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RESEARCH ARTICLE

Learning a non-neutral conditioned stimulus: place preference in the crab *Neohelice granulata*

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ABSTRACT

In the wild, being able to recognize and remember specific locations related to food sources and the associated attributes of landmarks is a cognitive trait important for survival. In the present work, we show that the crab *Neohelice granulata* can be trained to associate a specific environment with an appetitive reward in a conditioned place preference task. After a single training trial, when the crabs were presented with a food pellet in the target quadrant of the training arena, they were able to form a long-term memory related to the event. This memory was evident at least 24 h after training and was protein synthesis dependent. Importantly, the target area of the arena proved to be a non-neutral environment, given that animals initially avoided the target quadrant. In the present work, we introduce for the first time an associative one-trial memory paradigm including a conditioned stimulus with a clear valence performed in a crustacean.

KEY WORDS: Long-term memory, Contextual memory, Protein synthesis, Invertebrate, Classical conditioning

INTRODUCTION

Contextual memory is a very important cognitive function, as it allows the storage and retrieval of specific cued-based locations which are of relevance to animals (Mery, 2013; Shettleworth, 2010). The crab *Neohelice granulata* lives in the intertidal flats of narrow coastal inlets (rias) in Atlantic shores, where its populations form dense patches, with up to 70 burrows per square meter (Angeletti and Cervellini, 2015). Therefore, this species' orientation skills are a key component of successful navigation in this rich environment (Fathala and Maldonado, 2011). Several studies performed in maze and place preference arenas indicate that decapods are able to acquire and make use of learned information to orient themselves in rich environments (Davies et al., 2019; Huber et al., 2018; Tierney and Lee, 2011). In particular, *N. granulata* is a strong model to study the neurobiology of memory (Tomsic and Romano, 2013; Feld et al., 2020). In the present work, we introduce for the first time a place preference long-term memory paradigm in which *N. granulata* crabs are able to form an association between an identifiable striped quadrant of a circular arena and an appetitive reinforcement, which led to an increase in their relative presence in the target quadrant 24 h post-training. Interestingly, we show that the striped quadrant is a non-neutral stimulus and that the initial amount of time spent on the first day in the quadrant can be modulated by the animal's hunger state. This novel paradigm allows animals to form a long-term memory that can be modulated by the duration of the pairing between stimuli and is dependent on protein synthesis.

MATERIALS AND METHODS

Animals

Adult male intertidal crabs, *Neohelice granulata* (Dana 1851) (formerly *Chasmagnathus granulatus*), were collected from water <1 m deep in rias of San Clemente del Tuyú, Buenos Aires Province, Argentina. Only animals measuring 2.7–3.0 cm across the carapace and weighing ∼17 g were selected to perform experiments and transported to the laboratory. This was done to narrow down the age range of the animals and ensure they were all adults. Crabs were housed in plastic tanks (35×48×27 cm) filled to a depth of 2 cm with diluted marine water (Red Sea Fish Pharm) with a salinity of 1.0–1.4% and a pH of 7.4–7.6. The water was changed and the tank sanitized every 2 days. The housing room was maintained on a 12 h:12 h light:dark cycle (lights on from 07:00 h to 19:00 h) and temperature between 22 and 24°C. Animals were food-restricted for 6–10 days depending on the experiment. These are opportunistic animals that can go through several days without feeding in the wild (Sarapio et al., 2017). Experiments were performed only in males to avoid disrupting the natural population of crabs as females carry the fertilized eggs in the first stages of development and capturing them might affect the size of the population. Another consideration is that a natural population has many sources of variability and selecting male animals restricts variables such as size range. The reported research was conducted in accordance with the local regulations for the care and use of laboratory animals. All experiments were done in accordance with local regulations to minimize animal suffering and the number of animals used.

