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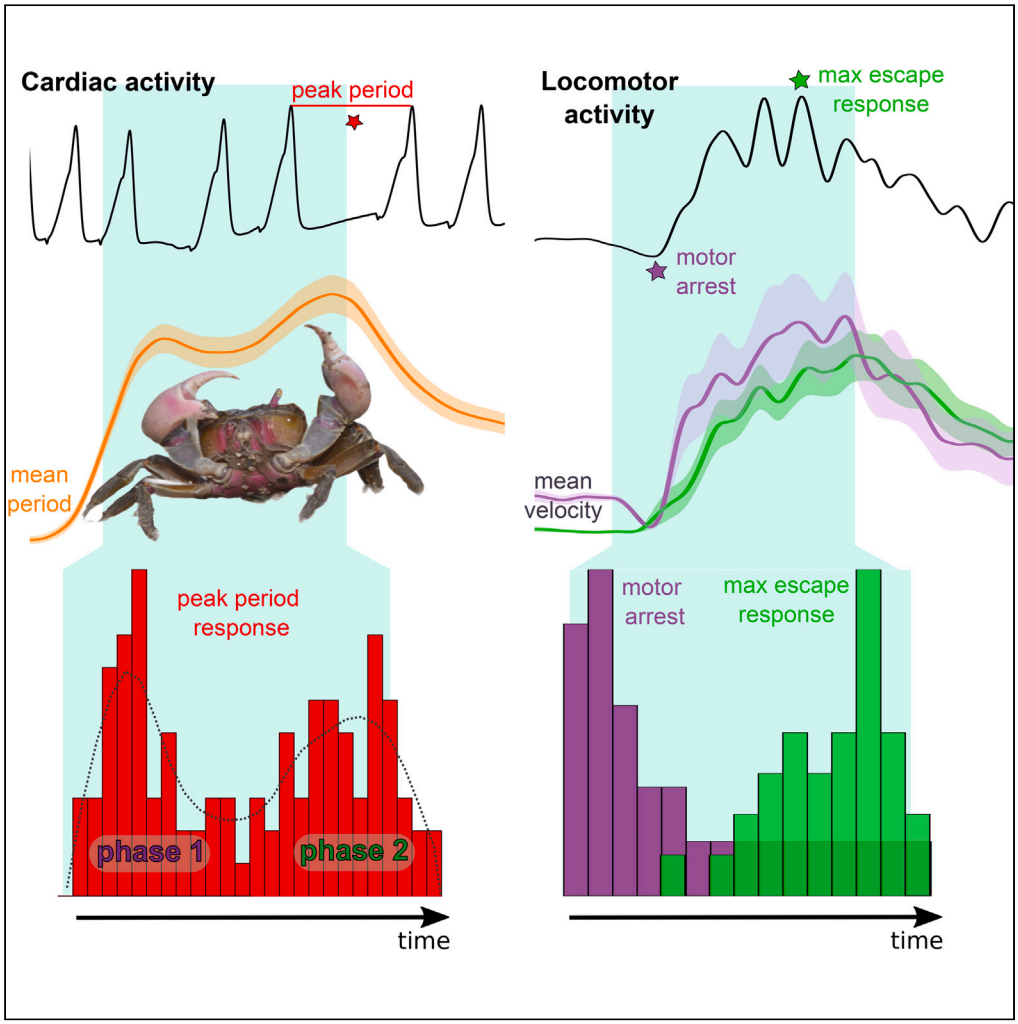

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Highlights

Threatening visual stimuli evoke two sequential cardiac response phases in arthropods

Low and high salience stimuli differentially modulate first and second cardiac phases

First cardiac phase would be related to animal's motor stop while second to escape

Cardiac activity modulation underlying defensive responses is rapid and flexible

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Threatening stimuli elicit a sequential cardiac pattern in arthropods

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SUMMARY

In order to cope with the challenges of living in dynamic environments, animals rapidly adjust their behaviors in coordination with different physiological responses. Here, we studied whether threatening visual stimuli evoke different heart rate patterns in arthropods and whether these patterns are related with defensive behaviors. We identified two sequential phases of crab's cardiac response that occur with a similar timescale to that of the motor arrest and later escape response. The first phase was modulated by low salience stimuli and persisted throughout spaced stimulus presentation. The second phase was modulated by high-contrast stimuli and reduced by repetitive stimulus presentation. The overall correspondence between cardiac and motor responses suggests that the first cardiac response phase might be related to motor arrest while the second to the escape response. We show that in the face of threat arthropods coordinate their behavior and cardiac activity in a rapid and flexible manner.

INTRODUCTION

When faced with a potential threat, an animal's nervous system first processes the available sensory information and then, as a consequence of this processing, orchestrates a particular motor plan to minimize animal damage together with the physiological actions that support it. These physiological responses typically include changes in heart and ventilation rate and metabolic adjustments.[1] A thorough understanding of these coordinated motor and physiological responses has been achieved mainly in vertebrates, particularly in mammalian species.[2] In contrast, the study of the defensive response in invertebrates has been principally focused on the motor component of the response[3–5] and to a lesser extent on the physiology.[6–9]

After the occurrence of a sensory stimulus that could indicate a threat, animals commonly increase the cardiac output to cope with the rise in the metabolic demands involved in the traditional fight-or-flight response.[10–12] Additionally, animals across many taxonomic groups can also produce an alternate physiological response to a threat. This response consists of a decrease of the cardiac activity (e.g., anurans[13]; fishes[14]; decapod crustaceans[15]). Different hypotheses have been stated to explain its functional role in different animal species (revised in King and Adamo[9]). The main proposals are that: the decrement in heart rate concomitant with behavioral freezing would reduce movement and noise from the animal which would help it hide from predators[16,17]; in preparation for flight, the reduction of the heart rate would be a compensatory response which may prevent blood pressure from rising too high as a consequence of the increase in the peripheral resistance that takes place with the stress response[14,18]; and the cardiac deceleration helps the brain increase sensory processing.[19–21]

In arthropods, a variety of optical and tactile stimuli induce transient cardiac arrests together with changes in animals' behavior.[15,22,23] Additionally, different studies have shown that even though no observable behavioral responses were elicited, heart rate was also affected by small environmental disturbances and by social interactions.[24–26] Given the remarkable sensitivity of this parameter to a variety of sensory modalities it has been posited that the cardiac response can serve as an indicator of perception in arthropods.[27]

In crustaceans, in crabs in particular, studies from our laboratory have shown that the saliency of threatening visual stimuli modulate animals' cardiac and escape responses.[28,29] Weak sensory stimuli – such as a light pulse or an air puff – elicit weak transient cardiac arrests with no overt behavioral responses; whereas stronger or more salient threat stimuli produce a stronger cardiac arrest together with vigorous escape responses.[7] Moreover, when visual stimuli of different dynamics, velocities or contrasts were presented, the latency, dynamic and intensity of cardiac and escape responses remained tightly correlated.[7,28]

