



Escape response of the crab *Neohelice* to computer generated looming and translational visual danger stimuli



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ABSTRACT

Historically, arthropod behavior has been considered to be a collection of simple, automaton-like routines commanded by domain-specific brain modules working independently. Nowadays, it is evident that the extensive behavioral repertoire of these animals and its flexibility necessarily imply far more complex abilities than originally assumed. For example, even what was thought to be a straightforward behavior of crabs, the escape response to visual danger stimuli, proved to involve a number of sequential stages, each of which implying decisions made on the bases of stimulus and contextual information. Inspired in previous observations on how the stimulus trajectory can affect the escape response of crabs in the field, we investigated the escape response to images of objects approaching directly toward the crab (looming stimuli: LS) or moving parallel to it (translational stimuli: TS) in the laboratory. Computer simulations of moving objects were effective to elicit escapes. LS evoked escapes with higher probability and intensity (speed and distance of escape) than TS, but responses started later. In addition to the escape run, TS also evoked a defensive response of the animal with its claws. Repeated presentations of TS or LS were both capable of inducing habituation. Results are discussed in connection with the possibilities offered by crabs to investigate the neural bases of behaviors occurring in the natural environment.

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1. Introduction

Insects and crustaceans are traditional models for neuroethological studies. Investigations on these animals have provided insights that are of general neurobiological interest concerning matters as diverse as the coincidence detection as a mechanism of behavioral responses (Edwards et al., 1998), the effects of social status and social hierarchy on the properties of individually identified neurons (Gaten et al., 2012; Herberholz et al., 2001; Yeh et al., 1996), the mechanisms of circadian rhythms (e.g. Cavanaugh et al., 2014; Yao and Shafer, 2014); the neural processes involved in visual motion (e.g. Borst, 2014; Cuntz et al., 2013; Oliva and Tomsic, 2014), visually guided navigation and collision avoidance (e.g. Fotowat et al., 2011; Oliva and Tomsic, 2012; Rosner and Homberg, 2013), and the mechanisms of learning and memory (e.g. Liu et al., 2006; Menzel, 2013; Tomsic and Maldonado, 2014; Tomsic and Romano, 2013). Some of these achievements were possible because of the particular advantages for the neurophysiological approach offered by arthropods, namely the presence of giant

neurons, which are easily accessible with microelectrodes, in combination with the animal's resilience to laboratory manipulations. However, behavioral and physiological studies in simplified laboratory conditions often tend to dismiss the complexity of the real world. An animal behaving in its natural environment has to relentlessly make behavioral decisions based on the flow of incoming information and its previous experience. Even the escape response to a sudden predator attack, which may appear a simple reflex behavior, can entail considerable complexity. Upon visual detection of the predator, animals often have to decide whether, when, in which direction and how intensely to perform an escape response. Each one of these decisions is known to be strongly affected by stimulus features, such as its size, speed and trajectory, as well as by environmental and behavioral contexts, such as the animal's position relative to a refuge. Additionally, decisions also depend on the animal's learnt experiences (e.g., Lima and Dill, 1990; Herberholz and Marquart, 2012; Hemmi and Tomsic, 2012). But, if the natural environment is so important in shaping the avoidance behavior, why to study it in simplified and rather artificial laboratory conditions? It is because as long as the essence of the behavior is preserved, characterizing the response to simple stimuli in the laboratory is a requisite for the identification of neurons subserving

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such behavior and for understanding the way these neurons perform their fundamental operations.