Training and testing procedures

The experimental arena comprised a cylindrical white PVC container with a 30 cm high wall, delimiting a 31.5 cm circumference arena, as shown in Fig. 1. One-quarter of the arena wall had a black and white striped vertical pattern (striped quadrant, SQ), in contrast with the rest of the wall, which was plain white. The floor of the arena was also plain white, with the exception of lines delimiting the SQ, which were made of 6-mm-wide strips of black electrical insulating tape (3M). For each experiment, the arena floor was covered to a depth of 0.5 cm with salt water and the arena was placed on top of a 130 cm stand to avoid visual contact with the experimenter. Crabs were individually placed in each arena. A structural scaffold was placed 150 cm above an array of 8 arenas, with two high-frequency fluorescent lamps

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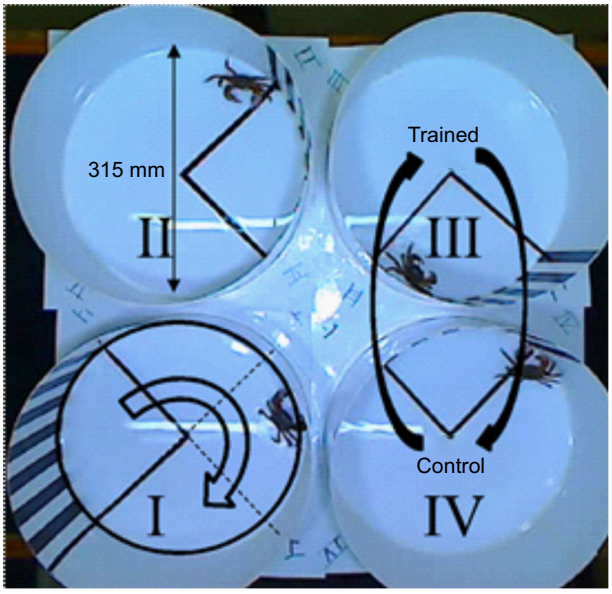

Fig. 1. Arrangement of a subset of four place preference arenas. The open arrow denotes the direction of rotation of the arena. The black arrows denote the exchange of experimental groups between arenas within the experiment.

and two 922 Logitech cameras (each one recording a subset of 4 arenas) attached. Animals were randomly assigned to each experimental group. Experimental groups were homogeneously distributed in all conditioning arenas and the orientation of each arena was rotated between trials, ensuring a distribution of the animal groups that takes into account the possible non-homogeneity of the experimental room (Fig. 1).

Video recordings of habituation, training and testing sessions were used to track each animal's position within the arena either manually and semi-automatically using Tracker 5.0.7, both in an offline manner (video analysis and modeling tool by Douglas Brown; https://physlets.org/tracker/). The percentage of time spent in the SQ, how much time it took each animal to start consuming the food pellet and the time devoted to its consumption were manually estimated by two observers who were blind to the experimental setup, whereas estimation of the distance to the wall (mm) was performed semi-automatically. Heat maps were compiled using ANY-maze behavioral tracking software. The training session lasted up to 15 min and consisted of 5 min habituation to the arena, followed by a variable period of time during which the animal was presented with one pellet of food (Labcon Bottom Fish) on the floor of the experimental arena in the center of the SQ. Then, animals were removed from the experimental arena and individually housed in plastic containers, covered to a depth of 0.5 cm with seawater and kept inside dimly lit drawers until the testing session, which took place 24 h later. The testing session consisted of 5 min exposure to the conditioning arena without the presence of a reward. The manual tracking consisted of evaluating the presence of the crab in the SQ every 10 s for the 300 s duration of habituation or testing sessions. This variable was expressed as a percentage of time the animal was present in the SQ out of the total duration of the session (n=30 measurements).

Drugs and injection procedure

The protein synthesis inhibitor cycloheximide (CHX, Sigma-Aldrich C7698) (Pedreira et al., 1995) was diluted in crustacean saline solution (450 mmol l^{-1} NaCl, 15 mmol l^{-1} $CaCl_2$, 21 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl) (Hoeger and Florey, 1989) and administered systemically at a final dose of 40 µg per animal in 50 µl volume. The drug solution or 50 µl vehicle control was injected through the right side of the dorsal cephalothoracic–abdominal membrane by means of a syringe needle fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was released in the pericardial sac. Given that crabs lack an endothelial blood–brain barrier (Abbott, 1970), and that blood is distributed by a capillary system in the central nervous system (Sandeman, 1967), systemically injected drugs readily reach the brain.

