Conditioning of an autonomic response in Crustacea

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A B S T R A C T

Reports on experience-dependent changes in invertebrate autonomic function are few. In the crab Chasmagnathus, repeated presentations of a visual danger stimulus (VDS) elicit long-term cardiac adjustments. Although these changes can be explained in terms of an associative process, they do not necessarily indicate an anticipatory conditioned response. In the present work, we investigated anticipation of the cardio-inhibitory response (CIR) after classical conditioning. We found that an initially seemingly neutral stimulus, which could trigger only a brief CIR as part of an arousal/orienting response, following pairing with the unconditioned stimulus, 24 h after a second exposure, triggered a significantly stronger CIR response compared to controls. We propose that, as a result of training, the conditioned stimulus acquires a different biological meaning, allowing the crab to anticipate the aversive stimulus.

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1. Introduction

In vertebrates, the Pavlovian conditioning causes many biological changes, regardless of the specific conditioned response (CR) assessed [1,2]. Somatomotor responses (e.g., eyelash, nictitating membrane response, and leg flexion) are the most often measured CRs during classical conditioning, but conditioning also elicits non-specific responses. Even though non-specific CRs may also consist of somatomotor behaviors [3], learned visceral changes, including salivary secretion, heart rate, systemic blood pressure, skin conductance, and changes in pupillary diameter, have been most frequently studied. As a result, conditioning of the autonomic function has been clearly demonstrated in mammals, birds and fishes [4–11]. In invertebrates, previous reports on experience-dependent changes in the autonomic function were first limited to sensitization of the heart rate in the mollusk Aplysia [12] and habituation of the cardiac response to visual stimuli in the fly Calliphora [13], until Watanabe and Mizunami [14,15] demonstrated the classical conditioning of salivation in Periplaneta americana, suggesting that the sophisticated neural control of the autonomic function is not specific to vertebrates, but also applicable to insects.

The context signal memory (CSM) paradigm developed in the crab Chasmagnathus granulatus is mediated by an association between the environmental features of the training site (the context) and the features of the movement of a screen above the animal, named visual danger stimulus (the signal) [16]. This long-term memory was usually assessed by a somatomotor response until our previous work examining both the behavioral response and the concomitant neuroautonomic adjustments, namely a cardio-inhibitory response (CIR) [17]. Our results supported the view that the same memory process brings about changes in both responses. Vigorous escape, reversible heart arrests and bradycardia were considered three parameters of the unconditioned response, while minor escape, no heart arrests and bradycardia attenuation were considered three parameters of the learned response. However, although the adjustments in the autonomic function can be explained in terms of the same associative process that explains the observed changes in the escape response, they do not necessarily indicate an anticipatory conditioned response. These adjustments are only apparent upon the presentation of the unconditioned stimulus, i.e.: the visual danger stimulus (VDS), 24 h after training and not upon re-exposure to the training context. Thus, the present investigation was aimed to find an anticipatory cardiac response after classical conditioning.

Several antecedents have proved valuable to accomplish the above-mentioned aim. Earlier studies have revealed that light (infrared, dim red and white) caused alterations in the heart rate of crayfish [18–20]. In addition, we have recently observed that in the crab a short light pulse elicits a brief CIR but no escape response [21], thus suggesting it is a relatively neutral stimulus. Taking into account that it has been suggested that, in crayfish — which are closely related to crabs — relatively “neutral” unexpected external stimuli might

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trigger some processing of the information about the “novel” stimulus and its possible consequences [22], we predicted that the light pulse might achieve a different meaning after its pairing with an unconditioned stimulus following classical conditioning.

Regarding the unconditioned stimulus, previous results have pointed to the utilization of visual cues as reinforcement in a conditioning paradigm. More than 20 years of work with Chasmagnathus did not, in our laboratory, have shown that the VDS suddenly presented above the animal is immediately recognized by the crab as an impending threat, and thus directional escape is elicited [23]. Recently, we have further characterized stimuli regarded as threatening (the VDS and virtual looming stimuli) by comparing their effects on both cardiac and locomotor activity [24]. Parallel observations were also carried out in the field, where escape is easily triggered by either natural threats or the presentation of a simulated menace (the rectangular screen) introduced in the real environment [24]. Furthermore, upon the repeated presentation of the VDS, the crab’s response declines and is replaced by a strong freezing, which persists over time [25]. Therefore, if the context-VDS association underlies the CSM paradigm, the VDS might act similarly as an appropriate reinforcement in a light pulse-VDS pairing.

