

The cardiac response of the crab *Chasmagnathus granulatus* as an index of sensory perception

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SUMMARY

When an animal's observable behavior remains unaltered, one can be misled in determining whether it is able to sense an environmental cue. By measuring an index of the internal state, additional information about perception may be obtained. We studied the cardiac response of the crab *Chasmagnathus* to different stimulus modalities: a light pulse, an air puff, virtual looming stimuli and a real visual danger stimulus. The first two did not trigger observable behavior, but the last two elicited a clear escape response. We examined the changes in heart rate upon sensory stimulation. Cardiac response and escape response latencies were also measured and compared during looming stimuli presentation. The cardiac parameters analyzed revealed significant changes (cardio-inhibitory responses) to all the stimuli investigated. We found a clear correlation between escape and cardiac response latencies to different looming stimuli. This study proved useful to examine the perceptual capacity independently of behavior. In addition, the correlation found between escape and cardiac responses support previous results which showed that in the face of impending danger the crab triggers several coordinated defensive reactions. The ability to escape predation or to be alerted to subtle changes in the environment in relation to autonomic control is associated with the complex ability to integrate sensory information as well as motor output to target tissues. This 'fear, fight or flight' response gives support to the idea of an autonomic-like reflexive control in crustaceans.

Key words: invertebrates, cardiac response, behavior, sensory perception.

INTRODUCTION

Perception is the process that enables animals to attain environmental information through the senses. Some method of observation is needed to determine how animals recognize a cue or a threatening signal in their environment. The examination of individual neurons and neural paths has allowed a thorough understanding of the primary detection process. However, this approach does not reveal how sensory information is integrated within the animal unless it is correlated to animal behavior. On the other hand, by only monitoring the animal through external observation one can be misled in determining if an animal senses an environmental cue when its conduct remains unaffected. Measuring an index of internal state may provide additional information about the perception of a stimulus (Li et al., 2000).

In decapod crustaceans many studies have used the changes in a vegetative index to monitor the effects of a wide range of environmental variables on the physiology: water currents (Larimer, 1964), P_{O_2} (Airriess and McMahon, 1994), ammonia (NH_3) (Bloxham et al., 1999), heavy metals (Aagaard et al., 2000), ambient CO_2/O_2 (Gannon and Henry, 2004), water temperature (Camacho et al., 2006). In addition, the responses to several types of stimuli (tactile and chemical cues) were examined for their effects on the heart and ventilatory rate of crayfish, finding a reflex inhibition in the majority of them (Larimer, 1964). More specifically, bradycardia or reversible heart arrests have been reported in crabs, lobsters and crayfish to a variety of optical and tactile stimuli (Cuadras, 1980; Cumberlandidge and Uglow, 1977; Florey and Kriebel, 1974; Grober, 1990a; Grober, 1990b; Larimer and Tindel, 1966; McMahon and Wilkens, 1972; Mislin, 1966; Shuranova and

Burmistrov, 2002; Uglow, 1973; Wilkens et al., 1974). Furthermore, another set of results also gathered in crustacea have shown that even though no observable behavioral responses were elicited, heart rate was measurably affected by small disturbances in the environment or by social interaction (Li et al., 2000; Listerman et al., 2000; Schapker et al., 2002). Given the remarkable sensitivity of this parameter to a variety of sensory modalities it has been posed that the cardiac response can serve as an indicator of perception in decapod crustaceans and could well be utilized in studies on perceptual physiology.

Our interest in altering the illumination stemmed from earlier studies which revealed that white light causes cave crayfish to seek shelter (Li and Cooper, 1999), and others which showed (Grober, 1990b; Li et al., 2000; Schapker et al., 2002) that light (infrared, dim red, and white) can induce alterations in crayfish's heart rate.

Both antennae of crustacea bear sensory flagella which carry mechanoreceptive sensilla (Derby, 1982) enabling them to use tactile and mechanical cues to extract information from the environment (Patullo and Macmillan, 2005). These cues enable animals to find resources, orient to water currents or escape predators (Weissburg, 1997). In addition, mechanoreceptive neurons responsive to stimulation have been found all over crabs, lobsters and crayfish (Arechiga et al., 1975). Thus, we planned to assess a mechanosensory cue, i.e. an air puff, by looking for changes in heart rate.

Visual cues, such as natural and artificial objects, including two-dimensional shapes, can influence or guide directional orientation of decapod crustaceans in specific situations (Chiussi and Diaz, 2002; Cuadras, 1980; Diaz et al., 1994; Diaz et al., 1995a; Diaz et

al., 1995b; Herrnkind, 1983; Langdon and Herrnkind, 1985; Orihuela et al., 1992). Furthermore, our own work in *Chasmagnathus* shows that upon the sudden presentation of a rectangular screen passing above the animal, the visual danger stimulus (VDS), the crab responds with a running reaction in an attempt to escape (Maldonado, 2002), while a cardiac response is also elicited by the same stimulus (Hermitte and Maldonado, 2006). Consequently, the effect of presenting a VDS as a visual cue was further explored.

Finally, the ability to detect and react to looming objects is present in most visual animals from insects to mammals even though their visual systems are largely different. Behavioral reactions elicited by looming stimuli have been studied in taxa as diverse as insects, amphibians, birds, and mammals (Jablonski and Strausfeld, 2000; Maier et al., 2004; Regan and Hamstra, 1993; Tammero and Dickinson, 2002; Yamamoto et al., 2003). Taking into account that in the crab *Chasmagnathus* a robust and reliable escape response can be elicited by computer-generated looming stimuli (Oliva et al., 2007), we decided to explore the possibility that virtual collision stimulus might elicit physiological responses as well.

Therefore, our working hypothesis is that a physiological parameter such as heart rate can constitute a sensitive index to assess crustacean perceptual capacity. The purpose of our study was to examine stimuli characterized as innocuous (a light pulse and an air puff) and those regarded as threatening (a visual danger stimulus and virtual looming stimuli), comparing their effect on both cardiac and locomotor activity. In addition, both escape and cardiac response latencies to looming stimuli were simultaneously recorded with the purpose of comparing them and examining the relationship between them. Similar to vertebrates, invertebrates may also need rapid cardiovascular and respiratory regulation to be primed for 'fear, fight or flight' when the need arises (Wilkins and McMahon, 1992). The ability to escape predation or to be alerted to subtle changes in the environment in relation to autonomic control is associated with the complex ability to integrate sensory information as well as motor output to target tissues. Very few previous studies have investigated the simultaneous occurrence of autonomic and somatic responses in invertebrates (Hermitte and Maldonado, 2006). Thus, a broader objective was to further investigate how these more integrated strategies work in invertebrates.

MATERIALS AND METHODS

Animals

Animals were adult male *Chasmagnathus granulatus* Dana 1985 crabs 2.7–3.0 cm across the carapace, weighing approximately 17 g, collected in the rias (narrow coastal inlets) of San Clemente del Tuyú, Argentina, and transported to the laboratory, where they were lodged in plastic tanks (35 cm × 48 cm × 27 cm) filled to 2 cm depth with diluted seawater to a density of 20 crabs per tank. Water used in tanks and other containers during the experiments was prepared using hw-Marinx (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 7:00 h to 19:00 h and 22–24°C. Experiments were run between 8:00 h and 19:00 h 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 (see below). During this recovery period, crabs were kept in individual tanks and fed rabbit food pellets (Nutrients, Argentina) daily. Following tests, animals were returned to the field and released in an area 30 km away from the capture area. Experimental procedures were in compliance with the Argentine laws for Care and Use of Laboratory Animals.