Data analysis

Animals displaying more than two standard deviations from the mean of the percentage of time spent in SQ during the habituation session were excluded from the analysis. This criterion was used for all experiments, with the exception of the assessment of memory retention for a conditioned stimulus with negative valence (see below). In that case, we only analyzed the behavior of animals with a negative preference index (PI). We based this criterion on the interpretation that these animals avoided the SQ and, therefore, this arena's section could play the role of a conditioned stimulus with negative valence. The SQ PI was calculated as: PI=percentage of SQ time−(percentage of WQ time/3), where WQ is white quadrant. Statistical analyses were performed using GraphPad prism software. Data were tested for normality using the Shapiro–Wilk test and analyzed with a repeated measures ANOVA (with Holm–Šidák *post hoc* test, when contrasts were pertinent), unless otherwise stated. Data are presented as means±s.e.m., unless otherwise stated in the cases when it deviated from normality. One-sample Wilcoxon signed rank test was applied to compare the median of the sample with a hypothetical median (in this case, zero).

RESULTS

Animals explore the arena asymmetrically

During their first exposure to the arena (habituation session), crabs showed a non-homogeneous distribution of exploratory behavior. In all four quadrants of the arena, animals spent most of the exploration time (5 min) in close proximity to the walls (Fig. 2A,B), suggesting thigmotaxis as a driver in their behavior. Nonetheless, the distance to the wall kept by the animals in the SQ was significantly larger than the distance shown in the directly opposite WQ, indicating that the striped pattern is a relevant stimulus for these crabs (Fig. 2B). The SQ PI denotes the exploration time distribution of the animals in the arena. During habituation, 77.75% of the animals presented a PI <0, indicating they spent less time in the SQ than in the rest of the arena, while 22.25% presented the opposite behavior (Fig. 2C).

When the SQ PI during habituation was calculated for 12 independent experiments, the resulting median was significantly lower than the null median expected for no preference (median=−10.14, P<0.001; Fig. 2D). This result indicates that avoidance of the SQ by the majority of the animals was the most frequent response to the initial presentation of the arena.

Considering the ever-changing relationship between an animal and its environment, we hypothesized that this initial avoidance of the SQ could be affected by a number of factors, including the animal's fasting period previous to the habituation session. An animal that has been food deprived will show an increased drive for food-seeking behaviors, overcoming mildly aversive stimuli such as the stripes (Dethier, 1976; Brown, 1992a,b). Fasting may also result in more robust learning experiences in crabs and other species (Klappenbach et al., 2017; Krashes et al., 2009). We found a significant positive correlation between the number of days animals were food deprived before their first encounter with the arena

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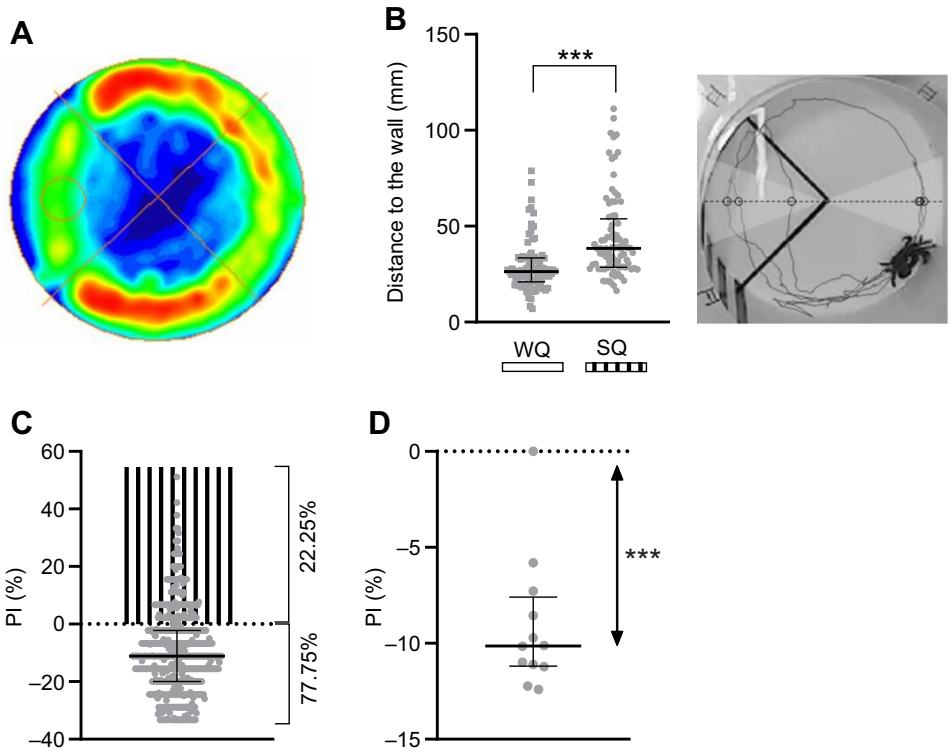