2. Materials and methods

2.1. Animals

Adult male C. granulatus crabs 2.7–3.0 cm across the carapace, weighing approximately 17 g, were collected in the rias (narrow coastal inlets) of San Clemente del Tuyú, Buenos Aires Province, Argentina, and transported to our laboratory, where they were lodged in plastic tanks (35×48×27 cm) filled with diluted seawater to a depth of 2 cm and a density of 20 crabs per tank. The water used in the tanks and other containers during the experiments was prepared using hw-Marine (Winex, Hamburg, Germany), salinity 10–14‰, at a pH of 7.4–7.6, and maintained within a range of 22–24 °C. The holding and experimental rooms were maintained on a 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) at 22–24°C. Experiments were run between 8:00 A.M. and 7:00 P.M. and performed within the first two weeks after the animals’ arrival. Each crab was used only in one experiment. All the recording experiments were conducted 2 or 3 days after the initial wiring of the animals. During this period, crabs were kept in individual containers and fed rabbit food pellets (Nutrients, Argentina) daily. Following the tests, animals were returned to the field and released in an area 30 km away from the capture area. Experimental procedures are in compliance with the Argentine laws for Care and Use of Laboratory Animals.

Prior to the experiment crabs were immobilized by enclosing them in close-fitting thick elastic bands, with the legs positioned in an anterior position and slightly under their bodies to restrict movement. This procedure allowed us to stabilize the electrocardiogram (ECG), making it easier to quantify the heart rate.

2.2. Electrocardiographic (ECG) recording

A small jack with two metallic pins where the electrodes were soldered was cemented with instant adhesive to the dorsal carapace in a position anterior to the heart. The metallic pins were made of silver wire (0.25 mm in diameter, VEGA & CAMJI S.A., Argentina) previously cut in 2.4 cm long sections. The free end of both wires was inserted in holes drilled in the cardiac region of the dorsal carapace placed to span the heart in a rostral-caudal arrangement and separated 4–5 mm. The electrodes easily pierced the hypodermis and were cemented in place with instant adhesive (Fig. 1). All the recording experiments were conducted 2–3 days after the initial wiring of the animals in order to allow them to recover from the stressful handling, which has been shown to alter the heart rate (HR) for a few days [26]. Prior to each experiment, the crab was lodged in a container called actometer: a bowl-shaped opaque container with a steep 12 cm high concave wall (23 cm top diameter and 9 cm floor diameter) covered with marine water to a depth of 0.5 cm and illuminated with a 10 W lamp placed 30 cm above the animal. A plug connected to the impedance converter (UFI, model 2991, California, USA) was slotted in each jack cemented on the animal in order to monitor the HR. The impedance converter measured the changes in the resistance between two electrodes, associated with the hemolymph movement after each heart contraction [19,20,26]. The output from the impedance leads was sent to the analog-to-digital converter of a computer data acquisition and analysis system (Fig. 1).

2.3. The conditioned stimulus (CS): a light pulse

A 2 s white light-emitting-diode (LED) light was presented 7 cm above the animal (Fig. 1). The specification for a 5 mm White LED was: luminous intensity 10,000 mcd and viewing angle: 23°. The illumination intensity measured in the actometer was 450.0 mW/m² and 1267.0 mW/m² before and after stimulation, respectively.

2.4. The unconditioned stimulus (UCS): a visual danger stimulus (VDS)

The VDS, which consisted of an opaque rectangular screen (25×7.5 cm²) positioned 6 cm above the animal, was moved horizontally from left to right and vice versa. Each VDS lasted 5 s and comprised two successive cycles of screen movement (Fig. 1).

2.5. Experimental set up

The experimental room had 20 actometers, separated from each other by partitions, and was dimly illuminated. Prior to each experiment, the wired animals were carried individually to the experimental room, located in their respective bowl and connected to the impedance converter. A computer was used to program trial sequences and stimuli presentation and to monitor the experimental events.

The computer scanned each actometer in an orderly and consecutive manner during an interval of 9 s during which stimuli were presented (a trial). A complete assessment around the 20 actometers was thus completed in 3 min (9 s×20 actometers).