The escape behavior of semiterrestrial crabs to visual stimuli has been extensively investigated both in the field and in the laboratory. Field studies have been mostly performed with fiddler crabs (e.g. Land and Layne, 1995a,b; Hemmi, 2005a,b; Hemmi and Merkle, 2009; Hemmi and Pfeil, 2010; Raderschall et al., 2011), whereas laboratory studies have been mostly carried out with the grapsid crab *Neohelice* (previous *Chasmagnathus*) *granulata* (e.g. Oliva et al., 2007; Oliva and Tomsic, 2012). The latter species proved to be highly suitable for neurophysiological studies and these studies provided a great deal of knowledge of the neural system and the processes involved in crustaceans visually guided behavior (Berón de Astrada et al., 2001, 2009, 2011, 2012, 2013; Medan et al., 2007; Oliva and Tomsic, 2014; Sztarker and Tomsic, 2008; Sztarker et al., 2005, 2009). Field conducted investigations showed that crabs assess the level of risk of predators attacks analyzing the visual image of the moving predator. As the risk level rises, crabs change the avoidance strategies, escalating from freezing reactions, to run home and eventually burrow descend responses (Hemmi, 2005a,b). One paradoxical result of these studies was the finding that objects approaching more directly toward a crab, i.e. representing a high-risk predator attack, induced later escape responses than objects moving less directly to the animal (Hemmi and Pfeil, 2010). The field experiments were performed using a dummy that moved toward crabs that were located at different distances of the stimulus tracking line. Thus, the stimulus did not approached in a direct collision course (known as looming stimulus) or moved in a perfect translational trajectory with respect to the crab.

In the present study we investigated the escape response of crabs to pure looming or translational computer generated stimuli in the laboratory. The objectives of this study were to see how the trajectory of dangerous visual stimuli affects the escape response of *Neohelice*, and to characterize the responses to computer generated stimuli that can be used later in neurophysiological investigations.

2. Materials and methods

2.1. Animals

Animals were adult male *Neohelice granulata* (previously *Chasmagnathus granulatus*) crabs 2.7–3.0 cm across the carapace, weighing approximately 17 g, collected in the rías (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 at a density of 20 crabs per tank. Water used in tanks and other containers during the experiments was prepared using hw-Marinex (Winex, Hamburg, Germany), salinity 10–14‰, pH 7.4–7.6, and maintained within a temperature range of 22–24 °C. The holding and experimental rooms were maintained on a 12 h:12 h light:dark cycle (lights on 07:00 h to 19:00 h) and the experiments were run between 08:00 h and 19:00 h. Experiments were performed within the first 2 weeks after the animals arrived. Crabs were fed rabbit pellets (Nutrients, Buenos Aires, Argentina) every 3 days and after feeding the water was changed. Following experiments, animals were returned to the field and released in a location separated 30 km from the capture area.

2.2. Behavioral recording setup

The locomotor activity of the crab was investigated in a walking simulator device that has been described in detail elsewhere (Oliva

et al., 2007). Briefly, it consisted of a floating styrofoam ball that could be freely rotated by the locomotor activity of an animal, attached in a standing position to a weightless rod through a piece of rubber glued to its dorsal carapace. The rod was introduced inside a metal guide, positioned vertically above the ball, where it could be slid up and down with little friction (Fig. 1A). This allowed the animal to feel its own weight and thus to adopt its