Fig. 2. Crabs avoid the striped quadrant (SQ). (A) Heatmap representing the exploration distribution of animals during the habituation session – a plot of the animals' center position maximum occupancy. The SQ is identified by a circle (n=20). (B) Left: distance to the wall in the SQ versus the white quadrant (WQ). Medians±interquartile range are shown. Wilcoxon test between quadrants: W=−2788, ***P<0.001. Right: circles represent the intersection of a single crab trajectory with the bisector of each quadrant angle, denoting distance from the wall for both quadrants in the same axis. (C) SQ preference index (PI); 77.75% of animals spent less time in the SQ than in the rest of the arena, while 22.25% of crabs presented the opposite behavior (n=701). Medians±interquartile range. (D) PI calculated for each independent experiment (n=12 experiments, 40–80 crabs/experiment). One-sample Wilcoxon signed rank test: W=−78, ***P<0.001.

(i.e. habituation session) and each animal's PI (Fig. 3A; r^2=0.54, P<0.01), although total walking distance in measured experiments did not show a significant correlation with the fasting period (data not shown). We also found a significant positive correlation between the number of days the animals were food deprived and the fraction of animals preferring the SQ (Fig. 3B; r^2=0.49, P<0.05). These results point to an important modulating role of the animal's motivational state by hunger in exploration strategies when encountering a novel environment.

Seeking and feeding behavior in *N. granulata*

Once crabs had gone through the habituation period in the arena, we randomly divided them into two groups. Animals from the trained group received one food pellet in the center of the SQ, to act as an appetitive reinforcement until animals were removed, 10 min later. The other half of the animals remained in their arenas the same amount of time as the trained group but without the appetitive reinforcement, serving as the control. On average, animals from the trained group took 88.68 s to find the pellet (Fig. 4A) and this resulted in an effective reinforcement period of 497.3 s (time spent in SQ eating, after finding the pellet; Fig. 4B). Animals that experienced less than half of the mean effective reinforcement time were excluded from further analysis (identified with a cross in Fig. 4A,B). This could be the result of taking a longer period of time to find the food pellet, abandoning it in order to further explore the environment or taking it with them away from the SQ. Nevertheless, this type of behavior was not usual, as after finding the pellet crabs spent 97.5±1.66% of the time eating within the SQ and 98.2±1.7% of this time they remained in the same position, as seen in Fig. 4C. These characteristics of the crabs' feeding behavior allowed a prolonged unforced association between the SQ and the appetitive reinforcement.

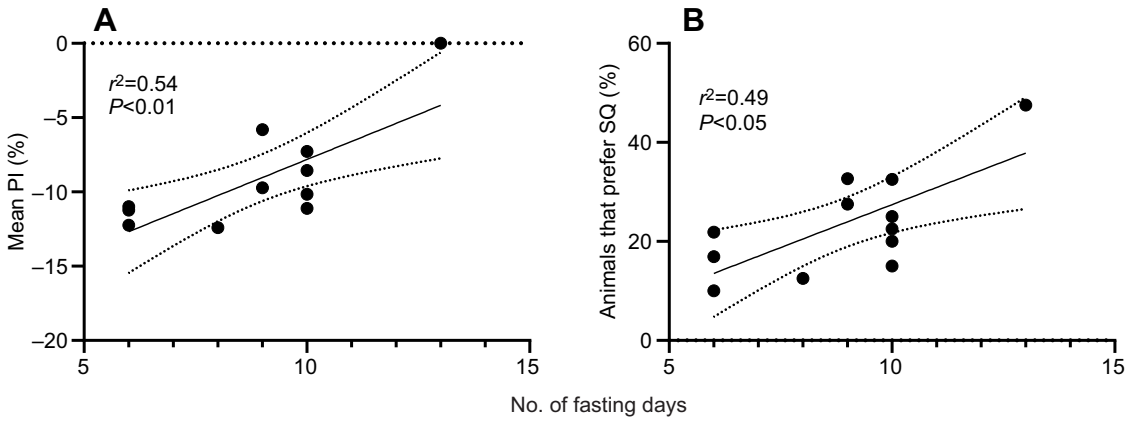

Fig. 3. Time in the SQ is modulated by the number of previous fasting days. (A) PI value of animals fasting for 6–13 days (n=12 independent experiments). (B) Percentage of animals that spent more time in the SQ when fasting for 6–13 days (n=12 independent experiments).