Both unrestrained and restrained animals were used in different experiments. The restrained condition was achieved by immobilizing crabs prior to the experiment 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 the quantification of heart rate easier.

Cardiac response: recording procedure

A small jack was cemented with instant adhesive to the dorsal carapace in a position anterior to the heart and had two metallic pins where the electrodes were soldered. These were made of silver wire (diameter 0.25 mm, VEGA & CAMJI S.A., Argentina) cut in sections 2.4 cm long. The free end of both wires was inserted in holes previously drilled in the cardiac region of the dorsal carapace placed to span the heart in a rostral-caudal arrangement and separated by 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 because it has been shown that it alters heart rate (HR) for a few days (Wilkins et al., 1985; Listerman et al., 2000). Prior to an experiment, the crab was lodged in the container called an actometer: a bowl-shaped opaque container with a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter) covered to a depth of 0.5 cm with sea water 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 HR. The impedance converter measures the changes in the resistance between two electrodes, associated with the hemolymph movement after each heart contraction (Li et al., 2000; Listerman et al., 2000; Schapker et al., 2002). The output from the impedance leads was sent to the analog-to-digital converter of a computer data acquisition and analysis system (Fig. 2Ai).

Escape response: recording procedure

The crab's escape response was recorded by means of four microphones cemented to the bottom of the container. The container vibrations induced electrical signals proportional to the amplitude

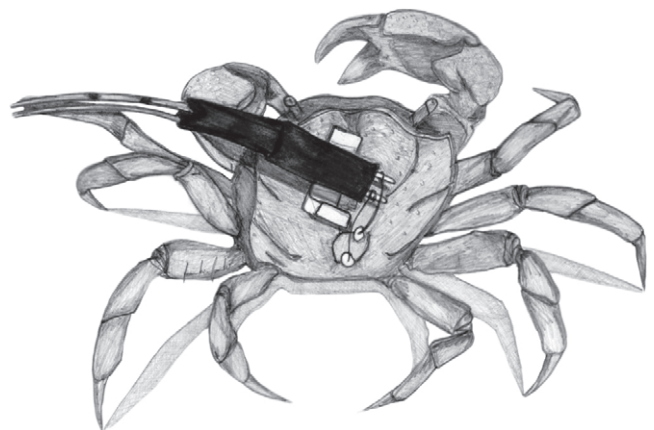


Fig. 1. A drawing of the placement of the recording leads for monitoring the heart of *Chasmagnathus*. On the dorsal carapace, two leads spanned the rostral-caudal axis of the heart to monitor heart rate. The free ends of both wires were soldered to the pins of a jack cemented to the dorsal carapace in a more anterior position.

and frequency of the signal that were amplified, integrated and processed by a computer (Fig. 2Ai).

Environmental disturbances

To test the crabs' perception of particular environmental alteration, the study was divided into four experimental conditions according to the different stimuli used in each one: (1) a 2 s white light pulse; (2) either a 3 s or 5 s air puff; (3) the projection of four different computer generated looming stimuli on a flat screen and (4) a moving visual danger stimulus (the VDS). Between 11 and 17 crabs were tested in each experimental condition. Both cardiac and locomotor activity could be simultaneously recorded. Unrestrained and restrained animals were used.

The light pulse stimulus

A 2 s pulse from a white-light-emitting diode (LED) was presented 7 cm from above the animal (Fig. 2Ai). The specification for a 5 mm white LED is: luminous intensity 10,000 mcd; viewing angle: 23°. The illumination intensity measured in the actometer was $450,06 \text{ mW m}^{-2}$ and $1.266,66 \text{ mW m}^{-2}$ before and after stimulation, respectively.

The mechanosensory stimulus

An air puff was generated by an air pump and delivered through a thin plastic transparent tube directed towards the animal and positioned 1 cm above the cephalothorax carapace (Fig. 2Ai). Stimulus duration could be precisely controlled, lasting either 3 or 5 s. Every animal in this experimental condition was tested with both stimuli, with a 3 min interval between them and varying the order in which the stimuli were presented to each animal.

The looming stimulus

Computer-generated visual stimuli were projected on a flat screen monitor (Phillips 107T, horizontal and vertical screen dimensions $32 \times 24 \text{ cm}$, respectively, refreshing rate 60 Hz), located 20 cm in front of the animal (Fig. 2B). ECG and/or locomotor activity records began after a black curtain was lowered in the front part of the cage and after the animal had remained visually undisturbed for 10 min. The illumination intensity measured in the setup was $250 \pm 5 \text{ mW m}^{-2}$ and $53 \pm 5 \text{ mW m}^{-2}$, before and after the expansion, respectively. All visual stimuli were generated from a single PC using commercial software (Presentation 5.3, Neurobehavioral Systems Inc., USA). Visual simulations generated by computer may differ in many ways from the visual input experienced under natural conditions. Nevertheless, no escape response differences were found in *Chasmagnathus* when comparing a black sheet of cardboard moving overhead with the computer-generated image (V. Medan and D. Tomsic, personal communication). The simulated looming stimulus used in the present study consisted of a 5 cm black square, which approached over a distance of 70 cm at different constant speeds of 5, 10, 20 and 40 cm s^{-1} , generating a different angular size for the virtual approaching stimulus as a function of time (Oliva et al., 2007).