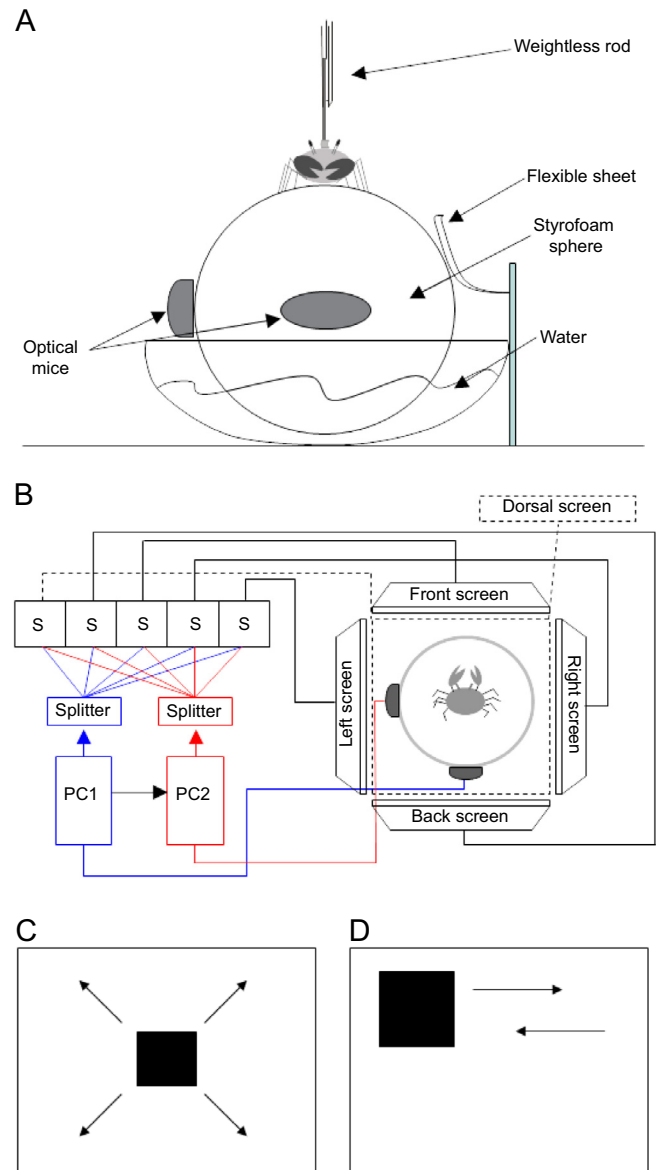


Fig. 1. Set up for measuring the escape response and visual stimuli. (A) Frontal view of the recording system. Locomotor activity was studied in a walking simulator device, which consisted of a floating sphere that could be freely rotated by the animal. The crab was held in position by a weightless rod attached to its carapace that could move freely up and down within a vertical guide located above the crab. Both the rod and the guide sleeve had square cross-sections, which prevented the animal from rotating around its yaw axis. The horizontal position of the floating ball was stabilized by four contact points separated by 90° and provided by two optical mice and two flexible sheets. Locomotion was assessed by recording the rotations of the ball with two mice. (B) Dorsal view of the stimulation and recording system. Five screens were located at 20 cm to the sides, in front, behind and above the animal (the dashed line represents the upper monitor). Stimulus signals generated by PC1 could be directed to any one of the five monitors through a selector switch, while PC2 generated a white screen (context). PC1 also provided a pulse to PC2 for synchronizing the recording of the mice. (C) Representation of looming stimulus (LS). (D) Representation of translational stimulus (TS).

natural posture while performing on the ball. The rod and the guide had both square sections, which prevented rotational movements and assured that the animal always saw the stimulus with the lateral pole of the eye, where sensitivity is highest (Berón de Astrada et al., 2012).

The styrofoam ball (16 cm in diameter) floated within a bowl-shaped container partially filled with water. Horizontal displacements of the ball were prevented by four set points provided by two optical mice and by two flexible sheets located at right angles from each other (Fig. 1A). Ball rotation was recorded by the two mice, with their optical reading systems protected by transparent acetate sheets, which also guaranteed the smooth movement of the ball. Locomotion signals were acquired using the recording facilities of the commercial software used to generate the visual stimuli (Presentation 5.3, Neurobehavioral Systems Inc., Albany, CA, USA). Two Presentation programs were run in two separate PCs (Fig. 1B). The PC that generated the visual stimuli (PC1) was used to record one mouse and to trigger the recording by the second mouse in the second PC (PC2). Hence, the program that generated the visual stimulus synchronized the recording of the two mice just before stimulus onset. The data from mice 1 and 2 during a trial generated two files, which contained a list of times associated with each data record and frame update. Further details on data recording can be found in Oliva et al. (2007). Behavior was also monitored by visually observing the animal on-line with a video camera.

2.3. Visual stimuli

Computer generated visual stimuli can be projected either simultaneously or alternatively on five flat screen monitors (Philips 107T; horizontal and vertical screen dimensions 32 cm and 24 cm respectively, refreshing rate 60 Hz), located at 20 cm in front, back, above and on both sides of the animal (Fig. 1B) (Oliva and Tomsic, 2012). The monitors were covered with anti-glare screens to reduce reflections between them. Visual stimuli were generated with a PC using Presentation 5.3. In this study the experiments were performed with stimuli presented only on the monitor located at the animal's right, in order to keep the visual danger stimuli at a fixed position on the lateral visual pole. We have previously shown that the initial response time is the same for stimuli approaching frontally or laterally (Oliva et al., 2007). However, when the stimulus is approaching frontally, the escape response includes a rotational component of the animal in attempt to run sideway. This rotation maneuver makes the analysis of the run velocity more difficult, a complication that for the purpose of the present study we wished to avoid.