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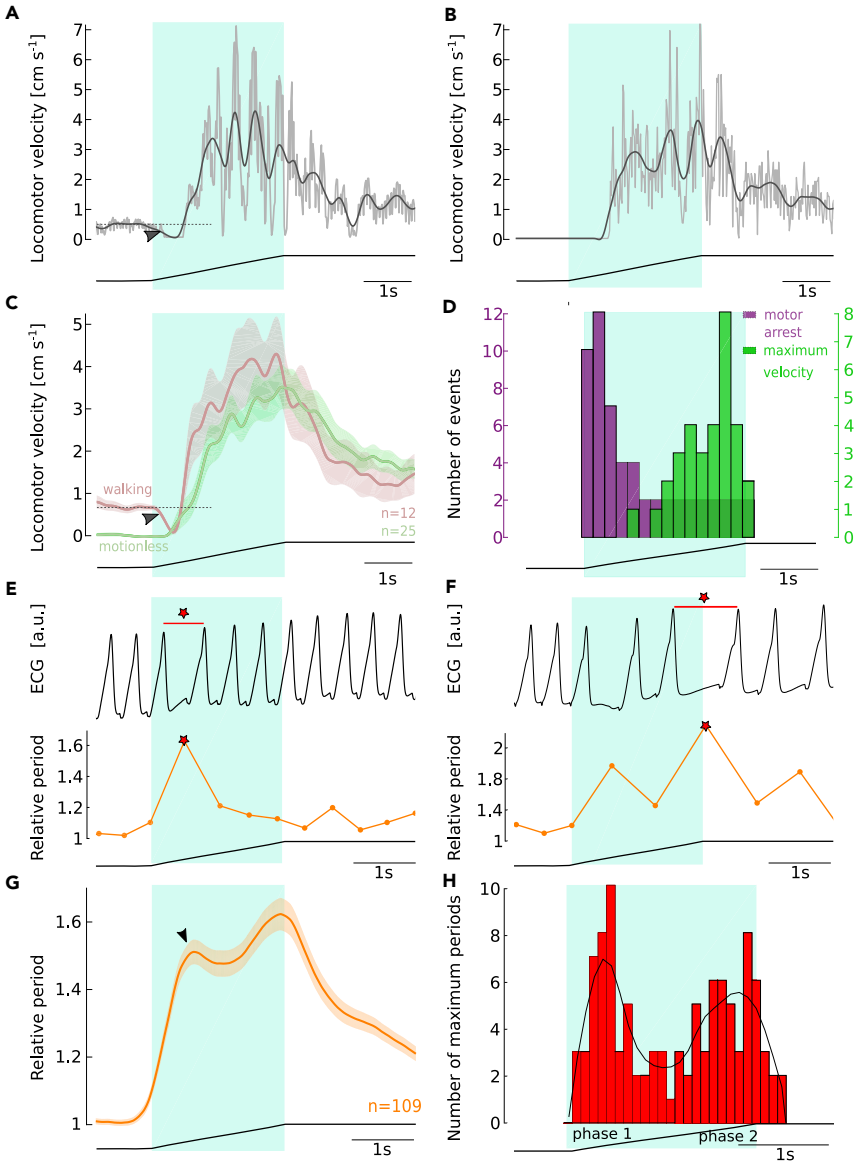

Figure 1. Behavioral and cardiac responses to moving edge stimuli

(A) Individual locomotor velocity as a function of time for a walking animal when faced to a moving edge (light blue shaded area). Dashed line indicates the average walking velocity previous to the stimulation and the arrowhead highlights animal's motor arrest.

(B) Individual locomotor velocity as a function of time for an animal that was motionless previous to stimulus presentation. For panels A and B the light gray line illustrates the original data and the dark gray line the smoothed data.

(C) Locomotor velocity of the animals that were walking (pale purple curve, mean ± s.e.m., n = 12) previous to stimulus presentation and of motionless animals (pale green line, mean ± s.e.m., n = 25).

(D) Peristimulus time histogram of animals that displayed motor arrest (purple histogram, n = 12) and peristimulus time histogram of the maximum locomotor velocity of escaping animals (green histogram, n = 37).

(E) Upper panel, representative electrocardiogram recording during visual stimulation with the moving edge. The red segment and the star highlight the maximum cardiac period occurring during stimulus presentation. Lower panel, quantification of the cardiac activity as explained in STAR Methods for this electrocardiogram. The orange dots represent the measured periods between two heart beats relative to the baseline period. The orange line is the linear interpolation between consecutive relative periods. Here, the maximum cardiac response period is also highlighted with a red star.

(F) Same representation as in E but here the maximum cardiac response period occurs by the end of visual stimulation.

(G) Relative cardiac period (means ± s.e.m., n = 109) as a function of time for animals that were visually stimulated with the moving edge. The black arrowhead indicates an inflection point in the average cardiac response curve.

Figure 1. *Continued*

(H) Peristimulus time histogram of maximum relative heart periods (red stars in panels E and F). The black line is the running average of the histogram values. Phase 1 and phase 2 were defined by clustering analysis (see text, k-means, squared Euclidean distance metric and two clusters).

In all the panels, the light blue shaded area indicates the time of presentation of the moving edge. The black line under the plots illustrates the horizontal position in the screen of the moving edge. The Michelson contrast of the moving edge in the experiments of current Figure was 0.8.

Interestingly, in vertebrates as well as in cephalopods, it has been observed that different sequential defensive behaviors such as startle, freezing or escaping are related with multiphasic heart-rate patterns.[9,30,31] Studies performed in crabs have shown that visual stimuli that mimic predators evoke first a brief animal motor arrest if the animals were already moving, and a subsequent escape response if the risk persists or increases.[5,32] Similar sequential defensive behaviors have been described in other arthropods species.[33,34] In arthropods it has been proposed that sequences of behaviors may be related to sequential heart-rate patterns as well.[8] However, this proposal has not been supported by experimental evidence so far (but see Barrios et al.[6]).

Here, we have studied whether sequential heart-rate patterns exist in arthropods and if the elements that compose these patterns may be related with sequential defensive behaviors. To this purpose we confronted animals with edge motion stimuli moving laterally at low velocities. By doing so, we were able to separate in time the animal's initial motor arrest from its later escape response; and we have identified two sequential phases of the crab's cardiac response that occur with a similar time pattern to that of the behavioral responses. The first phase of the cardiac response was mainly modulated by low salience stimuli (low contrast edge motion) and persisted along the repetitive spaced presentation of the stimulus. Instead, the second phase of the cardiac response was strongly modulated by high contrast stimuli and was reduced by repetitive stimulus presentation. The overall correspondence between the cardiac and motor responses suggests that the first component of the cardiac response could be related with the initial locomotor arrest while the second one with the animal's escape response. Finally, we discuss how the different heart rate phases could be recruited by neural circuits arising in the animals' optic ganglia.

RESULTS

The defensive responses of crabs evoked by visual stimuli have been thoroughly studied in the field and in controlled laboratory conditions (reviewed in Hemmi[35]; Tomsic et al.[36]). In both contexts visual figures translating tangentially or with looming dynamics provoke first a motor arrest if the animals were walking and, if the stimulus was sufficiently salient, a later escape response in opposite direction to that of the stimulus. Here, we stimulated the animals with a highly simple stimulus, a single edge translating laterally at constant angular velocity.[37,38] The stimulus was presented on a monitor screen located at the animal's lateral visual field. At the moment of stimulus onset, we identify two groups of animals, those that were walking and those that were motionless. In animals that were walking, the stimulus first evoked a motor arrest (Figure 1A, arrowhead) and a later escape response in the opposite direction of that of stimulus presentation (directions not shown). In motionless animals only the escape response was evident (Figure 1B). Figure 1C illustrates the average locomotor velocity of the animals that were walking (pale purple line, n = 12) or motionless (pale green line, n = 25) at the moment of stimulus presentation. As we have observed before for many different stimulation dynamics,[39] the average escape velocity increases throughout stimulus presentation and sharply decreases when visual stimulation ceases.