2.6. Conditioning design and procedure

The experiment lasted 2 days and included two phases on each day: an adaptation session (10 min) and a training session (20 trials) on Day 1, and an adaptation session (10 min) and a testing session (6 trials) on Day 2. During the adaptation session on each day, baseline cardiac activity was recorded and the animals were allowed to adapt to the experimental set up without being disturbed. Training and testing immediately followed the adaptation session on each day. The training and testing sessions lasted 60 min (9 s×20 actometers×20 trials) and 18 min (9 s×20 actometers×6 trials), respectively.

2.6.1. Training session

2.6.1.1. Day 1. Four groups, 30–40 crabs each, were formed and trained simultaneously (Fig. 2A). Two control groups were used to assess the effects of either the conditioned (CS) or the unconditioned (UCS) stimulus. The CS control group received 20 presentations of a 2 s white light-emitting-diode (LED) light pulse with an intertrial interval (ITI) of 3 min, whereas the UCS control group received 20 presentations of a 5 s VDS with an ITI of 3 min. In both control groups, the stimulus was delivered after a 3 s delay in order to allow the recording of the basal heart rate before stimulation. A third control group, the unpaired non-contiguous group (UP) was an associative
control group in which the crabs received 20 presentations of the CS and the UCS unpaired in such a way that the onsets of the CS and the UCS were 80 s apart. The fourth group, the paired contiguous group (PAI) was the experimental group in which crabs received forward paired presentations of both stimuli: 20 trials of the CS offset immediately followed by the UCS onset with an ITI of 3 min. Thus, the CS and US groups controlled the effects of the iterative presentation of either the conditioned or the unconditioned stimuli, while the UP group controlled the effects of the iterative presentation of both stimuli unpaired. In addition, the fact that both in the CS and UCS groups the stimulus was presented after a 3 s delay allowed examining the CIR habituation to each stimulus during training.

Immediately after the training session, crabs were moved from the training location and housed individually in the resting containers, i.e., plastic bowls covered with water to a depth of 0.5 cm and kept inside dimly lit drawers for 24 h.

Results of the UP group are not shown here because the development of the training could not be traced, as crabs in this group received both stimuli separated by 80 s, an interval that was longer than the 9 s recording interval (see Fig. 2A). For the other three groups, the 9 s trial time during which the CS, the UCS or both stimuli were dispensed, accurately overlapped with the recording interval.

2.6.2. Testing session

2.6.2.1. Day 2. After 24 h in the resting containers, all the crabs were placed in the training context and the 10-min adaptation session took place. Then, the CS (light pulse) was presented to the four groups after a 3-s delay during six trials with an ITI of 3 min (Fig. 2B). This procedure allowed the assessment of the long-term effects of the training during the re-exposure to the CS in the four groups. Rescorla [27] convincingly argued in favor of using this sort of analysis instead...
of a paired training-testing comparison, emphasizing the need to clearly distinguish between the time of input (training session) and the time of assessment (testing session).

2.7. Data collection

Cardiac activity was recorded during the trial period of 9 s during which the stimuli were presented. A number of previous studies have shown that the HR can vary widely, both between and within individuals as well as with the experimental conditions [17,28]. To control this variation, the HR was normalized by the baseline level by calculating a response index. The response index (%) was calculated as

\[ 100 \times \frac{\text{number of heart beats per minute during the first 3 s after the onset of the stimulus}}{\text{the basal heart rate}} \]

which was the number of heart beats per minute during the 3 s of the baseline period prior to the stimulus presentation. This normalization assessed the effects of the stimuli on the HR and also allowed comparisons between stimulus types. This method also controlled the variation in the BHR due to slight changes in ambient temperature or small differences in crab size. Since changes in the HR in response to sensory stimulation are usually rapid and very brief [14,17], the 3 s recording interval after the onset of the stimulus provided a suitable interval to measure cardiac responsiveness to sensory stimulation.

2.8. Statistical analysis

Data were analyzed using a complex design analysis of variance (ANOVA) with a between-subjects variable (treatment groups) and a within-subjects variable (four three-trial blocks for training and three two-trial blocks for testing) followed by Tukey’s post-hoc tests. Differences were considered significant at \( P \leq 0.05 \). All data presented in the figures are means and standard error (S.E.M.).