An object approaching in a direct collision course to an animal generates a symmetrical image expansion on its retina, which is usually referred as a looming stimulus (LS). An object moving parallel to the animal, a translational stimulus (TS), also generates a change in the apparent size, which first increases as the object nears the animal and then decreases as it moves away. These changes in the image size, however, are not symmetrical.

The simulated looming stimulus (Fig. 1C) was designed as described by Oliva and Tomsic (2012). The one used in the present study consisted of a 70 cm black square, which approached directly toward the crab over a distance of 9.7 m at a constant speed of 285 cm/s. For the crab's eye the stimulus had an apparent size of 4° at its stationary initial position and expanded until covering the entire screen (60°). The duration of the stimulus expansion was 3.4 s.

The translational stimulus (Fig. 1D) consisted of a black square subtending 34° (12 cm sides), which moved parallel to the animal from one side of the screen to the other at a constant speed of approximately 48°/s (18 cm/s on the lateral screen). Each TS

presentation initiated with the square moving backward until reaching the end of the screen where it changed direction and moved forward to reach the initial position. The backward and forward motion cycle encompassed in a TS presentation was completed in 3.4 s.

Visual simulations generated with the computer may differ from the visual input experienced under natural conditions in various respects. For example, the refresh rate of a monitor screen may impose a severe constraint on the study of the visual system of animals with a high flicker fusion frequency. We did not measure the fusion frequency in *Neohelice*; however, in fiddler crabs, this frequency was found to be below 50 Hz (Layne et al., 1997). In crayfish, responses to looming stimuli corresponding to real approaching objects or filmed representations projected at 24 frames-s⁻¹ yielded identical results (Glantz, 1974). The effectiveness of 2D computer images to elicit the crab's escape response has previously been demonstrated in *Neohelice* (Oliva et al., 2007; Oliva and Tomsic, 2012). Moreover, we found no differences between the escape response elicited by a black sheet of cardboard approaching the animal and the computer simulation of an object of the same size and approaching speed (Oliva, 2010).

2.4. Conditions of stimulation and recording

We began stimulation after the animal had remained visually undisturbed inside the setup for 10 min. The locomotor responses to both LS and TS were recorded during 10 s after the stimulus began. Only one stimulus (either LS or TS) per trial was presented and recorded in each animal, except in the habituation experiment where each animal received 9 trials separated by 1 min. Brightness on the monitor screen was 4 mW/m² (black square) and 240 mW/m² (background).

2.5. Criteria for response selection, beginning of escape and data analyses

All animals challenged with the LS or the TS used in the present study displayed some level of behavioral reaction. However, we considered as escapes only those responses in which the animal moved more than 8 cm during the recording period. We defined the beginning of the escape run as the moment in which the animal's velocity exceeded 1.5 cm/s. Rational for choosing this threshold velocity criteria is because it is just above the maximum velocity recorded for any animal walking on the ball previous to the stimulus presentation. This is followed by a progressive increase in the animal's speed (see Fig. 4 in Oliva et al., 2007). Each recording was examined separately and the response delay was obtained for every animal. Chi square test was used to statistically analyze the proportion of responding animals, *t* test was used to analyze values of latency, velocity and distance of the escape response, and repeated measures ANOVA was used to analyze habituation of the escape response. Parametric tests were performed after checking for normality and homoscedasticity.

3. Results

3.1. Escape response probabilities to TS and LS

The effectiveness of computer simulated object approach (LS) to evoke the escape response of the crab in the laboratory has been already demonstrated (Oliva and Tomsic, 2012), but the efficacy of simulated objects moving parallel to the animal (TS) has never been explored. In order to determine the size of TS required to elicit consistent escape responses we performed a first experiment with TS of three different sizes ($n = 23$ crabs per stimulus size).

Square black objects with 3 or 6 cm sides evoked the escape response in only 17% and 24% of the animals respectively. The TS of 12 cm, however, evoked escape in most cases (70% of the animals). Therefore, the following experiments were performed with TS of 12 cm.