As walking velocity is rather slow, the reduction in the average locomotor velocity observed in animals that were walking upon stimulus presentation seems not to be a great behavioral change (Figure 1C). However, all the animals that were walking at the beginning of stimulation completely ceased their movement. To better assess the occurrence and temporal profile of this categorical response, we generated a peristimulus time histogram in which we analyzed for each temporal bin the number of animals displaying a motor arrest (Figure 1D, purple histogram). During the first 200 ms 10 out of 12 animals displayed a motor arrest and between 200 and 400 ms all animals had stopped moving; by 1–1.2 s all except two animals had abandoned the immobility and intended to evade the stimulus. We also assessed the time in which each animal produced its maximum escape velocity irrespectively if it was a walking or a motionless animal before stimulus onset (Figure 1D, green histogram, n = 37). The histograms of Figure 1D illustrate motor arrest and escape responses in the same graphical representation. Highly similar peristimulus histograms were obtained for different edge angular velocities ($20°s^{-1}$, $30°s^{-1}$ and $60°s^{-1}$; Figure S2).

Previous studies performed in crabs have shown that the appearance of a visual threat gives rise to a transient cardiac arrest.[7,28,40] The upper panels of Figures 1E and 1F show two representative electrocardiogram recordings obtained during visual stimulation with the moving edge at $30°s^{-1}$. Below each electrocardiogram the quantification of the relative period between consecutive peaks of cardiac activity is shown. The orange dots represent the value of the periods between two heartbeats relative to the baseline period (see STAR Methods) and the orange line is the linear interpolation between consecutive relative periods. Figure 1G displays the average cardiac response to visual stimulation (n = 109). As we have observed before for other threatening visual stimuli, relative heart rate period increases along with stimulus presentation and sharply decreases when visual stimulation ceases.[28,40] However, it was striking that despite the fact that in the current study the stimulus has a constant angular velocity and hence we would have expected a gradual increase of the relative heart period, here, we observed at the beginning of stimulation an inflection point in the average cardiac response curve (Figure 1G black arrowhead). When looking carefully at individual electrocardiograms we observed that maximum relative periods were located either in the first part of the stimulus presentation or by the end of the stimulation (red stars in Figures 1E and 1F respectively). Moreover, in many cardiac responses a local maximum at the beginning or at the end the stimulation period could also be appreciated. The case displayed in Figure 1F is one of these cases in which a local maximum is observed at the beginning of the stimulation period while the maximum response occurs at its end. To avoid uncertainties in the determination of local maximums, we simply assessed the time of occurrence of the maximum relative heart period along stimulus presentation. The resultant histogram (Figure 1H) indicates that the occurrence of maximum heart period is not unimodal (Dip test of

unimodality,[41] D = 0.07, p < 0.001, n = 109). We have defined two groups of responses using clustering analysis (k = 2, sumd1 = 4.0 and sumd2 = 5.5, n = 109). A first group of responses at the beginning of visual stimulation time (center id1 = 0.65 s) and the second group by the end of stimulation (center id2 = 2.0 s). When we reduced the stimulus velocity (15°s^{-1}), an initial group of maximum relative periods and more dispersed later responses could still be identified (Figure S3). However, when we doubled the edge velocity (60°s^{-1}), the stimulation time became too short to temporally disclose two separate cardiac components (Figure S3).

These results indicate that the evoked cardiac activity is composed of two components. We named these components as phase 1 and phase 2 cardiac responses (Figure 1H). We hypothesized that two different physiological processes underlie these two phases. To test this hypothesis, we performed the following experiments aimed to modulate differentially the two components.

Modulation of the cardiac response by the visual saliency of the threatening stimulus

A simple way to modulate the salience of a visual danger stimulus is to modify its contrast.[29,42] Increasing the contrast of the moving edge effectively led to an increase in the escape response (Figure S4) as well as an increase in the cardiac response (Figures 2A–2C). Figure 2A shows the cardiac activity of a representative animal when confronted with a low, medium and high contrast stimulus (Michelson contrasts 0.30, 0.62, 0.77 respectively). In these examples, at the beginning of the stimulation, for low contrast stimulus the cardiac response presents a global maximum (Figure 2A, star symbol) while for medium and high contrast stimuli presents a local maximum which seems not to be highly modulated by the stimulus contrast (Figure 2A, pentagon symbol). However, by the end of the stimulation, the medium and high contrast stimuli produced maximum relative periods that seem to be modulated by contrast (Figure 2A). At first glance, the average cardiac response of 35 animals to a broader set of contrasts confirms that stimulus contrast produces its principal effect by the end of the stimulation (Figure 2B). One difficulty we faced to properly quantify a differential effect of contrast upon the two phases is that both responses overlap at the middle of the simulation time and, thus, it is not possible to measure one response independently of the other. Therefore, we have performed different analyses that complement each other to find out if effectively there is a differential effect of the contrast on the two proposed cardiac response phases. Figure 2C shows the quantification of the area under the curve for phase 1 and phase 2 cardiac responses. The area of both phases increased significantly with contrast (phase 1: slope = 0.08, slope confidence interval = [0.05, 0.11], n = 35; phase 2: slope = 0.25, slope confidence interval = [0.20, 0.29], n = 35). However, the relative contribution of phase 2 response (rPh2 area, see STAR Methods) to the total cardiac response increases significantly with the contrast of the stimulus presented (Figure 2D, slope = 0.86, slope confidence interval = [0.58, 1.14], n = 35).

We have also analyzed the peristimulus time histograms of the maximum relative periods evoked by each contrast stimulus (Figure 2E). For high contrast stimuli, maximum relative periods occurred mainly by the end of visual stimulation, while for low contrast stimuli, they usually occurred at the beginning. The proportion of phase 2 events with respect to the total number of events (pPh2, see STAR Methods) correlates positively with the contrast of the stimulus (Figure 2F; pPh2 events = 0.47 x + 0.19; R^2 = 0.90, n = 8). As a whole, these results indicate that phase 1 and phase 2 responses are elicited by a wide variety of contrast stimuli; however, phase 2 response is more sensitive to stimulus contrast than phase 1 response.

Modulation of the cardiac response by spaced stimulus presentation

Animal defensive responses are greatly influenced by stimulus repetition.[43] Particularly in crabs, the repeated presentation of a visual danger stimulus gives rise to a reduction of the escape response (reviewed in Tomsic et al.[44]). Previous studies performed on *Neohelice* have shown that the cardiac response as a whole also decreases as a consequence of the repeated presentation of threatening visual stimuli.[45] Here, we studied if both cardiac phases are similarly affected by the spaced presentation of 5 stimulation trials with an intertrial interval of 10 min.

For simplicity, Figure 3A shows the cardiac response of an animal to three of the stimulation trials (trials 1, 2 and 4; T1, T2 and T4 respectively) while Figure 3B shows the average response to each of the five stimulation trials across all trained animals (n = 23). At the beginning of the stimulation we did not observe noticeable differences in the cardiac response across trials. However, by the end of the stimulation the cardiac response decreases with stimulus repetition. The quantification of the phase 1 and phase 2 responses across stimulation trials is shown in Figure 3C. Phase 1 response area is not modified across trials (Figure 3C; ANOVA, F = 0.67, p = 0.61; n = 23). In contrast, phase 2 response area decreases with stimulus repetition (Figure 3C; ANOVA, F = 2.44, p < 0.05; n = 23). Consistently, the area of phase 2 response with respect to the total response area decreases with stimulus repetition (Figure 3D; Wald test, chi square = 21.58, p < 0.001, odds ratio T1/T5 = 2.37, confidence interval = [1.39, 4.06], n = 23).

Figure 3E shows the peristimulus time histogram of maximum relative periods for the successive stimulation trials. For the first trial the maximum relative periods are similarly distributed in the first and second half of the stimulation time (Trial 1: 10 and 7 events respectively), while for the last trial maximum relative periods concentrate at the beginning of the stimulation time (Trial 5: 19 and 1 events respectively). The proportion of phase 2 events with respect to total events correlates negatively with stimulus repetition (Figure 3F, pPh2 events = −0.09 x + 0.49; R^2 = 0.99, n = 5). The results of the current experiment indicate that the spaced repeated presentation of the stimulus (10 min intertrial interval) has no effect on the phase 1 cardiac response but produces a reduction of the phase 2 response.

Modulation of the cardiac response by massed stimulus presentation

In *Neohelice*, as well as in other vertebrate and invertebrate species, massed stimulations with visual danger stimuli give rise to a faster but short lasting reduction of the escape response than spaced stimulation (reviewed in Tomsic et al.[44]). A large amount of evidence has shown that the neural sites of plasticity, the cellular and molecular mechanisms that underlie the behavioral modifications induced by spaced and

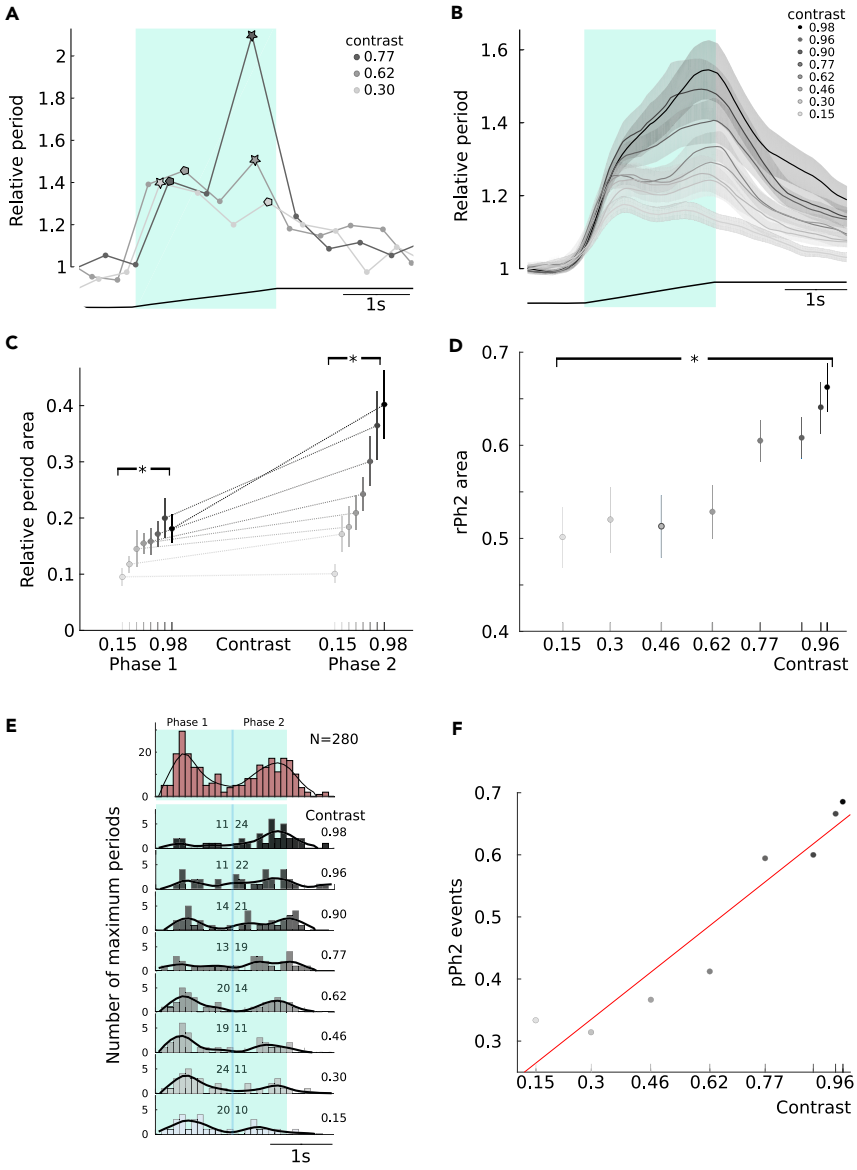

Figure 2. Modulation of the cardiac response by the visual saliency of the threatening stimulus

(A) Cardiac activity (relative period) recordings from a same animal to three stimuli with different intensity contrasts (Michelson contrasts = 0.30, 0.62, and 0.77). Stars represent the maximum relative heart periods while pentagons represent local maximum periods.

(B) Relative period (mean ± s.e.m., n = 35) as a function of time for animals that were visually stimulated with moving edges of different contrasts (Michelson contrasts = 0.15, 0.30, 0.46, 0.62, 0.77, 0.90, 0.96, and 0.98).

(C) Area under the curve (mean ± s.e.m., n = 35) of the individual cardiac responses for phase 1 and phase 2 time intervals.

(D) Relative contribution of phase 2 area (rPh2 = (Phase 2 area)/(Phase 1 area + Phase 2 area) as a function of stimulus contrast.

(E) Peristimulus time histograms of occurrence of maximum heart periods (e.g., stars in panel A) for the different edge contrasts. Top panel (light pink histogram) condenses the results for all the contrasts, 264 maximums were effectively detected from a total of 280 trials analyzed. Lower panels, the different gray histograms correspond to the different stimulus contrasts presented. The numbers in each panel indicate the number of maximums in each phase. The vertical cyan line indicates the separation of the two phases. The black lines are the running average of the histogram values.

(F) The graph illustrates the proportion of maximum heart periods events that occurred in the second half of visual stimulation with respect to the total number of events detected in both phases (pPh2 events = (# second half of stimulation)/(# first half of stimulation + # second half of stimulation) as a function of the stimulus contrast. The red curve represents the linear fit of the function pPh2 events = 0.47 x + 0.19 (R^2 = 0.90, n = 8).

In all the panels, the light blue shaded area indicates the time of presentation of the moving edge. The black line under the plot illustrates the horizontal position in the screen of the running edge. Increasing levels of gray represent a higher contrast. The sample size (n) for each experimental group in panels B–F is 35. Significant differences are indicated as follows: *p < 0.05.