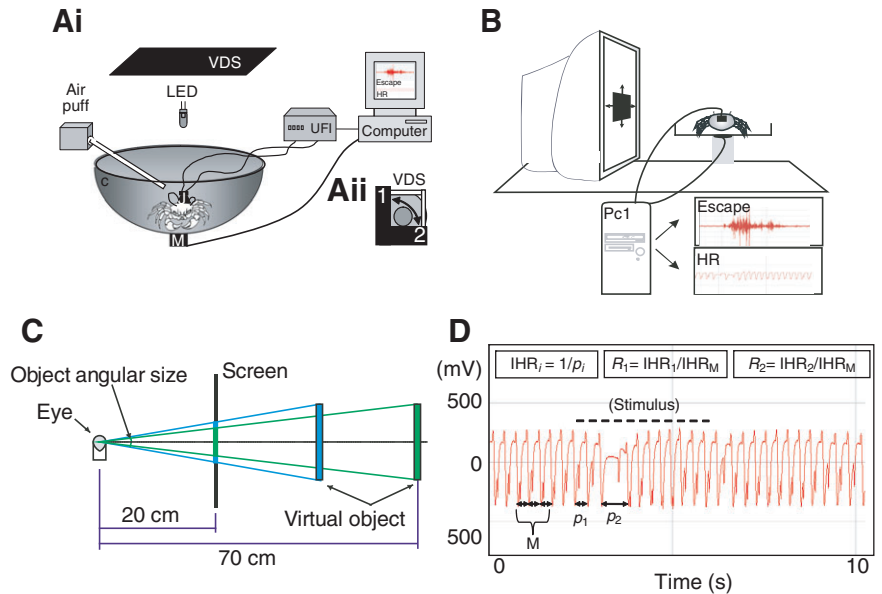


Fig. 2. (Ai) Representation of the set up used for recording the heart rate and locomotor activity during the presentation of the different stimuli. The crab is lodged in the actometer (a bowl-shaped opaque container, c) and connected to the impedance detector (UFI, model 2991) by means of a jack (J). The impedance output is sent to a computer to allow HR to be monitored. The crab's escape response is recorded by means of four microphones (M) cemented to the bottom of the container. This set up was used for the presentation of three different stimuli: a light pulse generated by a white light-emitting-diode (LED) placed 7 cm above the animal, an air puff presented in direction to the animal's cephalothorax carapace 1 cm from above, and an opaque rectangular screen (the VDS) placed 6 cm above the crab. (Aii) The VDS is a motor-operated screen (an opaque rectangular strip of $25.0 \text{ cm} \times 7.5 \text{ cm}$) moved horizontally over the animal from left to right and vice versa. (B) Representation of the set up used for the recording of the HR and locomotor activity during the presentation of different virtual looming stimuli. A single PC using commercial software is used to generate visual expansion stimuli projecting on a flat screen monitor located at 20 cm in front of the animal that is lodged in a transparent box and connected to the impedance detector (UFI, model 2991) by means of a jack. The impedance output is sent to a computer to allow HR to be monitored. The crab's escape response is recorded by means of four microphones cemented to the bottom of the box. (C) The looming stimulus consisted in a simulated projection of an approaching object from the monitor screen. The simulation corresponded to a black square object of 5 cm that approached from a distance of 70 cm at different constant velocities (see methods). (D) Representative recording of cardiac activity. The duration of the cardiac event or period (p) is measured and used to calculate the instant heart rate (IHR) as the inverse of the period ($\text{IHR} = 1/p$). The dashed line stands for a stimulus. IHR_M , IHR , R_1 and R_2 are also defined in the text.

Throughout the experiments, expansions were always directed towards the animal (see Fig. 2C). Every animal in this experimental condition was tested with the four looming stimuli, with a 3 min interval between them and counterbalancing the order in which the stimuli were presented to each animal.

The visual danger stimulus

An opaque rectangular screen ($25 \text{ cm} \times 7.5 \text{ cm}$), i.e. the visual danger stimulus (VDS), positioned 6 cm above the container, was moved horizontally from left to right and vice versa (Fig. 2Ai). A VDS lasted 5 s and consisted of two successive cycles of screen movement (Fig. 2Aii).

Data analysis

The cardiac activity was recorded during an interval of 9, 10 or 20 s, during which the stimulus was presented after a 3 s delay. The duration of the cardiac event or period was measured and used to calculate the instant heart rate (IHR) as the inverse of the period ($\text{IHR} = 1/p$). A number of previous studies in crustaceans have shown that heart rate can vary widely both between and within individuals and with

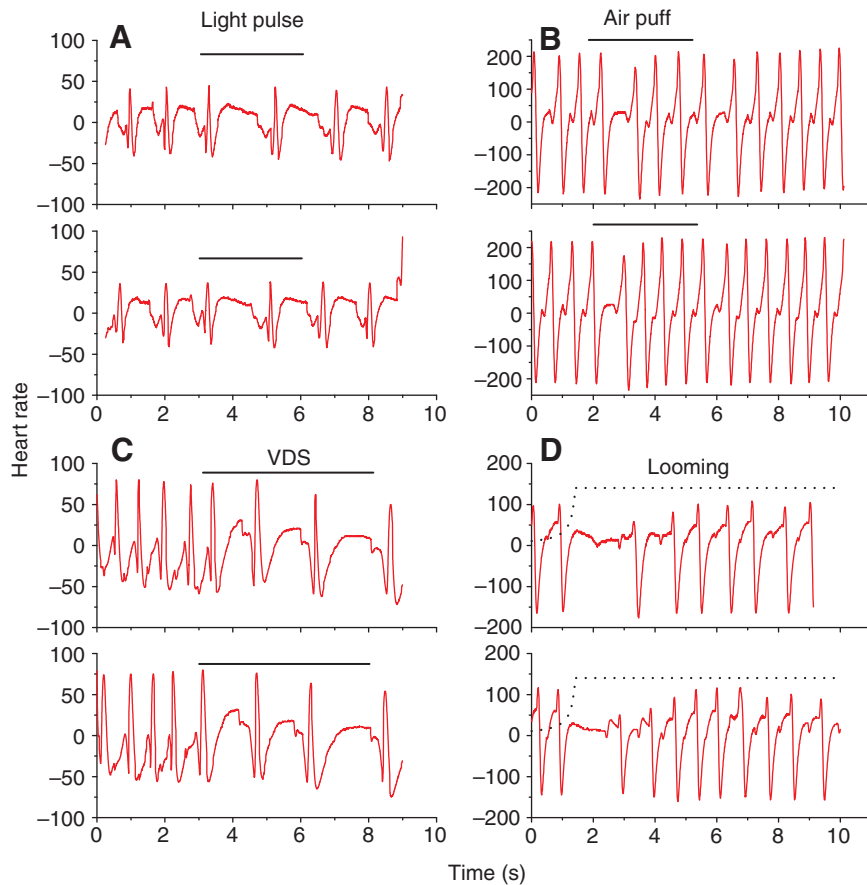


Fig. 3. Representative recording of heart rate prior to and during the presentation of stimuli regarded as innocuous: (A) light pulse, (B) air puff; and threatening: (C) visual danger stimulus (VDS), (D) looming stimuli. Upper trace: first presentation; lower trace: second presentation. The solid lines indicate the stimulus presentation.

the experimental conditions (Grober, 1990a; Hermitte and Maldonado, 2006). For this reason we normalized the IHR (normalized $IHR_i = IHR_i / IHR_M$; where IHR_i is the IHR of each single event and IHR_M is the mean IHR). To control that changes in the IHR due to the stimuli were significantly different from spontaneous changes in the IHR, two ratios (R_1 and R_2) were statistically compared (Fig. 2D). R_1 represents the quotient between IHR_1 (the IHR of one event randomly selected prior to stimulus on-set) and the IHR_M ($R_1 = IHR_1 / IHR_M$). R_2 represents the quotient between the IHR_2 (the IHR of one representative event during the cardiac response) and the IHR_M ($R_2 = IHR_2 / IHR_M$). Since changes in heart rate in response to sensory stimulation are usually rapid and very brief (Grober, 1990a), the 9, 10 or 20s recording time provided a suitable interval for measuring cardiac responsiveness to sensory stimulation.

Statistical analysis

Non-parametric statistics was used to compare the differences found between R_1 and R_2 as a result of sensory stimulation (Kruskal–Wallis ANOVA and the Multicompare test for *a posteriori* contrasts). A simple linear correlation analysis was performed to examine the association between the cardiac and escape responses to looming stimuli. A frequency analysis was performed by means of a G-test for homogeneity with the Yates correction to determine whether the probability of response to different stimuli is equally distributed across restrained and unrestrained animals.

RESULTS

Cardiac inhibitory response (CIR) to environmental disturbances

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. Upon the presentation of a light pulse or an air puff which are stimuli regarded as innocuous, a small but clear heart arrest or a brief bradycardia is recorded (Fig. 3A,B). However, stimuli considered to be threatening, such as looming stimuli or the VDS elicit deep heart arrests and sustained bradycardia (Fig. 3C,D). Upon a second presentation of the stimulus to the same animal a similar profile is obtained, revealing the consistency of the response (Fig. 3, lower traces).