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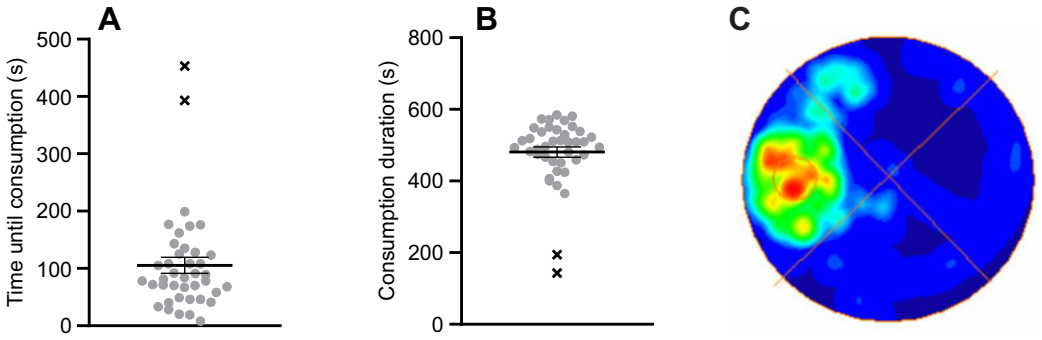

Fig. 4. Seeking and feeding behavior in the arena. (A) Amount of time it took each trained animal to find the food pellet in the SQ (n=38). (B) Duration of appetitive reinforcement of trained animals. (C) Heatmap representing the exploration distribution of animals during the habituation session – a plot of the animals' center position maximum occupancy. SQ is identified with a circle in the center of the quadrant. Animals identified with a cross in A and B were removed from the experiment, as their effective reinforcement time was less than half of the mean.

Neohelice granulata shows long-term memory retention in a place preference learning task

Animals from both the trained and control groups were tested 24 h after the training session, by exposure to their corresponding cleaned arenas from which all remnants of food had been removed, and their behavior was evaluated for 5 min (Fig. 5A). Contrary to their exploratory activity during habituation, animals that had received reinforcement in the SQ for 8 min (i.e. the trained group) spent more time in this quadrant when compared with non-reinforced crabs (the control group; P<0.01; Fig. 5B). This difference in behavior towards the SQ denotes the formation of a long-term memory which lasted at least 24 h after acquisition and was the result of only one training trial. In order to further characterize this type of memory, we evaluated whether its strength depends on the amount of time the animal was presented with the pairing between stimuli. For this, we performed another experiment in which the total amount of time available for the animals to eat the food pellet in the SQ was reduced to 2 min. When animals were able to eat for only 2 min, their change in behavior between the habituation and testing sessions significantly differed from the behavior presented by animals that had experienced reinforcement for an average of 8 min (Fig. 4C). This result shows a clear dependence of the strength of the memory formed on the length of the reinforcement period.

Long-term retention depends on protein synthesis

Probably the most characteristic feature of long-term memory is its dependence on *de novo* protein synthesis. In order to evaluate whether the memory trace resulting from one training trial depends on this process, we performed an experiment similar to the one described in Fig. 5A, but included systemic administration of either CHX or vehicle (saline) control immediately after the training session (trained+CHX and trained+saline group, respectively; Fig. 6A). CHX is a broadly used protein synthesis inhibitor which has proven effective in several animal models, including the crab *Neohelice* (Fustiñana et al., 2013; Kaczer and Maldonado, 2009; Pedreira et al., 1995; Tomsic et al., 1998). Additionally, one control group was injected with saline at this time point (control+saline). Trained+saline animals showed a higher percentage of time spent in the SQ, relative to the total duration of the training session, when compared with control+saline animals, which indicates the formation of long-term memory as expected from the first experiment. However, when animals from the trained+CHX group were re-exposed to the arena 24 h after training, they spent significantly less time in the SQ than crabs that were also trained but were administered with saline solution (trained+saline; Fig. 6B). To investigate whether a change in exploratory behavior due to protein synthesis inhibition could actually be caused by a difference in locomotion activity, we evaluated each animal's total distance