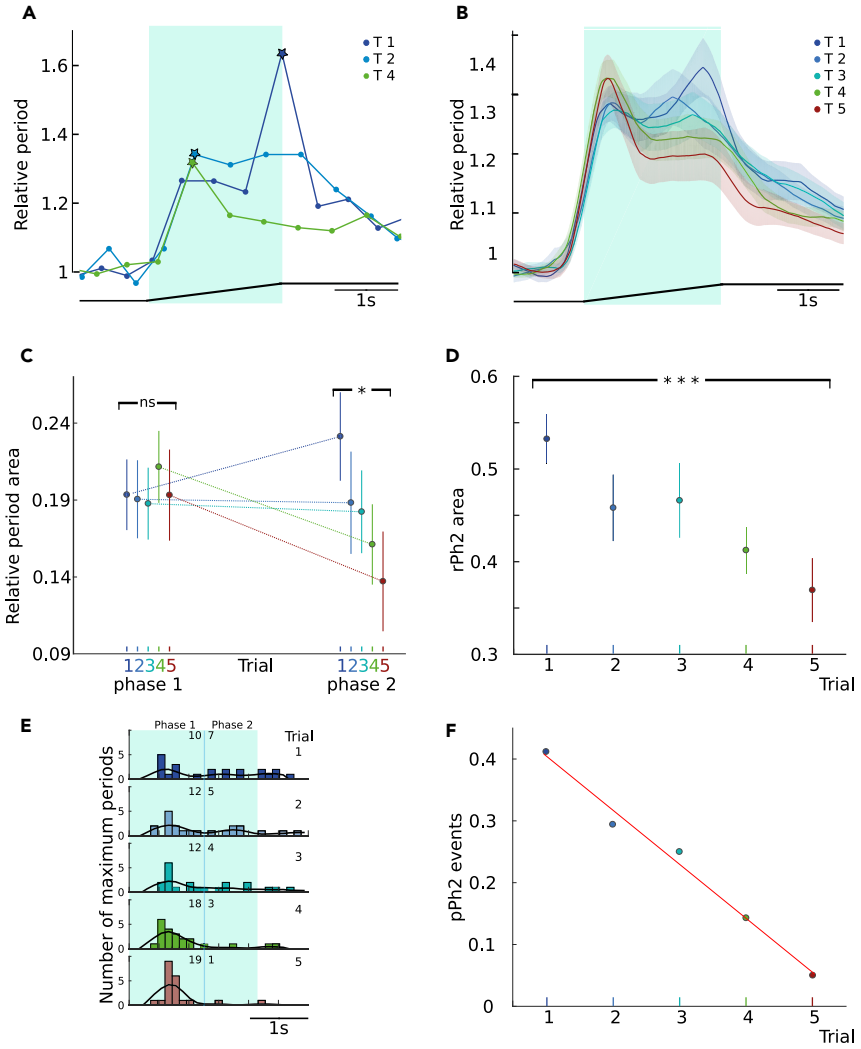

Figure 3. Modulation of the cardiac response by spaced stimulus presentation

(A) Cardiac activity response (relative period) recordings from the same animal to trial 1, 2 and 4 (blue, light blue and green respectively) with the same moving edge stimulus (intertrial interval = 10 min).

(B) Relative period (mean ± s.e.m., n = 23) as a function time for animals that faced five stimulation trials with the same stimulus.

(C) Area under the curve (mean ± s.e.m., n = 23) of the individual cardiac response for phase 1 and phase 2 time intervals for the trials one to five.

(D) Relative contribution of phase 2 area (rPh2 area) to the sum of phase 1 and phase 2 areas as a function of the trial number (n = 23).

(E) Peristimulus time histograms of occurrence of maximum heart periods for the successive stimulation trials (n = 23). The black lines are the running average of the histogram values. The numbers in each panel indicate the number of maximums in each phase.

(F) The graph illustrates the proportion of maximum heart periods events that occurred in the second half of visual stimulation with respect to the total number of events detected in both phases (pPh2 events) as a function of the trial number. The red curve represents the linear fit of the function pPh2 events = −0.09 x + 0.49 (R^2 = 0.99, n = 5).

The color code indicates the trial number (blue: trial 1; light blue: trial 2; green: trial 3; pale green: trial 4; and red: trial 5). The Michelson contrast of the moving edge was 0.8. Significant differences are indicated as follows: *: $p < 0.05$ and ***: $p < 0.001$; ns: non-significant difference. Other symbols as in previous figures.

massed training are different (reviewed in Tomsic and Romano[46]). Thus, we also evaluated if the massed presentation of the visual stimulus has a differential effect on phase 1 and phase 2 cardiac responses. To this purpose, we applied 5 stimulation trials with an intertrial interval of 7 s.

Figure 4A shows the cardiac response of a crab to a session of massed visual stimulation. The cardiac response was highest for the first stimulation trial and rapidly decreased for the following ones. Figure 4B shows the average cardiac response of 22 animals, while Figure 4C the quantification of phase 1 and phase 2 cardiac response areas across stimulation trials. In contrast to the results obtained for the spaced stimulation, massed stimulation produced a fast and significant reduction of the phase 1 response (Figure 4C; ANOVA, F = 28, 59, $p < 0.0001$, n = 22) as well as of the phase 2 response across stimulation trials (Figure 4C; ANOVA, F = 11, 16, $p < 0.0001$, n = 22). Unlike when presented with spaced stimulus repetition, the relative area of phase 2 response with respect to the total response area

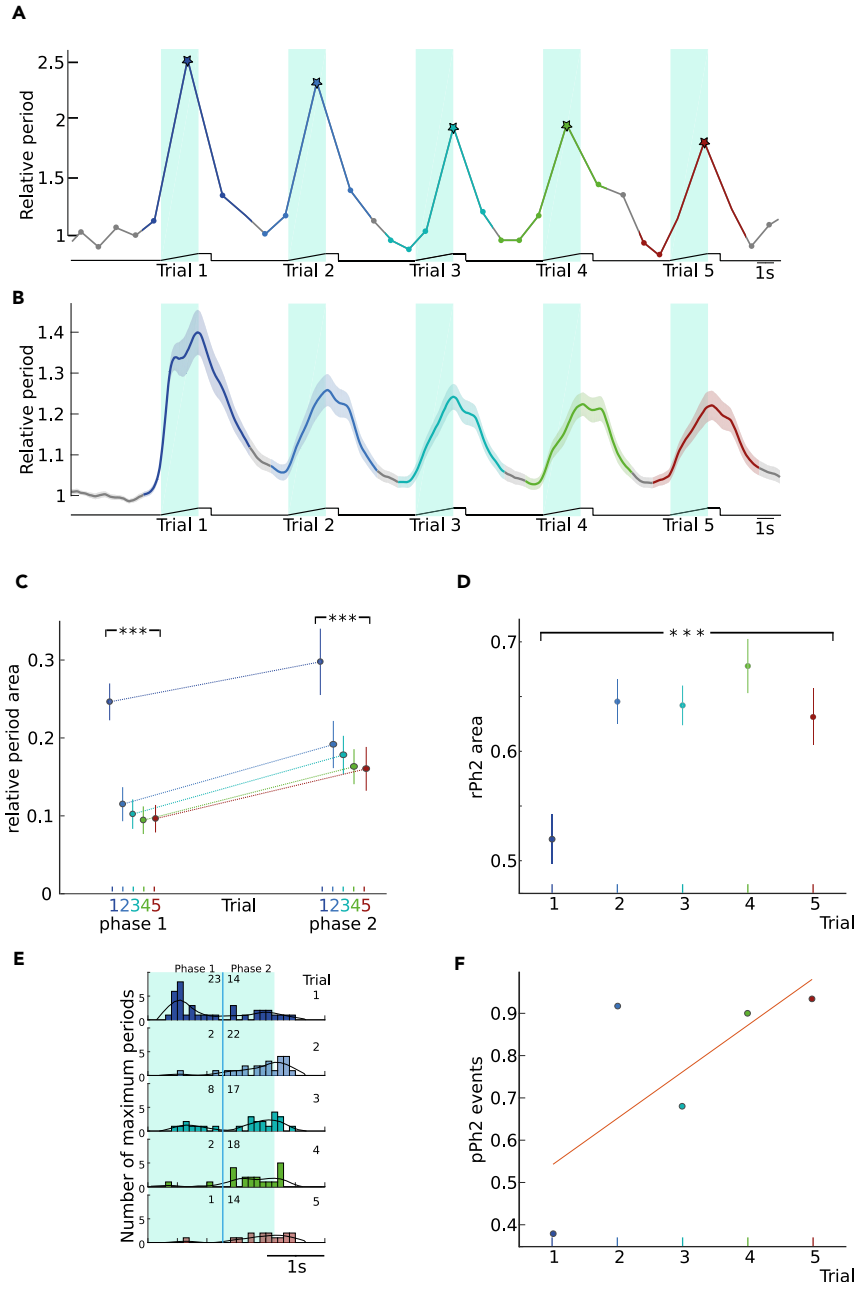

Figure 4. Modulation of the cardiac response by massed stimulus presentation

(A) Cardiac activity response (relative period) recordings from the same animal to five consecutive trials with the same moving edge stimulus (intertrial interval = 7 s).

(B) Relative period (mean ± s.e.m., n = 22) as a function of time for an animal that faced five stimulation trials with the same stimulus.

(C) Area under the curve (mean ± s.e.m., n = 22) of the individual cardiac responses for phase 1 and phase 2 time intervals for the trials one to five.

(D) Relative contribution of phase 2 area (rPh2 area) to the sum of phase 1 and phase 2 areas as a function of the trial number (n = 22).

(E) Histograms of occurrence of maximum heart periods for the successive stimulation trials. The black lines are the running average of the histogram values. The numbers in each panel indicate the number of maximums in each phase.

(F) The graph illustrates the proportion of maximum heart periods events that occurred in the second half of visual stimulation with respect to the total number of events detected in both phases (pPh2 events) as a function of the trial number. The red curve represents the linear fit of the function pPh2 events = 0.11 x + 0.43 (R^2 = 0.52, n = 5).

The color code indicates the trial number (blue: trial 1; light blue: trial 2; cyan: trial 3; green: trial 4; and red: trial 5). The Michelson contrast of the moving edge was 0.8. Significant differences are indicated as follows: ***: $p < 0.001$. Other symbols as in previous figures.

increased with massed stimulus repetition (Figure 4D; Wald test, chi square = 30.18, $p < 0.0001$, odds ratio T1/T2 = 0.59, confidence interval = [0.40, 0.88], n = 22). These results indicate that phase 1 is more sensitive to habituation than phase 2 when the animals are confronted with massed stimulus repetition.

Figure 4E shows the peristimulus time histogram of maximum relative periods for the successive stimulation trials. For the first stimulation trial, maximum relative periods are principally located in the first half of the visual stimulation time. In contrast, for the later trials maximum relative periods are principally located at the second half of the visual stimulation time. The proportion of phase 2 events with respect to the total number of events correlates positively with stimulus repetition (Figure 4F, pPh2 events = 0.11 x + 0.43; $R^2 = 0.52$, n = 5). Thus, both the analysis of rPh2 areas (Figure 4D) and the analysis of pPh2 events (Figure 4F) indicate that massed stimulus presentation produces a faster reduction of phase 1 than of phase 2 cardiac response.

DISCUSSION

It has long been proposed that in arthropods sequential behavioral patterns may correspond to sequential and multiphasic heart-rate patterns.[8] However, along with a recent study in flies,[6] this is one of the first studies to show that sequential multiphasic heart rate responses exist in arthropods and that these phases of the cardiac response may be related to sequential defensive behaviors. Here, by tuning visual stimulation, we have shown that two sequential cardiac responses can be elicited in crabs. In particular, we have observed that an edge moving laterally at constant angular velocity evokes a cardiac response that is indeed composed of two sequential phases, here named phase 1 and phase 2 cardiac responses (Figure 1). The phase 1 response is evoked by both low and high salience stimuli, whereas phase 2 responses are mainly evoked by high salience stimuli (Figure 2). The existence of two different phases in the cardiac response can be verified by the fact that the relative weight of each of these phases in the total cardiac response is differentially affected by two well-characterized habituation protocols that only differ in the intertrial interval[47] (Figures 3 and 4).

Relationship between behavior and cardiac activity

The experimental evidence obtained in arthropods has principally shown that low salience danger stimuli, regarded as innocuous, elicit weak heart rate changes with no accompanying behavioral responses.[7,24,25] Instead, more salient stimuli, apparently threatening ones, produce stronger cardiac responses together with accompanying modifications in behavior.[6,7] These observations may simply reflect gradations of the same cardiac response.[7,15] However, a recent study performed in flies has shown that upon an inescapable visual threat there is a cardiac acceleration during sustained animal running and a deceleration during animal freezing.[6] Here we have shown that upon a visual threat, the cardiac response of crabs presents two components and that these components can be evoked sequentially. In line with previous results in flies, our results suggest that arthropods coordinate their behavior and cardiac activity in a flexible manner.

In former research performed in *Neohelice* we have studied the cardiac responses of crabs when confronted with threatening stimuli that evoke evasive responses.[7,28,40] We had already observed that the magnitude of the overall cardiac response to these stimuli correlated with the magnitude of the escape response, even though in light of the current study the cardiac response as a whole must be reinterpreted. Here, we have observed that in particular the phase 2 cardiac response occurs around the same time as escaping and with a similar dynamic (Figures 1, S2, and S3). Consistently, the modification of stimulus saliency by increasing its contrast - known to increase crabs' escape response (e.g., Figure S4) - mainly increases the phase 2 response (Figure 2). Finally, when applying stimulation protocols known to habituate crabs' escape response[47] the phase 2 response was always reduced (Figures 3 and 4). Thus, throughout the current study we have observed a tight correlation between animal escape behavior and specifically the phase 2 cardiac response. Alternatively, it might occur that given that when recording the electrocardiograms the animals are immobilized, they display a freezing behavior when visually stimulated;[34,48] and, then, the cardiac responses we obtain correspond to this behavior.[6]

In the current study, we have principally found that there is an initial cardiac response phase that takes place at the beginning of visual stimulation, before the animal's escape response. This cardiac response phase occurs at a time highly similar to that of motor arrest (Figure 1). When presenting low contrast stimuli, a kind of stimulus that mainly evokes motor arrest (Figure S4), principally phase 1 responses were observed (Figure 2). Another stimulus that produces motor arrest but no escaping is a sudden change in illumination.[49] This stimulus produces just a brief heart deceleration at a timescale highly similar to the one obtained for the phase 1 cardiac response (data not shown). These observations suggest that the cardiovascular actions associated with phase 1 cardiac response could be related to the execution of motor arrest. Another possibility is that phase 1 cardiac response is related to actions that prepare the cardiovascular system to an eventual immediate evasive response.[8,9] Even when both hypotheses are not mutually exclusive, to discriminate between them, concomitant electrocardiograms and behavioral recordings should be performed in freely moving animals.

Neural control

Likely, the relations we observed between cardiac and motor responses originate in the underlying physiological mechanisms that orchestrate not only these actions but also the perceptual processes associated with defensive responses. Particularly, our results raise the question about which are the underlying physiological mechanisms that differentially recruit phase 1 and phase 2 cardiac responses. The fast and flexible manners in which these phases are evoked suggest their control would rely directly on the nervous system rather than on neurohemal mechanisms. In crustaceans, it has been shown that rapid heart rate decelerations are driven by a group of command interneurons located in the circumesophageal connectives whose activity is modified by sensory stimuli.[50,51] So far, these are our only candidate neurons for evoking

phase 1 and phase 2 cardiac responses. Furthermore, given the strong dependence of phase 1 and phase 2 responses on the visual attributes of the threat stimulus (e.g., Figure 2), another question that arises is which are the visual detection circuits involved in eliciting the cardiac response phases. Based on the relation observed between the cardiac activity and behavior it is reasonable to hypothesize that the same visual detection circuits underlie both kinds of responses. Visually evoked evasive behaviors in arthropods have been found to be guided by specific neural circuits arising in the third optic neuropil, the lobula.[52] In the crab we have identified a particular group of lobula tangential neurons involved in processing and conveying critical information to guide escape responses to impending threats.[53,54] The activity of these neurons reflect fundamental aspects of the crabs' escape performance.[29,55] These include modulations of the response intensity by stimulus contrast as well as the response modulation induced by spaced[56] or massed stimulus presentations.[57] So, as phase 2 cardiac response and escape behavior are tightly correlated, it is likely that lobula tangential neurons are also involved in eliciting this cardiac response phase. With respect to the phase 1 response, the visual circuits involved in eliciting orienting responses in arthropods, like the here described crab's motor arrest, have not been identified yet. Thus, it is not possible to postulate a visual detection circuit involved in eliciting this cardiac response phase.

Limitations of the study

Since we have performed the cardiac and behavioral studies in slightly different experimental conditions, the conclusions regarding the relationship between the behavioral and physiological outcomes require caution. However, the main conclusion of the paper – in crabs, the transient cardiac response induced by threatening visual stimuli is composed of two distinct, and separable, phases that can be modulated differentially by different parameters of a visual threat – is not conditioned by the experimental design used here.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108672.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.P.S., G.H., and M.B.A.; Methodology, V.P.S., G.H., and M.B.A.; Investigation, V.P.S., L.S., M.B., M.A.B., F.V.D., G.H., and M.B.A.; Writing – Original Draft, V.P.S., G.H., and M.B.A.; Writing – Review and Editing, V.P.S., G.H., and M.B.A.; Funding Acquisition, V.P.S. and M.B.A.; Supervision, V.P.S. and M.B.A.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Neohelice granulata male crabs	Captured in the rias of San Clemente del Tuyú.	Taxonomy ID: 53323
Software and algorithms		
Matlab	Matlab software	https://la.mathworks.com/products/matlab.html
R studio	RStudio, PBC (Delaware, USA)	RRID:SCR_000432

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martín Berón de Astrada (martin@fbmc.fcen.uba.ar).

Materials availability

Adult male *Neohelice granulata* crabs were collected from the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact (martin@fbmc.fcen.uba.ar) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Adult male *Neohelice granulata* crabs (previously *Chasmagnathus granulatus*) were used in the present study. Animals 2.7-3.2 cm width across the carapace were collected from the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina, and transported to the laboratory. The animals were kept in plastic tanks filled 1 cm deep with artificial marine water (Red Sea's Coral Pro Salt; salinity 10–14%, pH 7.4–7.6). The holding and experimental rooms were kept on a 12 h light/dark cycle (lights on 7:00 AM to 7:00 PM) and the experiments were run between 7:00 AM and 7:00 PM. All the experimental protocols were performed in accordance with relevant guidelines and ethical regulations of the School of Science, Universidad de Buenos Aires.

METHOD DETAILS

Experimental arenas

The arena for studying animal's locomotor activity consisted of a 40 × 40 cm area with a spherical treadmill located at its center (Figure S1A). Three sides of the arena were delimited with vertical white foam boards and the remaining side was occupied by a monitor screen that cast the visual stimuli (refresh rate 60 Hz; Philips 107T CRT, Suzhou, China). The spherical treadmill consisted of a 18 cm diameter Styrofoam ball floating on water that could be freely rotated by the animal.[29] A rod was fixed vertically with cyanoacrylate glue to the dorsal carapace of the animals and inserted into a guide that restricted rotational movements of the animals. The crabs were positioned 20 cm away from the monitor screen with the lateral pole of the eye looking at the center of it and, as they could not rotate, experienced all visual stimuli with this same region of the eye.[58] In the treadmill animals adopt their natural locomotor posture and freely move their legs, rotating the ball located beneath them while walking. Two position sensors (optic PC mice; Genius GM-04003P, Taipéi, Taiwan) recorded every 16.7 ms the "*x*" and "*y*" coordinates of the sphere. We used this information to reconstruct the attempted translational movements of the animals (see below). The delivery of the visual stimuli and the recording of the sphere positions were implemented with commercial software (Presentation 5.3, Neurobehavioral Systems Inc., Albany, CA, USA).

The recording of stable electrocardiograms of animals in the spherical treadmill proved difficult. Thus, heart rate was recorded in independent groups of animals. To perform these recordings the crab was held fixed by securing its claws and legs against its body with a rubber band. An adjustable clamp was used to hold the animal suspended in air at the center of an experimental arena highly similar to the one just described (Figure S1B). Three sides of the arena were delimited by vertical white foam and the remaining side was occupied by a monitor screen (refresh rate 60 Hz, LG-32LA613B, LG Electronics, Seoul, South Korea). The animals were also located 20 cm away from the monitor

screen with the lateral pole of the eye looking at its center. The delivery of the visual stimuli and the synchronization with the digitizer that recorded the cardiac activity was implemented using Matlab (MathWorks, Natick, MA, USA).

Visual stimuli and experimental protocols

The visual stimulus consisted of a vertical edge translating horizontally across the monitor screen at constant angular speed.[59,60] Edge motion stimuli are simple visual stimuli with only one border in motion. This simplifies the control of the effective angular size and angular velocity of the stimulus as well as the determination of the stimulation contrast given the stimulus possesses a single contrast transition.[37] The area of visual stimulation encompassed 78º x 69º in the locomotor activity arena and 66º x 58º in the arena where cardiac activity was studied. The edge occupied all the height of the stimulation area and advanced from the anterior- to the posterior-lateral visual field of the animals, an area where the horizontal sampling resolution of crab's eye is uniform.[58] Unless otherwise stated, the edge translated with a constant angular velocity of $30^{\circ}s^{-1}$.

Each crab was placed in the experimental arena and left 10 minutes undisturbed until the first stimulus presentation. Thirty seconds after the moving stimulus reached the end of the screen, the background illumination was eventually restored.