The relationship between escape and cardiac responses

Fig. 4 shows a representative example of two different animals whose heart rate and locomotor activities (LA) were simultaneously recorded. When no stimulus was presented, spontaneous walking activity was observed while no heart arrests were identified, revealing the independence of each pattern of activity in both animals (Fig. 4Ai,Bi). By contrast, upon the presentation of a different stimulus to each animal (Fig. 4Aii,Bii), a diverse pattern of activity could be observed. During the presentation of a seemingly innocuous stimulus such as an air puff, it was possible to record a small but clear cardiac response while no escape was elicited. Upon a looming stimulus presentation strong cardiac and escape responses were concurrently triggered.

Analysis of the effect of environmental disturbances on the instant heart rate

The instant heart rate (IHR) is the chosen parameter that allowed us to assess in the best possible way the corresponding observed changes in the cardiac activity as a result of sensory stimulation and to adequately describe the reversible heart arrests or brief bradycardia illustrated in the previous figures.

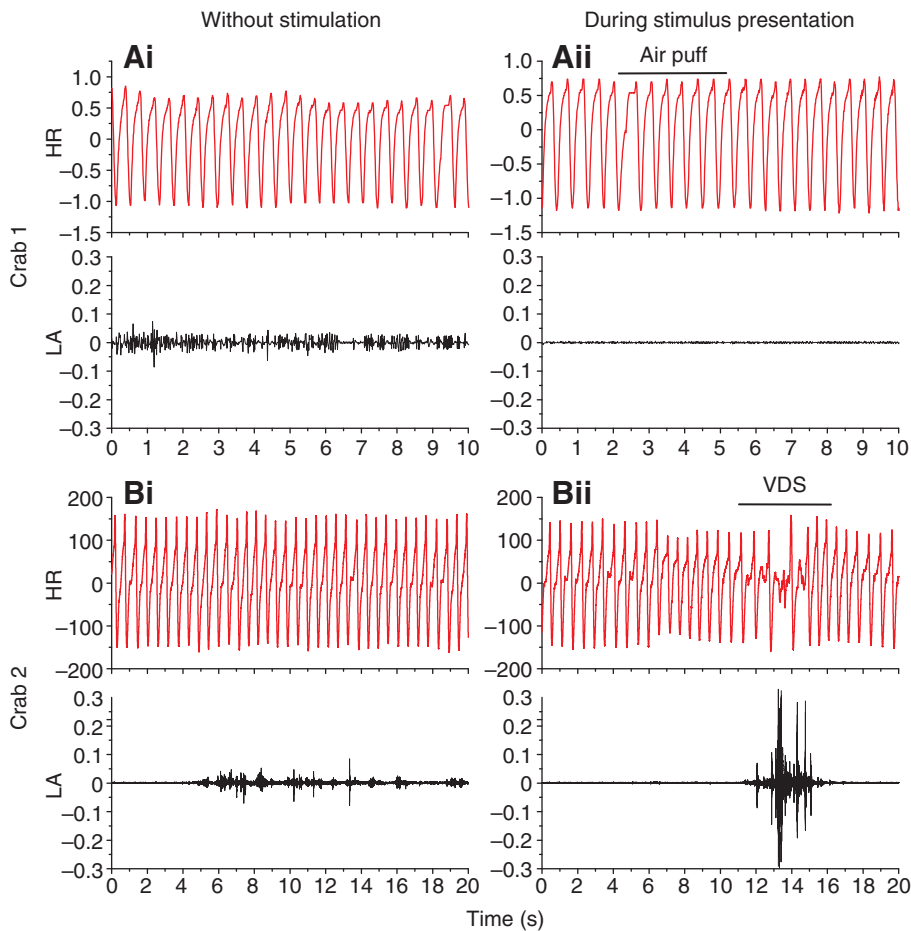


Fig. 4. Representative heart rate (upper trace) and locomotor activity (lower trace) recordings of two different animals (crab 1 and crab 2) without and during sensory stimulation. (Ai) no stimulation present, (Aii) during the presentation of an air puff to the same animal; (Bi) no stimulation present, (Bii) during the presentation of a VDS to the same animal. The solid lines indicate the stimulus presentation.

Upon the presentation of a 5 s air puff to restrained and unrestrained animals, a noticeable and consistent cardiac response could be observed as a decrease in the mean IHR curve immediately after stimulus started in both groups of animals (Fig. 5). A smaller but visible decrease in the IHR was also observed when the air puff ended. The restrained animals exhibited a more conspicuous response than the unrestrained animals. When a statistical analysis was performed to compare the IHR previous (R_1) and during stimulation (R_2), significant differences were found between them ($P < 0.01$), revealing the sensitivity of this parameter to an air puff in the two groups (Fig. 5A,B). Together with the cardiac recording, a locomotor activity record was performed, showing no escape response to this stimulus (data not shown).

When a different air puff stimulus of 3 s duration was presented to the same animals similar results were obtained. Here too, significant differences between base line (R_1) and stimulation (R_2) were found in restrained and unrestrained animals ($P < 0.01$) and no escape was triggered by this stimulus (data not shown).

The presentation of a light pulse could also be traced in the IHR mean curve of two other groups of crabs (restrained and unrestrained) as a small and clear response at the beginning of the stimulation and an off-response at the end, similar in magnitude to that previously detected for the air puff (Fig. 6). Once more, the restrained group displayed a larger response compared with that of unrestrained animals showing a tendency that was retained for all the stimuli used in this work. The decrease in the IHR due to the light pulse stimulation was comparable to that observed for the air puff, ranging from 10 to 15%. The statistical analysis revealed

significant differences between base line (R_1) and stimulation (R_2) for both groups ($P < 0.01$; Fig. 6A,B). Here again the simultaneous record of the locomotor activity could not reveal an escape response to this stimulus (data not shown).

Virtual stimuli as looming expansions triggered conspicuous changes in the IHR in restrained and unrestrained animals. When a 20 cm s^{-1} looming stimulus was presented, a stronger response was displayed, reaching a 30% decrease in IHR of unrestrained animals as can be observed in the mean curve in Fig. 7. This response almost duplicated, in magnitude, that obtained for both previous stimuli and reached its maximum at approximately 4.2 s in the animals in both conditions. The locomotor activity of unrestrained animals, which was also plotted in the same figure (lower trace in Fig. 7) also reached its maximum at about 4.2 s when escape was triggered, showing a close matching between both responses. The off-set of the looming stimulus causes a change of the set-up illumination that also elicited a small but noticeable response in both activities. The statistical analysis of the mean IHR of one event prior and during a looming stimulus presentation (R_1 and R_2 , respectively) revealed significant differences between them ($P < 0.01$) in both unrestrained and restrained animals (Fig. 7A,B).