3.2. Defensive response probabilities to TS and LS

Fig. 2A shows that despite the fact that 12 cm TS ($n = 43$) was quite effective in eliciting escapes, the response probability evoked by the LS ($n = 30$) was even higher (χ^2 : 9.53, $P = 0.002$). This difference can be caused by a difference in the intensity as well as in the meaning these two stimuli may have for the crab. The latter is supported by the fact that while escaping from the TS many crabs also performed a defensive response, which consisted of the animal raising the claws toward the stimulus. This protecting component

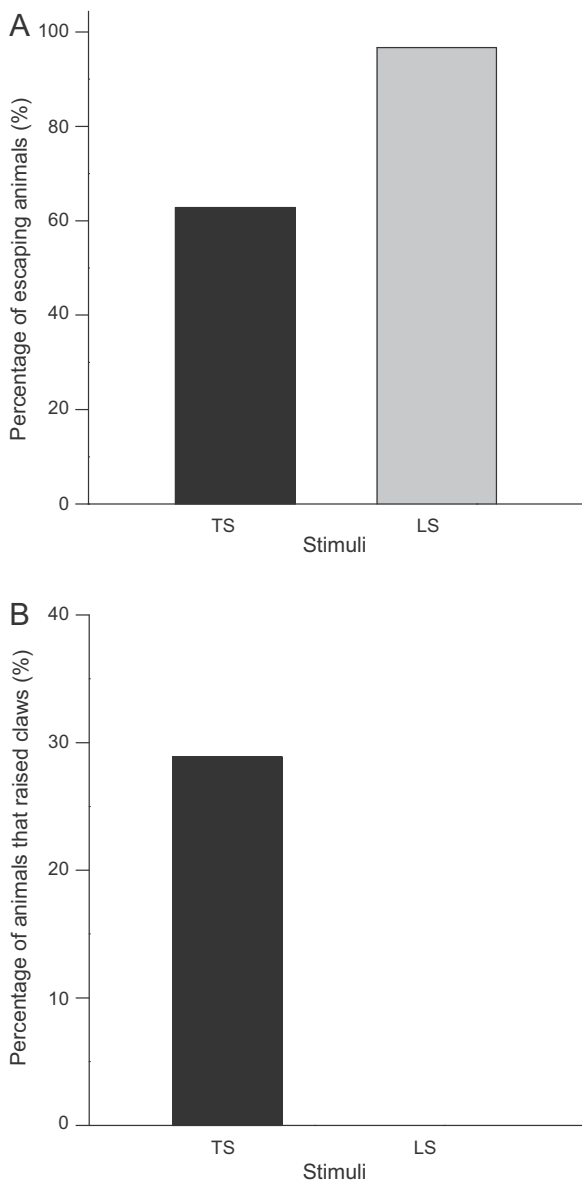


Fig. 2. Percentage of animals displaying escape and defensive responses to TS and LS. (A) The percentage of animals that escape from LS ($n = 30$) is significantly higher than from TS ($n = 43$) ($\chi^2 = 9.53$; $p = 0.002$). (B) The percentage of animals that reacted raising their claws was significantly higher with TS than with LS ($\chi^2 = 8.56$; $p = 0.003$).

was observed in 30% of the escapes to the TS, but in none of those performed to the LS (Fig. 2B). Thus, it appears that crabs employ different strategies to deal with LS and TS, suggesting they may consider the two stimuli as qualitatively different.

We then performed an experiment where the same 12 cm TS was presented at three different elevations, namely, slightly below the animal, slightly above the animal (our standard elevation, Fig. 1D), and right above the animal (using the dorsal screen). Interestingly, when we presented the TS across the dorsal screen the probability of defensive reactions was only 7%, it increased to 30% with the standard elevation, and reached 40% when the TS moved across the lower part of the screen. Thus, with lower TS elevation the defensive strategy is increasingly recruited. These results indicate that in addition to the stimulus trajectory (TS or LS), the animal considers the elevation of moving objects to organize its avoidance and defensive strategies.

3.3. