can be interpreted as the component related with 'anxiety', with increasing levels of anxiety to the positive values (Fig. 5A). The second component was mainly describing 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. 5B). When evaluating group separation, TR and TR-VEH were the only groups separated from the UN group (ratio of the between-group variance to the global variance, permutation tests UN vs TR and UN vs TR-VEH: $p<0.05$; UN vs 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 on the light arms, a shorter latency to enter the dark arms, and an increased time spent immobilized in the dark zones (Fig. 2). The serotoninergic 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 minutes (Fig. 3). Principal component analysis 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 principal component analysis (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 anxiogenesis in the light/dark 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 to 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; Pedetta and Maldonado, 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, 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; Bacqué-Cazenave et al., 2019; Fossat et al., 2014; Fossat et al., 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, crabs' 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 now in supplemental video S1, 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 by freezing outlasts the training session, differentiating it from simple stimulus-response reflexes since it persists in the absence of the visual danger stimulus and in the presence of the new context of the light/dark 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, they 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 danger visual stimulus, five trials (instead of fifteen) 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 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 bodies' activity (Maza et al., 2016a, Maza et al., 2016b, Maza et al.,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. 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.

Acknowledgments

We thank A. Vidal for technical assistance and V. Treutel and N. Fernández Larrosa for the initial preparation of the dark/light arms plus-maze.

Competing interests

The authors declare no competing or financial interests.

Contribution

F.J.M. and A.D. conceptualized and designed experiments. F.J.M performed experiments and analyses. F.J.M., A.D. and F.J.U. interpreted results. The manuscript was written by F.J.M., A.D., and F.J.U.

Funding

This work was supported by grants PICT 2016-1875, PICT 2019-00284, Agencia Nacional de Promoción Científica y Técnica de Argentina and UBA-UBACYT 2018 20020170100119BA.

Data availability

Data for this paper can be found within the article and its supplementary information. Custom scripts can be obtained upon request.

References

Agren, T. (2014). Human reconsolidation: A reactivation and update. *Brain research bulletin* **105C**, 70–82.

Anderson, D. J. and Adolphs, R. (2014). A Framework for Studying Emotions across Species. *Cell* **157**, 187–200.

Appel, M. and Elwood, R. W. (2009). Motivational trade-offs and potential pain experience in hermit crabs. *Applied Animal Behaviour Science* **119**, 120–124.

Bacqué-Cazenave, J., Cattaert, D., Delbecque, J.-P. and Fossat, P. (2017). Social harassment induces anxiety-like behaviour in crayfish. *Scientific Reports* **7**, 39935.

Bacqué-Cazenave, J., Berthomieu, M., Cattaert, D., Fossat, P. and Delbecque, J. P. (2019). Do arthropods feel anxious during molts? *Journal of Experimental Biology* **222**, jeb186999.

Baracchi, D., Lihoreau, M. and Giurfa, M. (2017). Do Insects Have Emotions? Some Insights from Bumble Bees. *Frontiers in Behavioral Neuroscience* **11**, 157.

Barreiro, K. A., Suarez, L. D., Lynch, V. M., Molina, V. A. and Delorenzi, A. (2013). Memory expression is independent of memory labilization/reconsolidation. *Neurobiol Learn Mem* **106C**, 283–291.

Caffaro, P. A., Suarez, L. D., Blake, M. G. and Delorenzi, A. (2012). Dissociation between memory reactivation and its behavioral expression: scopolamine interferes with memory expression without disrupting long-term storage. *Neurobiol Learn Mem* **98**, 235–45.

Crump, A., Browning, H., Schnell, A., Burn, C. and Birch, J. (2022). Sentience in decapod crustaceans: A general framework and review of the evidence. *Animal Sentience* **7**,.

Cumming, G. (2014). The New Statistics: Why and How. *Psychol Sci* **25**, 7–29.

Curran, K. P. and Chalasani, S. H. (2012). Serotonin circuits and anxiety: what can invertebrates teach us? *Invertebrate Neuroscience* **12**, 81–92.

Davis, M., Walker, D. L., Miles, L. and Grillon, C. (2010). Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. *Neuropsychopharmacology* **35**, 105–135.

de Abreu, M. S., Maximino, C., Banha, F., Anastácio, P. M., Demin, K. A., Kalueff, A. V. and Soares, M. C. (2020). Emotional behavior in aquatic organisms? Lessons from crayfish and zebrafish. *Journal of Neuroscience Research* **98**, 764–779.

de la Fuente, V., Federman, N., Zalcman, G., Salles, A., Freudenthal, R. and Romano, A. (2015). NF-κB transcription factor role in consolidation and reconsolidation of persistent memories. *Frontiers in Molecular Neuroscience* **8**,.

de Waal, F. B. M. and Andrews, K. (2022). The question of animal emotions. *Science* **375**, 1351–1352.

Delorenzi, A. (1999). Modulación de la memoria y osmorregulación en chasmagnathus : relación funcional y papel del sistema angiotensinérgico.

Delorenzi, A., Pedreira, M. E., Romano, A., Pirola, C. J., Nahmod, V. E. and Maldonado, H. (1995). Acute administration of angiotensin II improves long-term habituation in the crab Chasmagnathus. *Neurosci Lett* **196**, 193–6.

Delorenzi, A., Dimant, B., Frenkel, L., Nahmod, V. E., Nässel, D. R. and Maldonado, H. (2000). High environmental salinity induces memory enhancement and increases levels of brain angiotensin-like peptides in the crab Chasmagnathus granulatus. *J Exp Biol* **203**, 3369–79.

Delorenzi, A., Maza, F. J., Suarez, L. D., Barreiro, K., Molina, V. A. and Stehberg, J. (2014). Memory beyond expression. *Journal of physiology, Paris* **108**, 307–322.

Farhadi, A., Liu, Y., Xu, C., Wang, X. and Li, E. (2022). The role of the renin-angiotensin system (RAS) in salinity adaptation in Pacific white shrimp (Litopenaeus vannamei). *Frontiers in Endocrinology* **13**,.

Federman, N., Zalcman, G., de la Fuente, V., Fustiñana, M. S. and Romano, A. (2014). Epigenetic mechanisms and memory strength: A comparative study. *Journal of Physiology-Paris* **108**, 278–285.

Fossat, P., Bacque-Cazenave, J., De Deurwaerdere, P., Delbecque, J.-P. and Cattaert, D. (2014). Anxiety-like behavior in crayfish is controlled by serotonin. *Science* **344**, 1293.

Fossat, P., Bacque-Cazenave, J., De Deurwaerdere, P., Cattaert, D. and Delbecque, J. P. (2015). Serotonin, but not dopamine, controls the stress response and anxiety-like behavior in the crayfish Procambarus clarkii. *The Journal of experimental biology* **218**, 2745–52.

Frenkel, L., Freudenthal, R., Romano, A., Nahmod, V. E., Maldonado, H. and Delorenzi, A. (2002). Angiotensin II and the transcription factor Rel/NF-kappaB link environmental water shortage with memory improvement. *Neuroscience* **115**, 1079–87.

Frenkel, L., Maldonado, H. and Delorenzi, A. (2005a). Memory strengthening by a real-life episode during reconsolidation: an outcome of water deprivation via brain angiotensin II. *Eur J Neurosci* **22**, 1757–66.

Frenkel, L., Maldonado, H. and Delorenzi, A. (2005b). Retrieval improvement is induced by water shortage through angiotensin II. *Neurobiol Learn Mem* **83**, 173–7.

Frenkel, L., Dimant, B., Portiansky, E. L., Maldonado, H. and Delorenzi, A. (2008). Both heat shock and water deprivation trigger Hsp70 expression in the olfactory lobe of the crab Chasmagnathus granulatus. *Neurosci Lett* **443**, 251–6.

Frenkel, L., Dimant, B., Portiansky, E. L., Imboden, H., Maldonado, H. and Delorenzi, A. (2010a). Neuroanatomical distribution of angiotensin-II-like neuropeptide within the central nervous system of the crab Chasmagnathus; physiological changes triggered by water deprivation. *Cell Tissue Res* **341**, 181–95.

Frenkel, L., Suarez, L. D., Maldonado, H. and Delorenzi, A. (2010b). Angiotensin modulates long-term memory expression but not long-term memory storage in the crab Chasmagnathus. *Neurobiol Learn Mem* **94**, 509–20.

Frenkel, L., Dimant, B., Suarez, L. D., Portiansky, E. L. and Delorenzi, A. (2012). Food odor, visual danger stimulus, and retrieval of an aversive memory trigger heat shock protein HSP70 expression in the olfactory lobe of the crab Chasmagnathus granulatus. *Neuroscience* **201**, 239–51.

Fustiñana, M. S., Carbo Tano, M., Romano, A. and Pedreira, M. E. (2013). Contextual Pavlovian conditioning in the crab Chasmagnathus. *Anim Cogn* **16**, 255–72.

Gisquet-Verrier, P. and Riccio, D. C. (2018). Memory integration: An alternative to the consolidation/reconsolidation hypothesis. *Progress in Neurobiology* **171**, 15–31.

Gonzalez, H., Bloise, L., Maza, F. J., Molina, V. A. and Delorenzi, A. (2020). Memory built in conjunction with a stressor is privileged: Reconsolidation-resistant memories in the crab Neohelice. *Brain Research Bulletin* **157**, 108–118.

Hall, M. R. and van Ham, E. H. (1998). The Effects of Different Types of Stress on Blood Glucose in the Giant Tiger Prawn Penaeus monodon. *Journal of the World Aquaculture Society* **29**, 290–299.

Hamilton, T. J., Kwan, G. T., Gallup, J. and Tresguerres, M. (2016). Acute fluoxetine exposure alters crab anxiety-like behaviour, but not aggressiveness. *Scientific Reports* **6**, 19850.

Hermitte, G. and Maldonado, H. (1992). Conditioned facilitatory modulation of the response to an aversive stimulus in the crab Chasmagnathus. *Physiol Behav.* **51**, 17–25.

Ho, J., Tumkaya, T., Aryal, S., Choi, H. and Claridge-Chang, A. (2019). Moving beyond P values: data analysis with estimation graphics. *Nature Methods* **16**, 565–566.

Johansen, J. P., Diaz-Mataix, L., Hamanaka, H., Ozawa, T., Ycu, E., Koivumaa, J., Kumar, A., Hou, M., Deisseroth, K., Boyden, E. S., et al. (2014). Hebbian and neuromodulatory mechanisms interact to trigger associative memory formation. *Proceedings of the National Academy of Sciences* **111**, E5584–E5592.

Kaczer, L., Pedetta, S. and Maldonado, H. (2007). Aggressiveness and memory: subordinate crabs present higher memory ability than dominants after an agonistic experience. *Neurobiol.Learn.Mem.* **87**, 140–148.

Kaczer, L., Klappenbach, M. and Maldonado, H. (2011). Dissecting mechanisms of reconsolidation: octopamine reveals differences between appetitive and aversive memories in the crab Chasmagnathus. *Eur J Neurosci* **34**, 1170–8.

Klappenbach, M., Nally, A. and Locatelli, F. F. (2017). Parallel memory traces are built after an experience containing aversive and appetitive components in the crab Neohelice. *Proc Natl Acad Sci USA* **114**, E4666.

Kravitz, E. A. (1988). Hormonal control of behavior: amines and the biasing of behavioral output in lobsters. *Science* **241**, 1775–81.

Larrosa, P. N. F., Ojea, A., Ojea, I., Molina, V. A., Zorrilla-Zubilete, M. A. and Delorenzi, A. (2017). Retrieval under stress decreases the long-term expression of a human declarative memory via reconsolidation. *Neurobiology of Learning and Memory* **142**, 135–145.

Lukowiak, K., Sunada, H., Teskey, M., Lukowiak, K. and Dalesman, S. (2014). Environmentally relevant stressors alter memory formation in the pond snail Lymnaea. *Journal of Experimental Biology* **217**, 76–83.

Maldonado, H. (2002). Crustacean as model to investigate memory illustrated by extensive behavioral and physiological studies in Chasmagnathus. In *The crustacean nervous system* (ed. K.Wiese, pp. 314–327. Berlin: Springer.

Maza, F. J., Locatelli, F. F. and Delorenzi, A. (2016a). Neural correlates of expression-independent memories in the crab Neohelice. *Neurobiol Learn Mem* **131**, 61–75.

Maza, F. J., Sztarker, J., Shkedy, A., Peszano, V. N., Locatelli, F. F. and Delorenzi, A. (2016b). Context-dependent memory traces in the crab's mushroom bodies: Functional support for a common origin of high-order memory centers. *Proceedings of the National Academy of Sciences* **113**, E7957–E7965.

Maza, F. J., Urbano, F. J. and Delorenzi, A. (2022). Contextual memory reactivation modulates Ca2+-activity network state in a mushroom body-like center of the crab N. granulata. *Scientific Reports* **12**, 11408.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science* **287**, 248–251.

McGaugh, J. L. (2013). Making lasting memories: Remembering the significant. *Proceedings of the National Academy of Sciences* **110**, 10402–10407.

Merlo, S. A., Santos, M. J., Pedreira, M. E. and Merlo, E. (2020). Identification of a Novel Retrieval-dependent Memory Process in the Crab Neohelice granulata. *Neuroscience* **448**, 149–159.

Nader, K. (2009). New approaches to amnesia. *Learn. Mem.* **16**, 672–675.

Ojea Ramos, S., Andina, M., Romano, A. and Feld, M. (2021). Two spaced training trials induce associative ERK-dependent long term memory in Neohelice granulata. *Behavioural Brain Research* **403**, 113132.

Osorio-Gómez, D., Guzmán-Ramos, K. and Bermúdez-Rattoni, F. (2017). Memory trace reactivation and behavioral response during retrieval are differentially modulated by amygdalar glutamate receptors activity: interaction between amygdala and insular cortex. *Learning & Memory* **24**, 14–23.

Osorio-Gómez, D., Saldivar-Mares, K. S., Perera-López, A., McGaugh, J. L. and Bermúdez-Rattoni, F. (2019). Early memory consolidation window enables drug induced state-dependent memory. *Neuropharmacology* **146**, 84–94.

Pedetta, S. and Maldonado, H. (2008). Effect of the serotonergic system on consolidation and reconsolidation of memory in an invertebrate. In *Gordon Research Conference - Genes and Behavior*, p. Lucca, Italy.

Pedetta, S. and Maldonado, H. (2009). Serotonin and appetitive memory. In *First Joint Meeting of the Argentine Society for Neurosciences (SAN) and the Argentine Workshop in Neurosciences (TAN)*, p. Argentina.

Pereyra, P., Gonzalez, P. E. and Maldonado, H. (2000). Long-lasting and context-specific freezing preference is acquired after spaced repeated presentations of a danger stimulus in the crab Chasmagnathus. *Neurobiol.Learn.Mem.* **74**, 119–134.

Perez-Cuesta, L. M. and Maldonado, H. (2009). Memory reconsolidation and extinction in the crab: mutual exclusion or coexistence? *Learn Mem* **16**, 714–21.

Perrot-Minnot, M.-J., Banchetry, L. and Cézilly, F. (2017). Anxiety-like behaviour increases safety from fish predation in an amphipod crustacea. *Royal Society Open Science* **4**, 171558.

Perry, C. J. and Baciadonna, L. (2017). Studying emotion in invertebrates: what has been done, what can be measured and what they can provide. *The Journal of Experimental Biology* **220**, 3856.

Salzet, M., Deloffre, L., Breton, C., Vieau, D. and Schoofs, L. (2001). The angiotensin system elements in invertebrates. *Brain Res.Brain Res.Rev.* **36**, 35–45.

Sánchez Beisel, J. M., Maza, F. J., Justel, N., Fernandez Larrosa, P. N. and Delorenzi, A. (2022). Embodiment of an Emotional State Concurs with a Stress-Induced Reconsolidation Impairment Effect on an Auditory Verbal Word-List Memory. *Neuroscience* **497**, 239–256.

Sandi, C. (2013). Stress and cognition. *WIREs Cognitive Science* **4**, 245–261.

Santos, E. A., Keller, R., Rodriguez, E. and Lopez, L. (2001). Effects of serotonin and fluoxetine on blood glucose regulation in two decapod species. *Braz J Med Biol Res* **34**, 75–80.

Santos, M. J., Merlo, S. A., Kaczer, L. and Pedreira, M. E. (2021). Social context shapes cognitive abilities: associative memories are modulated by fight outcome and social isolation in the crab Neohelice granulata. *Animal Cognition* **24**, 1007–1026.

Schroyens, N., Beckers, T. and Luyten, L. (2022). Appraising reconsolidation theory and its empirical validation. *Psychonomic Bulletin & Review*.

Sierra, R. O., Cassini, L. F., Santana, F., Crestani, A. P., Duran, J. M., Haubrich, J., de Oliveira Alvares, L. and Quillfeldt, J. A. (2013). Reconsolidation may incorporate state-dependency into previously consolidated memories. *Learning & memory (Cold Spring Harbor, N.Y.)* **20**, 379–87.

Soares, M. C., Banha, F., Cardoso, S. C., Gama, M., Xavier, R., Ribeiro, L. and Anastácio, P. (2022). Hemolymph Glycemia as an Environmental Stress Biomarker in the Invasive Red Swamp Crayfish (Procambarus clarkii). *Physiological and Biochemical Zoology* **95**, 265–277.

Tomsic, D. and Romano, A. (2013). A Multidisciplinary Approach to Learning and Memory in the Crab Neohelice (Chasmagnathus) granulata. In *Invertebrate Learning and Memory* (ed. Menzel, R.) and Benjamin, P. R.), pp. 337–355. Amsterdam: Academic Press.

Vallat, R. (2018). Pingouin: statistics in Python. *Journal of Open Source Software* **3**, 1026.

Vogel, E. H., Ponce, F. P. and Wagner, A. R. (2019). The development and present status of the SOP model of associative learning. *Quarterly Journal of Experimental Psychology* **72**, 346–374.

Wolf, O. T. (2019). Memories of and influenced by the Trier Social Stress Test. *Psychoneuroendocrinology* **105**, 98–104.

Figures and Table

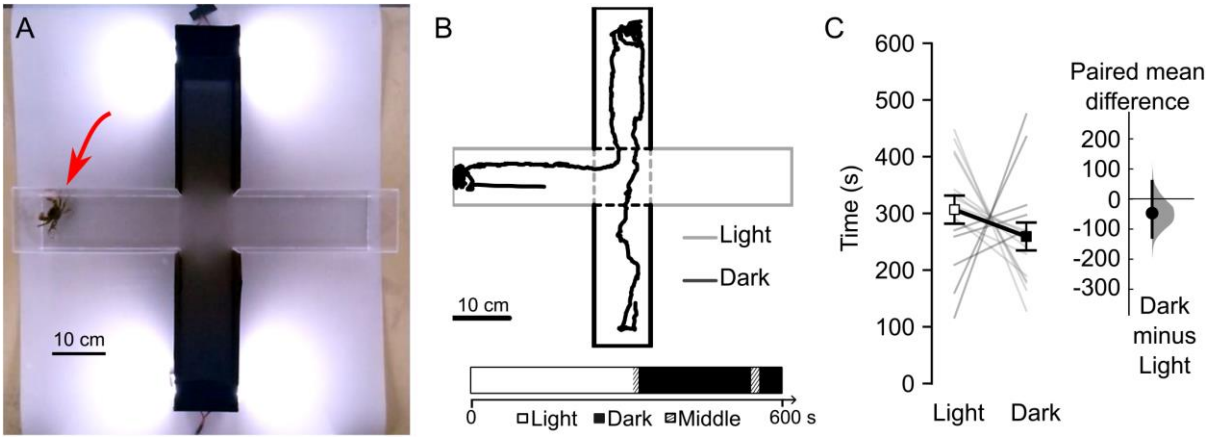


Fig. 1. Dark/light arms plus-maze. (A) Overview of the maze with a crab in it (arrow). (B) Example of a crab's tracked path. Below is the dynamic of maze exploration during the 10-minute (600 s) evaluation. (C) Left, time (seconds) spent in light and dark zones for naïve crabs (n=15). Each gray line corresponds to a crab, the black line to the mean and error bars to the SEM. Two-tailed paired t-test, $p>0.05$. Right, effect size (mean differences between dark and light zones exploration time) calculated using bootstrap-coupled estimation statistics. Curve shows the resampled distribution of mean differences and error bar 95% confidence interval.

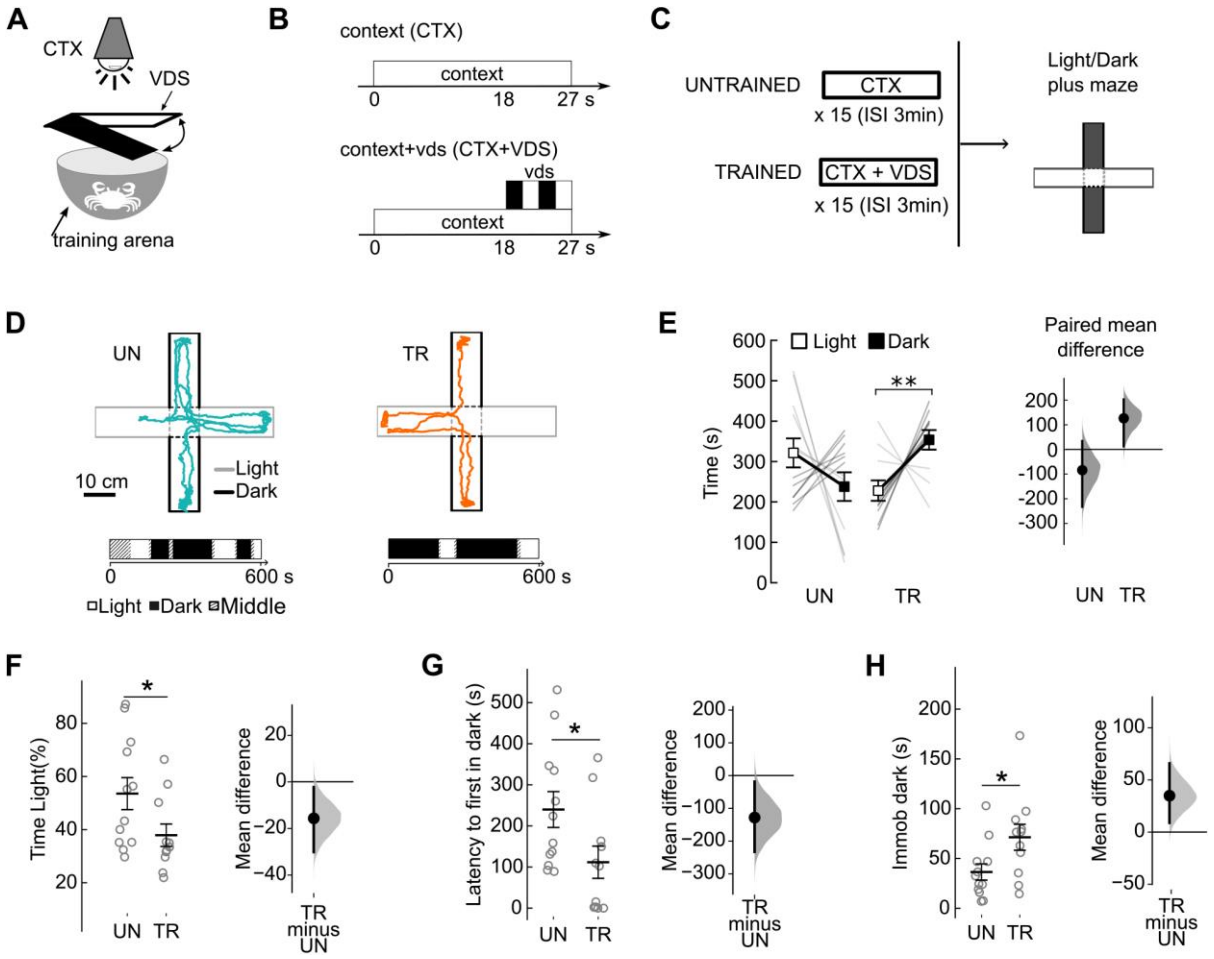


Fig. 2. Visual danger stimulus training and anxiety-like state in the dark/light plus-maze. (A) Scheme of the training arena and the visual danger stimulus (VDS). A crab is inside the actometer that is illuminated from below. Phasic presentation of the training context is done by a change in setup illumination from above (CTX). The VDS comprises a black rectangular panel moving above the horizon of the crab. (B) Context and VDS trials scheme. Context presentation involves a phasic change in setup illumination during 27 s. The VDS is presented during the last nine seconds of the context presentation moving back and forth twice in each trial (vds, black bars). (C) Experimental groups. Crabs were evaluated in the dark/light plus-maze immediately after taking them from the training arena. (D) Examples of crabs' tracked paths (UN: untrained, TR: trained). Below is the dynamic of maze exploration during the 10-minute (600 s) evaluation. (E) Left, time (seconds) spent in light and dark zones. Each gray line corresponds to a crab, the black line to the mean and error bars to the SEM. One-tailed paired t-test, **p<0.01. Right, effect size (mean differences between dark

and light zones exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars 95% confidence intervals. (F, G, and H) Time in light (%), Latency to first in dark (s), and Immobilization in dark (s). UN, TR: n=12, 11. Error bars over dot plots represent mean + / - SEM. One-tailed Welch t-test, *p<0.05.

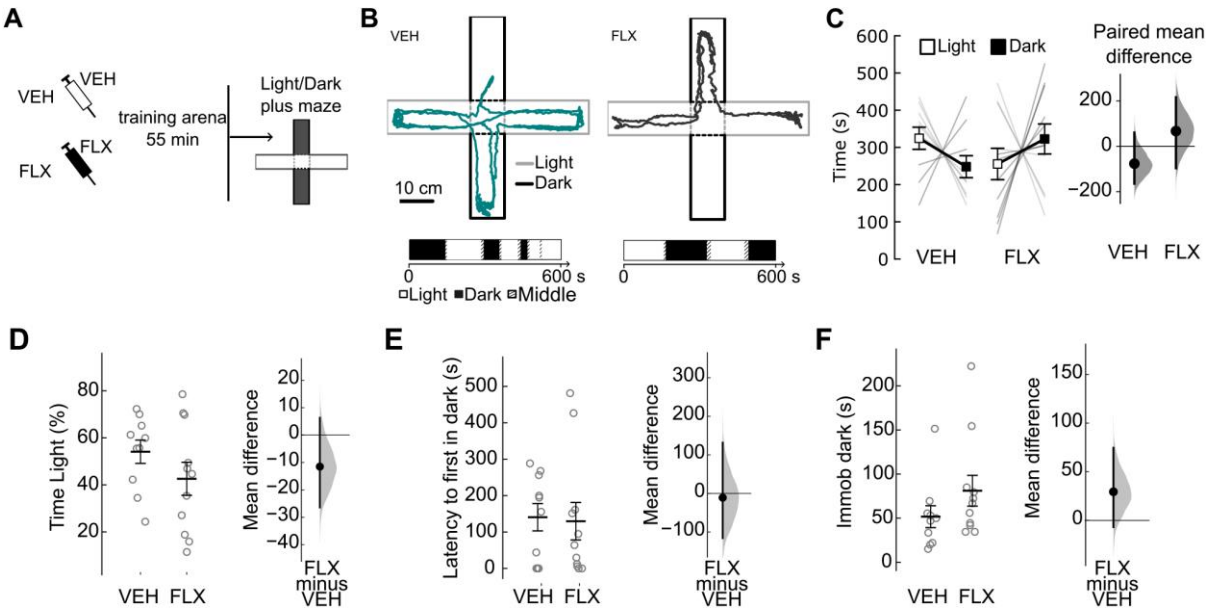


Fig. 3. Acute fluoxetine treatment. (A) Crabs injected with fluoxetine (FLX, 0.52 microg/crab) or vehicle (VEH) were isolated in the training arena for 55 minutes. Immediately after, we evaluated their behavior in the dark/light maze. (B) Examples of crabs' tracked paths. Below is the dynamic of maze exploration during the ten-minute (600 s) evaluation. (C) Left, time (seconds) spent in light and dark zones. Each gray line corresponds to a crab, the black line to the mean and error bars to the SEM. One-tailed paired t-test, $p>0.05$. Right, effect size (mean differences between dark and light zones exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars 95% confidence intervals. (D, E, and F) Time in light (%), Latency to first in dark (s), and Immobilization in dark (s). VEH, FLX: n=10, 11. Error bars over dot plots represent mean + / - SEM. One-tailed Welch t-tests, $p>0.05$.

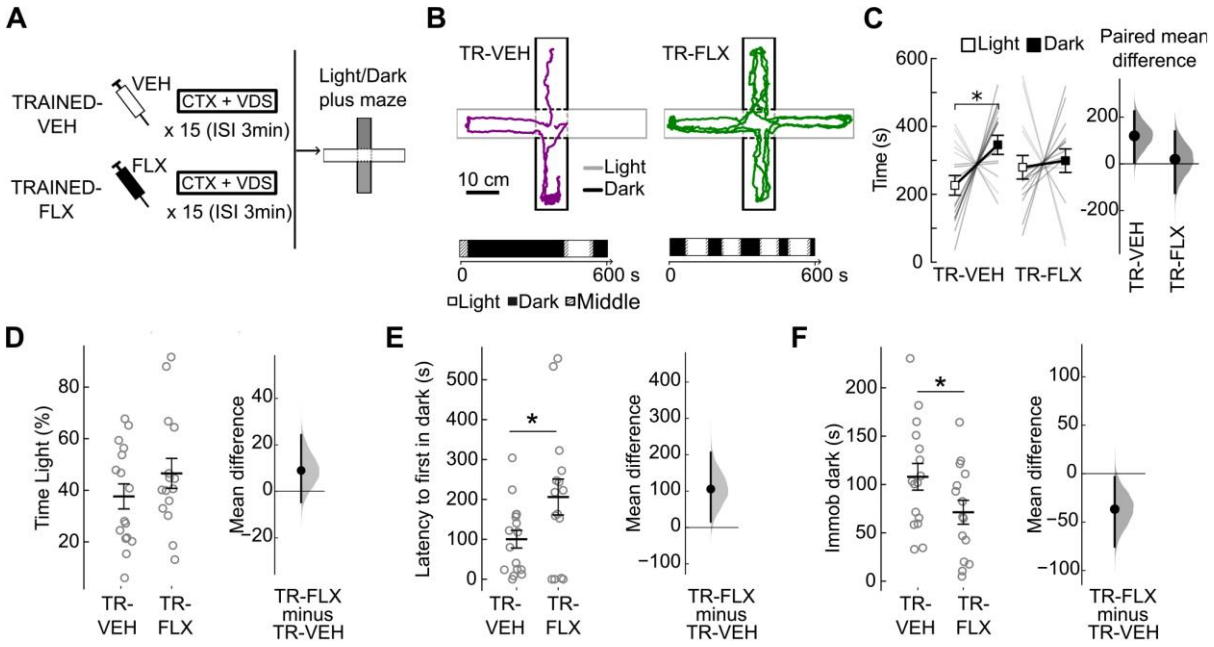


Fig. 4. Acute fluoxetine and anxiety-like behavior. (A) Crabs injected with fluoxetine (FLX, 0.52 microg/crab) or vehicle (VEH) were trained with the VDS. Immediately after, we evaluated their behavior in the dark/light maze. (B) Examples of crabs' tracked paths. Below is the dynamic of maze exploration during the 10-minute (600 s) evaluation. (C) Left, time (seconds) spent in light and dark zones. Each gray line corresponds to a crab, the black line to the mean and error bars to the SEM. One-tailed paired t-test, *p<0.05. Right, effect size (mean differences between dark and light zones exploration time) calculated using bootstrap-coupled estimation statistics. Curves show the resampled distribution of mean differences and error bars 95% confidence intervals. (D, E, and F) Time in light (%), Latency to first in dark (s), and Immobilization in dark (s). TR-VEH, TR-FLX: n=16, 15. Error bars over dot plots represent mean + / - SEM. One-tailed Welch t-test, *p<0.05.