Interestingly, upon the presentation of a 5 cm s^{-1} looming stimulus to the same group of animals the mean IHR showed a similar percentage decrease to that obtained for the 20 cm s^{-1} stimulus, which now reached its maximum response at approximately 13 s for both conditions (Fig. 8). This delay in the time response could be expected for a slower expansion. In the unrestrained group the locomotor activity (Fig. 8, lower trace) showed an escape response that again closely matched the cardiac response. When R_1 and R_2 were

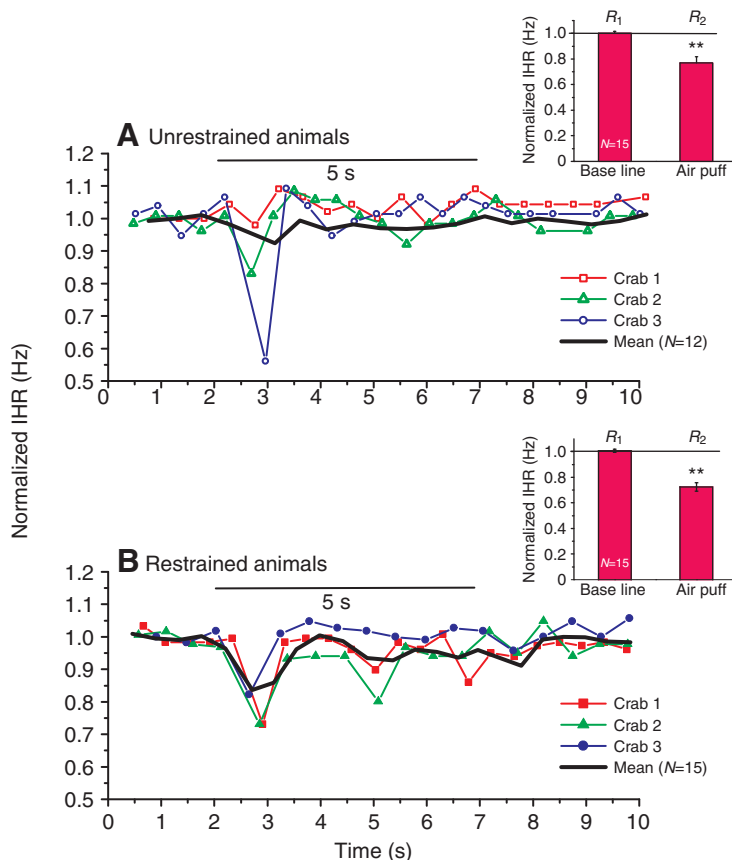


Fig. 5. Representative recordings of the normalized IHR of three crabs prior to and during the presentation of a 5 s air puff. The black line is the mean of the total number of animals. The bar graph in each panel shows, for all animals, the mean IHR of one event prior to and during stimulation (R_1 and R_2 , respectively) revealing significant differences between them ($P < 0.01$). (A) Unrestrained condition. (B) Restrained condition. The solid line indicates the stimulus presentation.

statistically compared significant differences were found between them ($P < 0.01$; Fig. 8A,B).

Two other looming stimuli were also assessed in this group of animals yielding similar results; a faster one (40 cm s^{-1}) and one in between the previously described looming stimuli (10 cm s^{-1}), which produced maximum responses at about 2.8 s and 6.5 s, respectively. These differences in time response can be accounted for in terms of the different expansion velocities. The statistical analysis for both stimuli revealed significant differences between base line (R_1) and stimulation (R_2) in restrained and unrestrained animals ($P < 0.01$; data not shown). On the whole, the results regarding looming stimuli reveal that the IHR proved useful to estimate the perceptual capacity in these animals even when these stimuli were virtual.

From the results of these various stimulations a clear-cut conclusion can be drawn: all the stimuli tested could trigger measurable cardiac responses. In addition, from the results in Figs 7 and 8 it can be observed that the cardiac response occurred at different times for the different expansion velocities of the stimulus, showing an earlier response when the expansion was fast and a later response for intermediate and slow expansions. A similar profile was observed for the mean escape response revealing the association between both responses. This issue is discussed further in the section 'Analysis of the cardiac and escape response latencies'.

Validation of the virtual looming stimulus with a real danger stimulus

In Fig. 9 the representative normalized IHR of three different crabs during the presentation of the VDS is shown together with the mean recording from a total of 13 animals. The bar graph in each panel shows, for all animals, the mean IHR of one event prior to and during a VDS presentation (R_1 and R_2 , respectively) revealing significant differences between them ($P < 0.01$) in the restrained condition. Some

comments are pertinent here: (1) this result strengthens those obtained with looming stimuli showing comparable magnitude responses; (2) previous work with the VDS was assessed as beats per minute (Hermitte and Maldonado, 2006). Our present results validate those preceding results, using a more accurate parameter, the IHR; (3) only animals in restrained condition are shown here because the VDS elicits a strong escape response that complicates heart rate observation in unrestrained crabs.

Overall results reveal that the instant heart rate is an adequate parameter to infer an animal's internal state and to assess *Chasmagnathus* perception of those stimuli that elicit a behavioral response as well as those that do not. Heart rate showed a significant decrease revealed by the response ratio comparison (R_2 vs R_1) for all the examined stimuli in both restrained and unrestrained crabs.

The cardiac response in restrained and unrestrained animals

Some animals would not always respond to stimulus presentation, thus a detailed analysis on response probability was performed for the whole population previously described. Fig. 10Ai,Aii compares the probability of cardiac response to the examined stimuli (looming stimulus, air puff, light pulse) in restrained and unrestrained crabs. In restrained animals the response probability was always higher than 60% and for threatening stimuli near 100%. In unrestrained animals it was lower and the response to innocuous stimuli such as the light pulse declined to near 30%. A frequency analysis with χ^2 -test for homogeneity was performed on the pooled data of the three stimuli together stating that the probability of response between both conditions was significantly different ($G_{\text{yates}} = 10.35 < \chi^2_{(1; 0.095)} = 3.84$).

To compare the cardiac response thresholds between restrained and unrestrained animals we calculated the response latency to the looming stimuli with different expansion velocities (Fig. 10Bi,Bii). Latencies showed an increase associated with the decrease in the

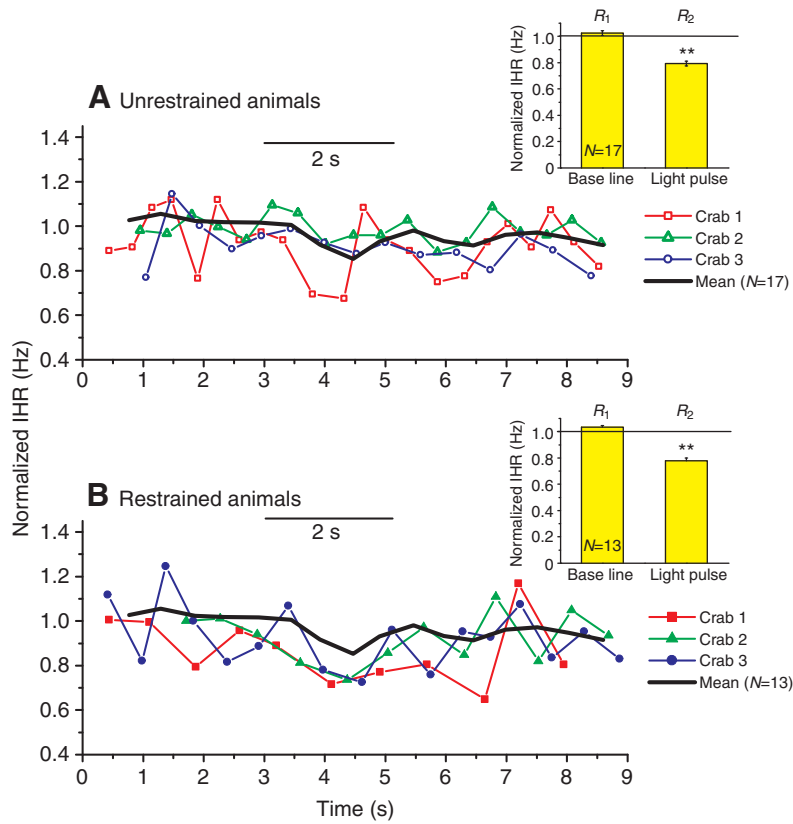


Fig. 6. Representative recordings of the normalized IHR of three crabs prior to and during the presentation of a light pulse. The black line is the mean of the total number of animals. The bar graph in each panel shows, for all animals, the mean instant heart rate (IHR) of one event prior to and after stimulation (R_1 and R_2 , respectively) revealing significant differences between them ($P < 0.01$). (A) Unrestrained condition. (B) Restrained condition. The solid lines indicates the stimulus presentation.