3. Results

3.1. Training: short-term changes in the cardiac response

A heart arrest can be generally observed as an increase in the duration of the interval between two beats although small differences can be readily distinguished between ECG profiles in different animals. Upon the light pulse onset (CS group), which is a stimulus regarded as innocuous, a small CIR, namely a weak heart arrest or a brief bradycardia, was recorded (Fig. 3). However, stimuli considered to be threatening, such as the VDS (UCS group), elicited a strong CIR, revealed by prolonged arrests of the heart and sustained bradycardia (Fig. 4). The same profile was observed in the PAI group, in which the strong response elicited by the VDS almost completely overshadowed the short-lived CIR triggered by the light pulse (Fig. 5).

The changes observed in the HR all along the training session are shown in Fig. 6A. Crabs in the CS group underwent a HR reduction of 15% in the first trial, a response that then rapidly declined. Crabs in the UCS group displayed a larger HR reduction of 50% in the first trial, a response that somewhat attenuated in the course of the training session, never reaching the baseline level. The response profile of the PAI contiguous group was very similar to that of the UCS and both differed substantially from the CS group. As reported above, training results of the UP group are not shown in this figure. Nevertheless, during training when the CS was presented, a small response indistinguishable from that recorded and shown for the CS group was observed during the first three trials that disappeared rapidly. Furthermore, as that found in the CS group, no other change was observed during training.

To analyze these results, data were clustered into four blocks of three trials in order to reveal the main trends in the data [29]. Fig. 6B describes the ANOVA performed per block of trials corresponding to four stages of the training session (First trial block = Trials 1 to 3; Second trial block = Trials 5 to 7; Third trial block = Trials 10 to 12 and Fourth trial block = Trials 18 to 20). A significant effect of treatment and time was
found \( F(2, 114) = 32.99, p < 0.0001 \) and \( F(3, 342) = 72.23, p < 0.0001 \), respectively. Furthermore, an interaction effect was found between them \( F(6, 342) = 6.92, p < 0.0001 \). The Tukey’s post-hoc test showed significant differences between groups, revealing significant differences in the first three-trial block \( (p \leq 0.0001) \) and in the second three-trial block \( (p < 0.005) \) between the UCS and the CS, and between the PAI and the CS, but no significant differences for the following trial blocks \( (p > 0.05) \). Additionally, no significant differences were found between the PAI and the UCS \( (p > 0.05) \).

3.2. Testing: long-term changes in the cardiac response

Testing results are shown in Fig. 7. An ANOVA test performed in three blocks of two trials (First trial block = Trials 1 to 2; Second trial...
Trials 3 to 4; Third trial block = Trials 5 to 6) revealed a significant effect of treatment and time \( F(3, 148) = 6, p < 0.001 \) and \( F(2, 296) = 11.20, p < 0.0001 \), respectively, but no interaction between them. When a Tukey’s post-hoc test was performed between the four groups, significant differences were found in the first two-trial block \( (p < 0.05) \) between the PAI and the other three groups (UCS, CS, and UP). Additionally, no significant differences between the UCS, CS and UP groups were found \( (p > 0.05) \). Differences between the PAI and the other three groups (UCS, CS and UP) were no longer significant for the second and third two-trial blocks \( (p > 0.05) \).

4. Discussion

The main finding of the present report is that an anticipatory response emerges upon CS re-exposure during testing, thus supporting conditioning of the cardiac response in the crab Chasmagnathus. This is the first evidence describing the classical conditioning of an autonomic response in Crustacea. The fact that those animals that experienced the VDS during training (UCS and PAI groups) are indistinguishable from those in the CS group during testing discards a more general explanation in terms of sensitization to the threatening stimulus. Additionally, the differences found between these three groups underestimate the possibility that an association between the VDS and the context has taken place during training.

After a first presentation of either a light pulse or a VDS in the animal’s environment, rapid cardio-inhibitory alterations in the heart rate can be induced. However, stimuli regarded as innocuous elicit weaker heart arrests and brief bradycardia, while stimulus considered threatening produce prolonged heart arrests and sustained bradycardia; escape is elicited only in the latter circumstance [21]. Similarly, the magnitude of reversible cardiac arrests or bradycardia in fish depends on the biological importance of the stimuli [30]. In our case, a screen moving over the animal (VDS) may be interpreted by the crab as an approaching aerial predator and therefore its biological significance may be greater than that of a non-specific stimulus like the onset of a light.

That in the short term during training the cardiac response to the CS (i.e.: CS group) habituated promptly is in accordance with the proposal stated above, while the VDS (i.e.: UCS group) continued to elicit a strong CIR until trial 10 (Fig. 6A), showing resistance to habituation. Rooney and Laming [31] also found that more intense stimuli were correlated with increased initial physiological (cardiac and respiratory) response, and correlated this with the number of stimulus presentations required for habituation.

However, during testing, the CIR to the presentation of the CS had probably different biopsychological meanings in the four groups assessed. Animals in the UCS, CS and UP control groups exhibited a weak CIR to the light pulse, but animals in the PAI experimental group were responsive to the CS on the testing day, showing a stronger CIR significantly different from the other three groups.

Presumably, after the presentation of the light pulse in temporal proximity with the VDS during training, the CS may have probably acquired a different biological meaning as a result of the CS-UCS pairing. Similar results have been reported in fish [11]. In contrast, animals in the UCS control group, which had never come into contact

Fig. 6. A: Time course of the normalized HR (Response index %) during the training session for three groups (CS, UCS and PAI groups). The vertical bars represent the S.E.M. B: Response index (%) during four blocks of three training trials of the three groups compared to the basal heart rate (BHR). Tukey’s post-hoc test showed statistical differences for the 1st and 2nd block between UCS and CS and between PAI and CS. The vertical bars represent the S.E.M. and the asterisks indicate significance (*** \( P \leq 0.0001 \); ** \( P = 0.001 \)).

Fig. 7. Response index (%) during three blocks of two testing trials of the four groups (CS, UCS, UP and PAI) compared to the basal heart rate (BHR). Tukey’s post-hoc test showed significant differences for the 1st block between the PAI group and the three other groups (UCS, CS, and UP). Statistical differences are no longer observed in the following blocks, although a tendency remains. The vertical bars represent the S.E.M. and the asterisk indicates significance (* \( P \leq 0.05 \)).
with the light pulse, are indistinguishable from those in the CS control group. Interestingly, crabs in the UP group trained with a light pulse unpaired with the UCS, differed significantly from the PAI group and not from the former two groups. Results from the UCS and UP groups suggest that no sensitization has occurred.

Although it is well-accepted that most animals engage in the traditional fight-or-flight response by increasing ventilation rate and cardiac output after a sudden environmental stimulus as a defensive reflex [32], many animals may temporarily reduce (bradycardia) or interrupt the heart rhythm, inducing reversible cardiac arrests [33] in taxa as diverse as mollusks [34], crustaceans [35], fish [36], amphibians [37], birds [38] and mammals [39].

We hypothesize that cardioinhibition is universally adaptive, based on its distribution among vertebrate and invertebrate groups [40]. Although these cardiac changes have been identified as indices of emotion by many researchers [41,42], they may also be an adaptive response to environmental contingencies. That cardiac inhibition has been associated with attentional phenomena [43–46] and that a stressful situation or a reduction of alertness inhibits the arousal/orienting response may be intended to evaluate a novel stimulus.

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Similarly, these may seem simple gradations of the same response: small, non-threatening stimuli are subthreshold to trigger escape, but large stimuli elicit a strong cardiac response and escape. However, another possibility is altogether tenable: the cardiac response to the CS (light pulse) simultaneous with an arousal, or orienting response may be intended to evaluate a novel stimulus. Similar results have been reported in other invertebrates [22,54]. In contrast, the cardiac response to the VDS is regularly associated with a non-directional “arousal” that does not involve escape, fright or startle [53]. At first glance, these may seem simple gradations of the same response: small, non-threatening stimuli are subthreshold to trigger escape, but large stimuli elicit a strong cardiac response and escape. However, another possibility is altogether tenable: the cardiac response to the CS (light pulse) simultaneous with an arousal, or orienting response may be intended to evaluate a novel stimulus.

Using being alert to subtle environmental changes or escaping predation is associated with complex sensorimotor integration. This “fear, fight or flight” response gives support to the idea of an autonomic-like reflexive control in crustaceans. The responses of the cardiovascular and respiratory systems in crustaceans to environmental and socially imposed alerting stimuli are comparable to the responses of vertebrates mediated by the autonomic nervous system [56]. It is likely that the selective pressures which promote the development and maintenance of these autonomic responses in invertebrates are the same for higher animals.

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