In the experiment presented in Figure 2 we have modified the saliency of the stimulus by varying the contrast of the advancing edge. The background was always white (RGB grayscale = 255) and the translating figure adopted different intensities (RGB grayscale = 224, 192, 160, 128, 96, 64, 32, 0). To calculate the stimuli contrast we measured the light casted by the monitor screen by placing an irradiance sensor (Tektronix J17 photometer, Wilsonville, OR, USA) at the position where crabs are located in the experimental arena pointing to the center of the monitor screen. The stimuli resulted in the following eight different edge contrasts: 0.15, 0.30, 0.46, 0.62, 0.77, 0.90, 0.96, 0.98 (contrasts calculated with Michelson's equation[61]). In order to reduce the number of experimental subjects each animal was presented with different stimuli belonging to an experimental series in randomized order. The intertrial interval was 10 min[56]. Because there is some habituation of the cardiac response with the repeated visual stimulation (see Figure 3), we presented half of the contrast stimuli one day and the other half the following day. One group of animals received the stimuli corresponding to contrasts 0.15, 0.46, 0.77 and 0.96 on the first day and the rest of the stimuli the following day. The other group of animals received the stimuli corresponding to contrasts 0.30, 0.62, 0.90 and 0.98 on the first day and the rest of the stimuli the following day. The sequence of group stimuli presented each day was randomized.

In the experiment of Figure 4 we presented five stimulation trials with an intertrial interval of 7 s. We call this a massed training protocol.[57] With this protocol, the cardiac responses to trials 2-5 were highly variable. Thus, to obtain a more confident temporal profile of the cardiac response and to reduce the number of the experimental subjects, two training sessions separated by 15 minutes were applied to each animal and an averaged cardiac response per animal was calculated. However, since we did not find notable differences between the two stimulation sessions, we used the responses obtained in both sessions to determine the maximum relative periods in each trial.

Electrocardiogram recordings

A small jack with two metallic pins where the electrodes were soldered was cemented with cyanoacrylate glue to the dorsal carapace of the crabs just above the heart.[7] The electrodes used to monitor heart rate were made of silver wire (diameter 0.25 mm, A-M Systems, Carlsborg, USA). The free ends of the electrodes were inserted in holes drilled 4-5 mm apart in the cardiac region of the dorsal carapace and cemented in place. The experiments were conducted at least 2 days after electrode positioning. The metallic pins of the jack were connected to an impedance converter (model 2991, UFI, California, USA) to monitor the heart rate. The impedance converter allowed recording the crab's heart rate as a measure of dynamic resistance.[62] Its output was digitized (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA) and the electrocardiogram (ECG) was recorded with compatible software (AxoScope 10, Molecular Devices, California, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Locomotor activity

With the information provided by the spherical treadmill we reconstructed the instantaneous velocity of locomotion of the animals in cm s^{-1} every 16.7 ms (for more details see Oliva et al.[29]). To smooth the data we calculated the value of the velocity by performing a 20-frame local linear regression. The analysis of the instantaneous velocities of locomotion allowed us to characterize the motor arrest in animals that were walking and escaping behaviors by performing behavioral peristimulus time histograms. As mentioned above, when moving crabs are presented with sudden visual stimuli, their first behavioral response is to stop any bodily movement. This behavior has been termed originally freezing response[63] but recent studies suggest that motor arrest could be a more accurate terminology for this response.[34] We have considered a motor arrest to occur when the locomotor velocity of a walking crab reduces to zero for more than 66.8 ms on the raw data, i.e. more than 4 mice consecutive readings. We have split the data into 200 ms temporal bins and computed the number of animals displaying a motor arrest per bin in response to the presentation of the moving edge for those animals that were moving at the moment of stimulus presentation.[35] On the other hand, if the stimulus is sufficiently threatening, as stimulation progresses both crabs that were immobile at the time of stimulus presentation and those that had arrested attempt to escape by running in the opposite direction of the stimulus.[5] To categorically characterize the escape response, we have assessed for each animal the temporal bin in which the escape response was most intense, i.e. the bin that corresponds to the time when the maximum instantaneous velocity is developed.[29]

Cardiac activity

To quantify the cardiac activity, the period between two beats was measured and normalized to the mean period during the 10 s preceding the stimulation. We have called this time interval 'relative period' (e.g., y axes in the graphs of Figures 1E and 1F). The population cardiac cardiac activity has a mean relative period of 1.002 ± 0.048 (mean $\pm$ sd). Thus, we have considered a cardiac arrest to occur when the value of a relative period is above 1.09 (mean + 2*sd). For analytical purposes, we have assigned the relative period value at a time point equidistant to the two beats (time positions of the markers of the curves in the graphs of Figures 1E and 1F). To analyze the temporal profile of the cardiac response for each animal and average it across individuals, we linearly interpolated the data between every two consecutive relative periods for each ECG recording (lines linking dot markers in lower panels of Figures 1E and 1F). While linearly interpolating between two beats introduces slight temporal artifacts, e.g. it gives rise to a slightly elevated relative period before stimulus onset, it allows a temporal and quantitative analysis of the response over large numbers of individuals. Thus, reducing the uncertainty derived from biological variability or noise recording.

We have also analyzed the time of occurrence of the maximum cardiac arrest, i.e. the maximum relative period in response to visual stimulation, to characterize the time distribution of the maximum cardiac responses. This time is marked with red stars in the ECG recordings of Fig. E, F which correspond to red stars in the lower graphs of the Figure. The maximum relative period was evaluated during the time of stimulation plus 1 s. With these events we produced peristimulus time histograms to characterize the time of occurrence of the maximum cardiac response in response to the different visual stimuli presented here (e.g., Figure 1H).

In the current study we have identified two phases in the cardiac response. To differentiate them we have performed a k-means clustering analysis of the time of occurrence of the maximum cardiac response by using a squared Euclidean distance metric and two clusters. To quantify both phases we have calculated for each temporal profile of the cardiac response (e.g., graphs in the lower panels in Figures 1E and 1F) the area under the curve in a 0.8 s interval centered at the center id of each cluster (histogram of Figure 1H: center id of phase 1 = 0.65 s and center id of phase 2 = 2.0 s). Thus, phase 1 area was calculated as the area under the relative period curve in the time interval [0.25, 1.05] s and phase 2 as the area in the interval [1.6, 2.4] s. Additionally, we have estimated the relative contribution of these response phases to the total cardiac response. Particularly, we have estimated the relative contribution of phase 2 area (rPh2 area) as rPh2 = (Phase 2 area) / (Phase 1 area + Phase 2 area). We have also estimated the relative distribution of the maximum relative periods. In particular, we have calculated the proportion of maximum relative period events that occurred in the second half of visual stimulation (pPh2 events) with respect to the total number of events occurred along the whole stimulation time (pPh2 events = (# second half of stimulation) / (# first half of stimulation + # second half of stimulation)).

Statistical analysis

In the visual saliency experiment (Figure 2) we used a Linear Mixed Model (LMM) to model the area under the curve for each phase of the cardiac response. Instead, to model the rPh2 modulation, we used a Generalized Linear Mixed Model (GLMM) with Beta distribution and logit link function. We have used the Michelson contrast as a fixed effects variable. For the spaced and massed stimulation experiments (Figures 3 and 4 respectively) the same statistical modeling was used using the stimulus trial number (categorical variable with five levels) as the fixed effects variable.

All analyses were performed using R Statistical Software[64] (v4.2.2 "Innocent and Trusting"). In order to fit the models we used nlme y glmmTMB packages.[65,66] Finally, to perform Tukey contrasts, the emmeans function of the emmeans package was used.[67]