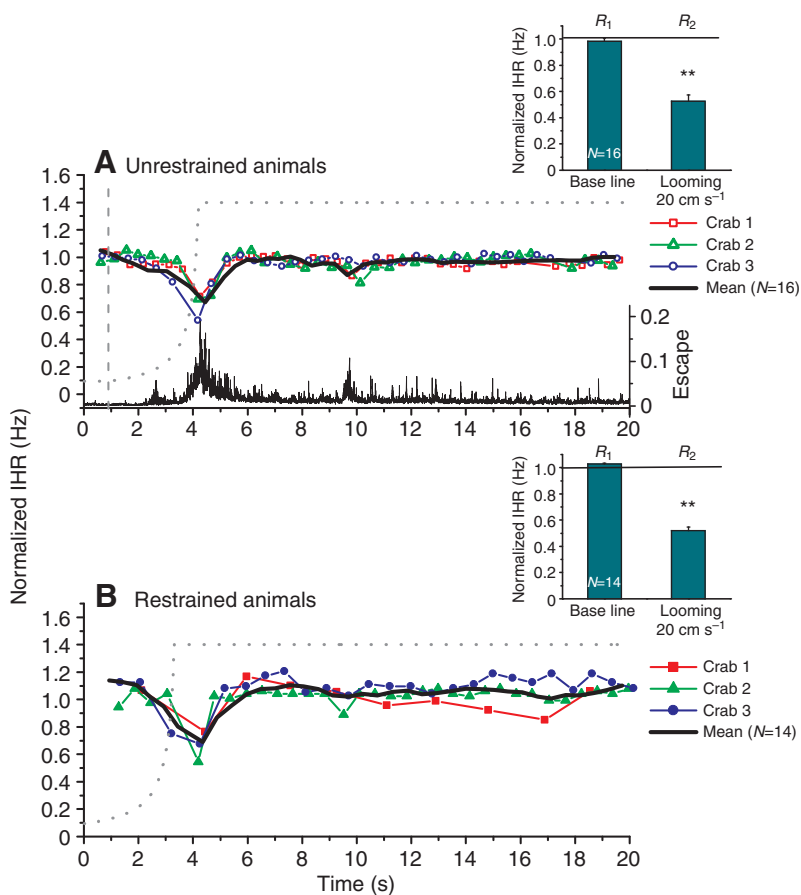


Fig. 7. Representative recordings of the normalized instant heart rate (IHR) of three crabs prior to and during the presentation of the looming stimulus of 20 cm s^{-1} . The black line is the mean of the total number of animals. The bar graph in each panel shows, for all animals, the mean IHR of one event prior and after stimulation (R_1 and R_2 , respectively) revealing significant differences between them ($P < 0.01$). (A) In the unrestrained condition the looming stimuli were initiated after a 0.9s delay relative to the heart rate recording (vertical dashed line). The mean escape response of freely moving animals is shown in the black lower trace. (B) In the restrained condition looming stimuli were initiated simultaneously with the heart rate recording. The dotted line represents the expansion of the virtual approaching stimulus plotted against time.

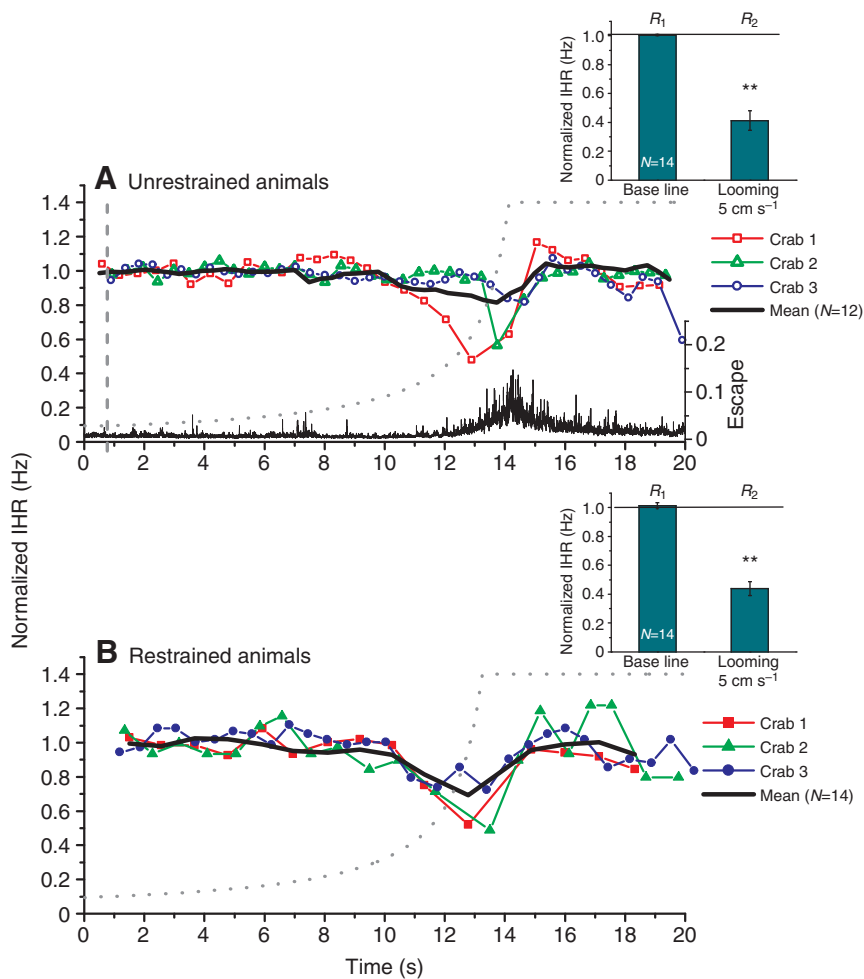


Fig. 8. Representative recordings of the normalized HR of three crabs prior to and during the presentation of the looming stimulus of 5 cm s⁻¹. The black line is the mean of the total number of animals. The bar graph in each panel shows, for all animals, the mean IHR of one event prior and after stimulation (R₁ and R₂, respectively) revealing significant differences between them ($P < 0.01$). (A) In the unrestrained condition the looming stimuli were initiated after a 0.9s delay relative to the heart rate recording (vertical dashed line). The mean escape response of freely moving animals is shown in the black lower trace. (B) In the restrained condition looming stimuli were initiated simultaneously with the heart rate recording. The dotted line represents the expansion of the virtual approaching stimulus plotted against time.

velocity of the looming expansion. No significant differences in the response latencies were found between unrestrained and restrained animals. An overall conclusion can be drawn from these results: the restrained condition seem to determine a change in the probability of response whereas no modifications were produced in the response threshold.