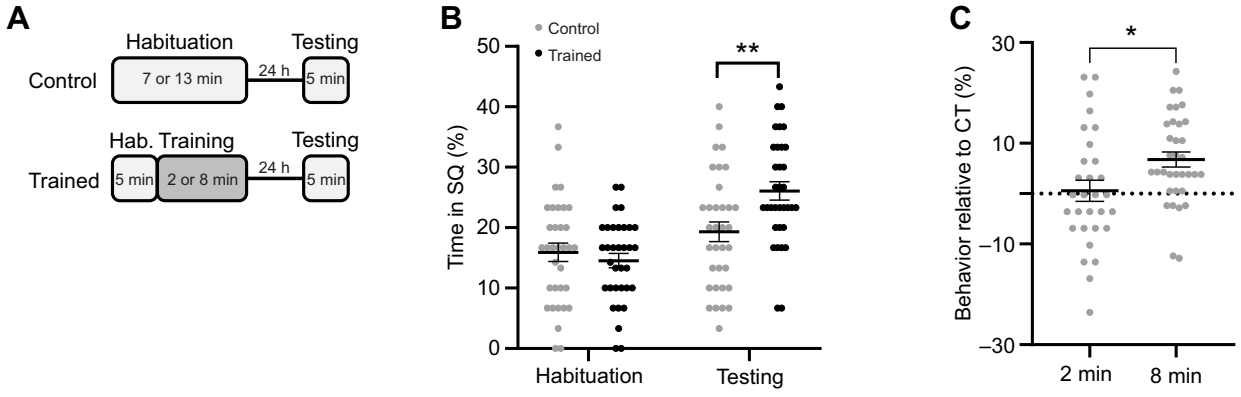

Fig. 5. *Neohelice granulata* shows long-term memory retention in a place preference learning task. (A) Experimental design. (B) Percentage of time spent by crabs in the SQ by the control and trained groups (n=34 per group). Two-way repeated measures ANOVA: session effect: $F_{1,66}$=25.69, P<0.001; experimental group: $F_{1,66}$=3.41, P=0.069; interaction: $F_{1,66}$=7.612, P<0.01; Holm–Šidák contrasts: control versus trained group at habituation: P=0.510; control versus trained group at testing: **P<0.01. (C) Group difference (trained−control) for 2 and 8 min of reinforcement. Paired two-tailed t-test: t_{62}=2.440, P<0.05 (2 min n=30, 8 min n=34). Means±s.e.m.

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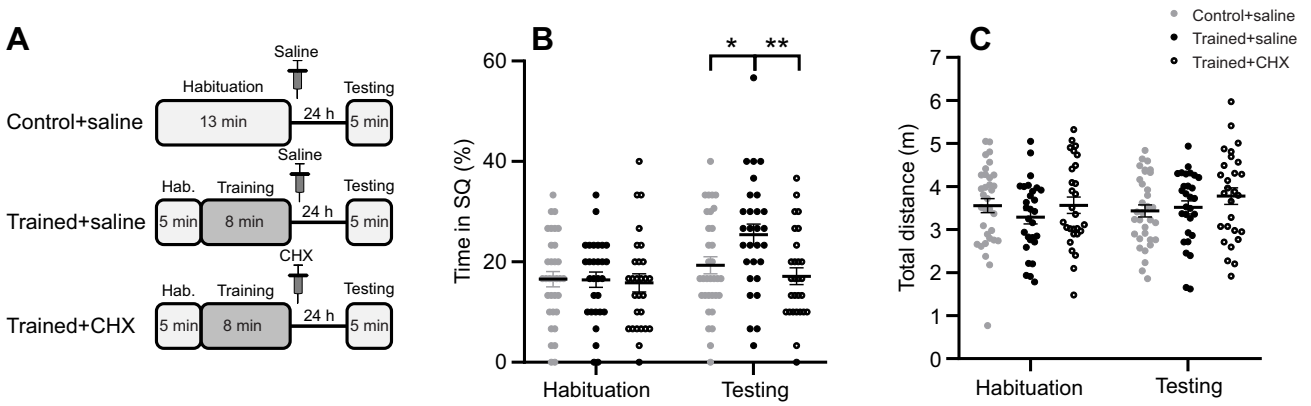

Fig. 6. Long-term memory retention is cycloheximide (CHX) sensitive. (A) Experimental design. (B) Percentage of time spent by each animal in the SQ. Two-way repeated measures ANOVA: session: $F_{1,87}$=13.26, P<0.001; experimental group: $F_{2,87}$=2.50, P=0.088; interaction: $F_{2,87}$=3.78, P<0.05. Holm–Šidák contrasts: within habituation: control+saline versus trained+saline P=0.99, trained+saline versus trained+CHX P=0.99, control+saline versus trained+CHX P=0.99; within training session: control+saline versus trained+saline *P<0.05, trained+saline versus trained+CHX **P<0.01 (control+saline n=33, trained+saline n=28 and trained+CHX n=28). (C) Total distance traveled by each animal in the testing and habituation sessions. Two-way repeated measures ANOVA: session: $F_{1,87}$=0.90, P=0.344; experimental group: $F_{2,87}$=0.97, P=0.384; interaction: $F_{2,87}$=1.13, P=0.329. Means±s.e.m.

traveled during the testing session (Fig. 6C). No significant differences were apparent between experimental groups, indicating that there was no effect of CHX on locomotion and that memory retention is susceptible to disruption when a protein synthesis inhibitor is injected immediately after training.

Memory retention on a conditioned stimulus with negative valence

As seen in Fig. 2B, animals could be separated by their initial predisposition to spending a greater amount of time in the SQ or in the WQ of the arena (initial preference). During habituation, 22.25% of the crabs actually spent more time in the SQ; therefore, in order to better understand and specifically evaluate the effect of a single training trial in animals with a negative initial preference, we analyzed the behavior of the subset of crabs characterized by spending less than 25% of the habituation time in the SQ (i.e. less than chance; animals exposed to a conditioned stimulus with an initial negative valence). We performed this type of analysis in 7 independent experiments, 3 involving non-injected animals and 4 involving animals that were injected with saline immediately after training, to check that this condition had no significant effect on the time spent in the SQ (P=0.551). As is evident in Fig. 7, animals were able to overcome the initial strong aversion to the SQ and form and retain a place preference long-term memory related to a conditioned stimulus which was initially avoided (P<0.01).

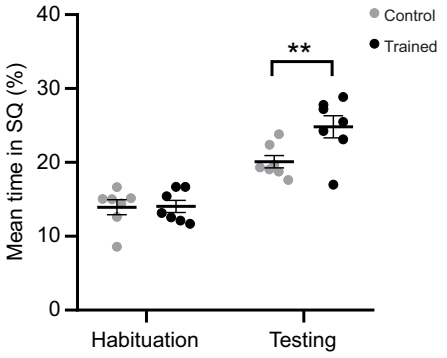

Fig. 7. Memory retention on a conditioned stimulus with negative valence. Mean time in the SQ of control and trained animals (n=7 experiments, 7–20 control crabs/experiment and 7–19 trained crabs/experiment). Three-way repeated measures ANOVA: session: $F_{1,10}$=128.5, P<0.001; experimental group: $F_{1,10}$=2,45, P=0.148; injection: $F_{1,10}$=0.38, P=0.551; interaction of session×injection: $F_{1,10}$=0.028, P=0.870; interaction of session×group: $F_{1,10}$=9.13, P<0.05; interaction of injection×group: $F_{1,10}$=0.92, P=0.359; Holm–Šidák contrasts: control versus trained at habituation: P=0.947; control versus trained at testing: **P<0.01. Means±s.e.m.

DISCUSSION

When exposed to a novel experimental arena which comprises a plain white environment with the exception of a striped section, crabs expressed a non-homogeneous spatial distribution in their exploratory behavior. The animals reacted to the striped pattern on the walls of the arena during the first exposure session, presenting a thigmotactic behavior that was reduced in the target quadrant (Fig. 2B). Moreover, they seemed to avoid the quadrant delimitation on the arena's floor, judging from their spatial distribution (higher occupancy in areas adjacent to the quadrant limits; Fig. 2A). In addition, animals showed a strong avoidance of the SQ (Fig. 2C,D). From this type of behavior, we can speculate that the striped pattern evokes some specific characteristic from the animal's original environment (for example, the interface between the intertidal flat and the striped pattern of plant bushes), but we cannot exclude the possibility that such a pattern is merely an attention attractor with negative valence. Given that the initial avoidance faded as the crabs' fasting period increased, an effect related to an attentional bias towards food-related stimuli is suggested. Similar observations have previously been reported relating fasting periods with cognition (Benau et al., 2014). This type of modulation of avoidance behavior by the animal's internal state resembles the behavioral changes reported in the *Drosophila* desensitization by hunger to bitter taste and 'negative smells' (Lin et al., 2019), as well as the preponderant use of idiothetic over sensory cues for foraging and feeding in hungry flies (Kim and Dickinson, 2017). Another possible interpretation of the crab's hunger modulation of their behavior is a shift towards the acceptance of a greater risk when exploring a novel environment, as seen by Godin and Crossman (1994) in their study on risk assessment by fish. Although this acceptance does not appear to be evident in the total amount of exploration (total walking distance), crabs may alter the tradeoff between the need for safety and energy acquisition, resulting in an increase in the tolerance towards a mildly aversive stimulus (higher PI value). This kind of