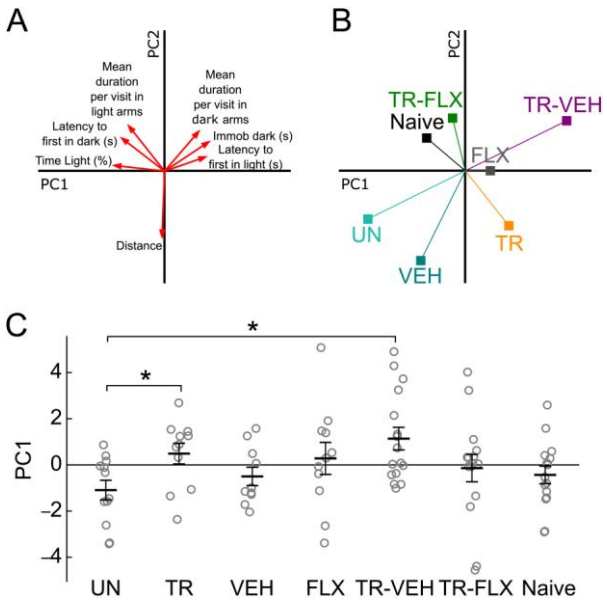
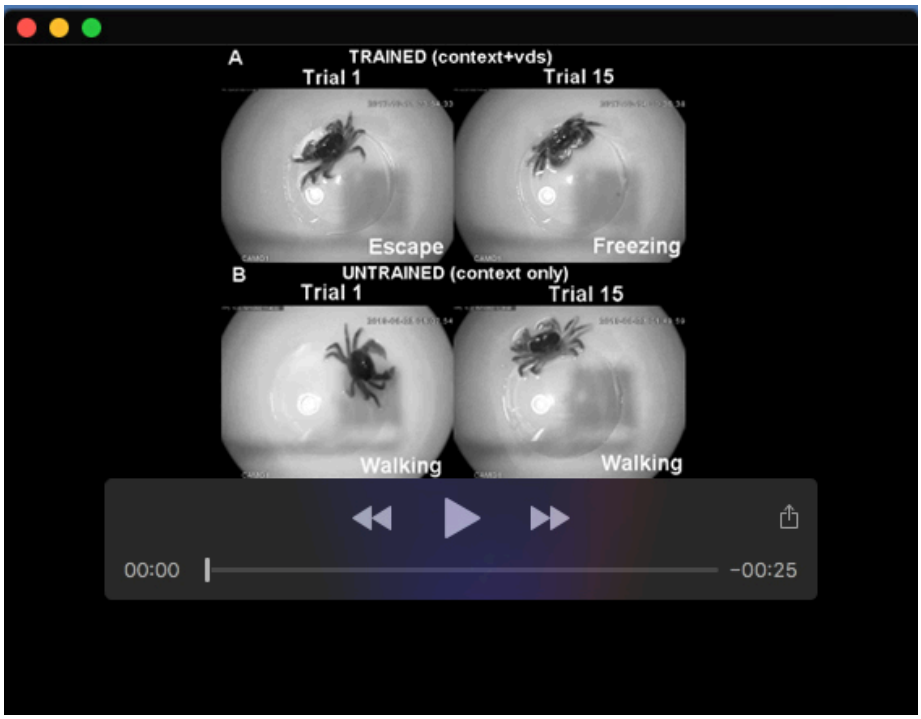


Fig. 5. Principal component analysis (PCA) with all experimental series. (A) Contribution of each variable to the variance in the first and second components of the PCA. Time in light (%), Latency to first in dark (s), and Immobilization in dark (s) were the variables that had more contribution to variance in the first component (PC1), which can be interpreted as the component related with 'anxiety'. Variance in the second component (PC2) is dominated by the walked distance, which can be interpreted as the component related with the 'locomotion'. (B) Location of each group mean in the plane defined by the first and second components of the PCA. Note that trained animals (TR and TR-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 UN (ratio of the between-group variance to the global variance). Mean and SEM are shown for each group.

Table 1. Resulting crabs after exclusion criteria.

Group	Total number	Final number	Training arena session	Did not explore L and D zones in maze
Naïve	19	15	-	4
UN	18	12	6 freezing at last trial	-
TR	17	11	1 walking response at last trial, 1 escape response at last trial, 1 no escape at first VDS trial	3
VEH	13	10	1 freezing at last trial	2
FLX	14	11	1 freezing at last trial	2
TR-VEH	21	16	1 no escape response at first VDS trial	4
TR-FLX	30	15	8 no escape response at first VDS trial	7



Movie 1. Inclusion criteria by behavior in the training arena. (A) Trained crab (from TR-VEH group). Left, escape response is displayed during the first training trial with the visual danger stimulus (shadow). Right, freezing response during the last training trial (trial 15). (B) Untrained crab (context presentation only). The animal is walking during the last trial.