Analysis of the cardiac and escape response latencies

A similar profile was found for the escape and cardiac responses (Figs 7 and 8) revealing an association between them which is worth further investigation. Fig. 11 compares the escape response and the cardiac response latencies to the presentation of different looming stimuli, and shows an equivalent increase in their latencies related to the decrease in the velocity of expansion of the stimuli (Fig. 11A). Interestingly, both responses showed a tight correlation ($r: 0.9742$; Fig. 11B).

DISCUSSION

Although rapid alterations in heart rate induced by changes in the animal's environment have been previously reported several times, our results for *Chasmagnathus*, combining behavioral and cardiac assays show that sometimes a brief change in heart rate to sudden stimuli is closely associated with overt behavior and sometimes it is not. In standardized experimental conditions of delivering stimuli, it became apparent that a physiological measurement, of heart rate, might be a more sensitive indicator of whether an animal perceives a stimulus, rather than behavioral measurements. Furthermore, the crab's integrated response is modulated by stimulus intensity.

Stimuli regarded as innocuous elicit weaker arrests of the heart and transient bradycardia and no overt behavioral response, whereas apparently threatening stimulus produce a prolonged cardiac arrest and sustained bradycardia together with a vigorous escape response. At first glance these may seem simple gradations of the same response. If the stimulus is small, and not threatening, it is subthreshold to trigger escape but if it is larger, it elicits a stronger cardiac response and an escape. However, another possibility is altogether tenable. Although a brief CIR to the presentation of the neutral cue may be considered to be part of an arousal or orienting behavioral response (Ide and Hoffmann, 2002) and investigative in nature, a strong CIR on perceiving a threat may be considered to be part of a startle response modulated by fear that may eventually contribute to removing the animal from the proximity of a potentially dangerous stimulus (Laming, 1981).

Transient cardiac inhibition in vertebrates has been identified as indicative of an emotional component by many investigators (Davis, 1992; Lang et al., 1972; LeDoux, 1993) although it has also been proposed that it may play a causal role in appropriate response selection, and thus have adaptive significance in its own right (McLaughlin and Powell, 1999). The fact that cardiac inhibition has been associated with attention phenomena (Lacey and Lacey, 1974; Graham and Clifton, 1966; Sokolov, 1963; Powell, 1994) suggests that the latter hypothesis has merit. Accordingly, it has been suggested that the invertebrate responses to different external stimuli, which strongly depend on the animal's functional state but less on the modality of the stimulus, appear to be similar to those characteristic of the so-called 'orienting reflex' of higher mammals

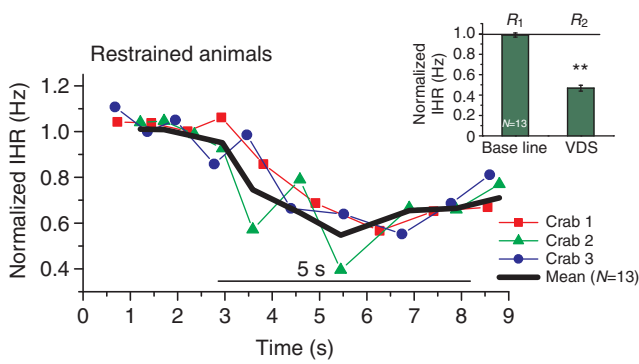


Fig. 9. Representative recordings of the normalized IHR of three restrained crabs prior and during the presentation of the visual danger stimulus (VDS). The thick line is the mean of the total number of animals. The bar graph in each panel shows, for all animals, the mean IHR of one event prior and after stimulation (R_1 and R_2 , respectively) revealing significant differences between ($P < 0.01$). The solid line indicates the stimulus presentation.

(Graham, 1979; Pavlov, 1923; Zernicki, 1987). Thus, relatively ‘neutral’ unexpected external stimuli might trigger in the crustacean brain some processing of the information about the ‘novel’ stimulus and its possible consequences (Shuranova and Burmistrov, 1996; Shuranova et al., 2006).

However, whereas the above explanation may account for the cardiac response to innocuous sensory stimulation, it is unlikely that it accounts for the bradycardia observed in crabs to threatening stimuli, as this physiological response is highly correlated with active movements away from the stimulus. Because of the correspondence

with avoidance behavior, this physiological response may provide a useful index to determine the specific characteristics of sensory stimuli that can elicit avoidance or startle behavior in crabs (Grober, 1990a). Thus, the cardiac responses of crabs to the VDS and looming stimuli may represent an example of the so-called ‘startle induced bradycardia’ of many animal species to rapid and intense sensory stimuli (Guirguis and Wilkens, 1995). Support for this proposal comes from earlier works showing that in most cases of intense sensory stimulation, both the heart and the scaphognathites of decapod crustaceans exhibit a coordinated and rapid decrease in beating (Larimer, 1964; McMahon and Wilkens, 1972; Cumberlandidge and Uglow, 1977). Furthermore, it was demonstrated that this coordinated response is the result of a command system of interneurons, located in the circumesophageal connectives innervating both the heart and gill bailers, whose activity can be altered by sensory inputs with parallel changes in HR and ventilation rate (Wilkens et al., 1974; Field and Larimer, 1975a; Field and Larimer, 1975b; Taylor, 1982; Miyazaki et al., 1985). Interestingly, the most common responses to the stimulation of the command fibers in these connectives are inhibitory in nature inducing bradycardia, arrhythmia or heart arrest. In addition, stimulation of this command system also elicits leg movements (Wilkens et al., 1974). This command system may be the primary neural pathway that enables the central pattern generators for ventilation and circulation to be overridden by sensory input.

The fact that cardiac and escape responses are not necessarily triggered together suggests a more complex relationship between them. When both responses were elicited on perceiving a threat, a tight correlation was found. However, bradycardia and not tachycardia was observed in *Chasmagnathus*. This may seem counterintuitive and maladaptive since the animals might be accumulating an oxygen debt when escape is starting. It is well