shift in exploratory strategy has already been reported in vertebrate and invertebrate models (Brown et al., 1992a,b; Ishihara et al., 2002; Wang et al., 2014; Ghosh et al., 2016).

The place preference memory paradigm described in the present work has the advantage of auto-administration of the reward by the animal, representing an unforced reinforcement, which could also be regulated in strength. A single training trial, which comprises a habituation period followed by exposure to the reinforcement, results in a significant change in the avoidance behavior of the animals towards the target quadrant, which lasts at least 24 h, implying the formation of a long-term place preference memory (Fig. 5B). This association between the reward and the SQ depends on the strength of the pairing between the two stimuli, expressed by the duration of availability of the reinforcement in the target zone. A short reinforcement period (2 min) is not enough to elicit the formation of long-term memory, whereas an 8 min reinforcement presentation period can achieve this (Fig. 5C). As other crustacean models require substantial iteration and stronger reinforcements such as opioids and psychostimulants in order to form a long-term memory (Dimant, 1991; Huber et al., 2018), we think that our paradigm offers the great advantage of exploring a one-trial naturalistic reward.

Further characterization of the memory resulting from this novel place preference paradigm confirmed that its consolidation depends on *de novo* protein synthesis (Fig. 6B), as has already been reported in several studies on this species (Fustiñana et al., 2013; Hermitte et al., 1999; Kaczer and Maldonado, 2009; Pedreira et al., 1995; Tomsic et al., 1998). Considering that animals were systemically administered with CHX immediately after the training trial, long-term stabilization of the memory trace is likely to be impaired by the inhibition of protein synthesis, rather than its acquisition. And, given that the total distance traveled in the experimental arena during the testing session did not differ between groups of animals that had been administered with CHX or saline control, we can conclude that the difference in exploratory behavior observed between these groups is due to an effect of the drug on memory retention, rather than a non-specific effect on the crabs' locomotion ability.

Even though the mean behavior expressed by each of the crab cohorts evaluated in the experimental arena showed an avoidance of the SQ on the habituation day (Fig. 2D), there remained a minor percentage of animals that expressed a preference for that quadrant (22.25% in Fig. 2C). When only those crabs that initially avoided the SQ were considered in the analyses, it was possible to unambiguously evaluate the association between a conditioned stimulus with an initial negative valence and an appetitive reinforcement (Fig. 7). The bases of memory retention of the association between a conditioned stimulus with an initially clear negative valence and an appetitive reinforcement could be further studied in this paradigm, opening the possibility of comparison with the animals that initially preferred the SQ, together with the modulation of this preference by hunger.

In summary, we present this one-trial place preference paradigm as a classical associative conditioning model, in which the crabs are able to associate a typical unconditioned stimulus (food pellet) with a clearly discernible part of the arena (SQ), which comprises a non-neutral conditioned stimulus. This particular trait of the conditioned stimulus is opposite to what is traditionally expected in this type of learning paradigm, which emphasizes the importance of a neutral stimulus in order to better understand and evaluate the association between the two (Pavlov, 1928). Nevertheless, it has been postulated that this type of approach is only representative of studies performed within specific laboratory conditions and fails to investigate the functional perspective of classical associative conditioning (Domjan, 2005). We strongly believe that the paradigm developed within the present work could provide a useful tool to study behavior in a naturalistic way, as introducing non-neutral conditioned stimuli allows evaluation of processes underlying animal conditioning as an adaptive trait.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.K., C.M., R.F.; Methodology: M.K., C.M., R.F.; Formal analysis: M.K., C.M., R.F.; Investigation: M.K., C.M.; Resources: R.F.; Data curation: M.K., C.M., R.F.; Writing - original draft: M.K., C.M., R.F.; Writing - review & editing: M.K., C.M., R.F.; Visualization: M.K., C.M., R.F.; Supervision: R.F.; Project administration: R.F.; Funding acquisition: R.F.

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