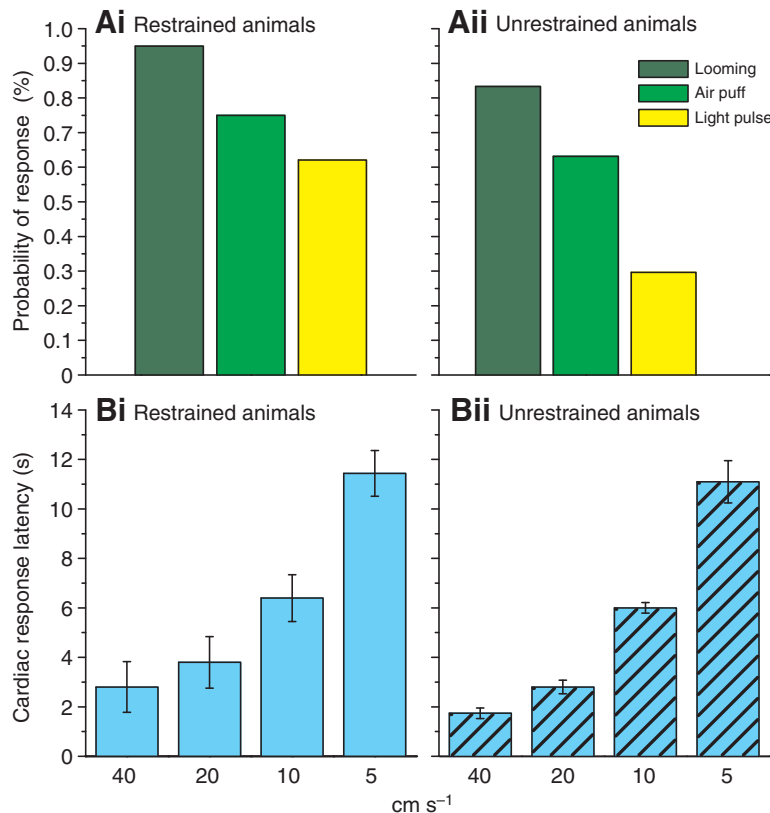


Fig. 10. (A) Probability of response to three stimuli (looming stimulus, air puff, light pulse). (i) Restrained condition. (ii) Unrestrained condition. (B) Cardiac response latency to the presentation of different looming expansions. (i) Restrained condition. (ii) Unrestrained condition.

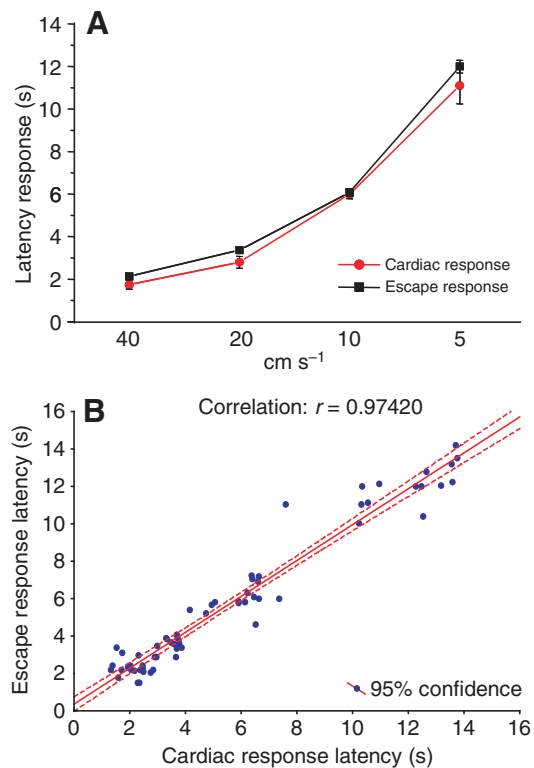


Fig. 11. (A) Comparison between the escape response and the cardiac response latencies to the presentation of different looming stimuli (40 cm s⁻¹; 20 cm s⁻¹; 10 cm s⁻¹; 5 cm s⁻¹). (B) Linear correlation between cardiac and escape responses.

accepted that animals increase their ventilation rate and cardiac output after a sudden stimulus (Wingfield, 2003) in a 'fight or flight' reaction, and although animals across many taxonomic groups have an alternate response to sudden stimuli that includes decreased ventilation rate and decreased cardiac output, these autonomic responses are normally correlated with behavioral freezing (King and Adamo, 2006). The prevalence of this alternate reaction across vertebrate and invertebrate groups invites hypotheses that assume it has a universally adaptive function related to 'death feigning behavior' (McMahon and Wilkens, 1974; Horridge, 1965); adaptive metabolic drops (Burnett and Bridges, 1981); blood redistribution in preparation for flight (Laming and Savage, 1980; Laming and Austin, 1981; King and Adamo, 2006).

Chasmagnathus, on the contrary, upon the presentation of sudden and threatening stimulus, exhibits an initial burst of activity or startle response presumably in an attempt to escape, though somewhat restricted by the actometer, together with a strong CIR. This short lived activity as well as the accompanying bradycardia lasts no longer than 10 s. Tachycardia may be developing later or in a more natural setting. Guirguis and Wilkens (Guirguis and Wilkens, 1995) have stated that crustacean heart rate response to exercise involves two phases. Phase I is rapid onset tachycardia, which occurs within the first 2–3 min of exercise. Most probably this occurs in *Chasmagnathus*, but we have not yet explored responses beyond 10 s after stimulus presentation.

The results obtained with virtual looming stimuli suggest that they are as effective as more natural stimuli in eliciting cardiac responses and applicable in research on the processes underlying the perceptual physiology of invertebrates. Although it is generally

acknowledged that many natural or artificial threatening stimuli do not only elicit immediate overt defensive or avoidance behavior, but also generate autonomic changes including rapid changes in heart rate and blood pressure, few studies have been conducted in invertebrates. In this work we found a clear correlation between cardiac and escape response in *Chasmagnathus* to computer-generated looming stimuli. Previous work on this crab had shown that a robust and reliable escape response could be elicited by computer-generated looming stimuli while two subclasses of previously identified movement-detector neurons from the lobula (third optic neuropil) exhibited robust and consistent responses to the same looming stimuli that trigger the behavioral response (Oliva et al., 2007). These effects were also studied in pigeons, showing a tight correlation between the activity of the rotundal looming-sensitive cells, the muscle activity and the heart rate measurements (Wang and Frost, 1992; Wu et al., 2005). These findings and ours strengthen the idea that in the face of impending danger the crab triggers several integrated defensive reactions.

Although no response threshold differences were induced by the restrained condition, restrained crabs are more likely to respond to sensory stimulation. Although animals in both conditions showed a significant decrease in R_2 for all the stimuli examined, differences in the probability of cardiac response were found between them. These might be related to differential attention states, as well as stress or sensitization imposed by the restrained condition.

The responses of the cardiovascular and respiratory systems in crustacea to environmental and socially imposed alerting stimuli are very similar to the responses of vertebrates mediated by the autonomic nervous system (ANS) (Astley et al., 1991; Cuadras, 1979; Cuadras, 1980; McMahon, 1995; McMahon and Wilkens, 1983; Li et al., 2000; Listerman et al., 2000; Schapker et al., 2002; Shuranova and Burmistrov, 2002; Shuranova et al., 2006). It is probable that the selective pressures that promoted the development and maintenance of autonomic responses in the invertebrates are the same for vertebrates. Recently it has been argued that although there is no structural counterpart to the ANS in the invertebrates, the basic functional properties of the ANS may have been established very early in metazoan evolution (McMahon, 1995; Miller, 1997; Shimizu and Okabe, 2007). How the body plan developed such a system may have been different in different taxa, but one would expect some similarities since the crustacean autonomic responses are also neurally driven and regulated. In addition, blood-borne hormones or compounds are released that influence many target tissues at once. The involvement of the cardiovascular and respiratory systems in this function has been conserved in evolution.

LIST OF SYMBOLS AND ABBREVIATIONS

ANS	autonomic nervous system
CIR	cardio-inhibitory response
CR	cardiac response
HR	heart rate
IHR	instant heart rate
IHR _M	mean IHR
LA	locomotor activity
LED	light emitting diode
R_1	the quotient between the IHR of one event randomly selected prior to stimulus on-set and the IHR _M ($R_1 = \text{IHR}_1 / \text{IHR}_M$)
R_2	the quotient between the IHR of one representative event during the cardiac response and the IHR _M ($R_2 = \text{IHR}_2 / \text{IHR}_M$)
VDS	visual danger stimulus

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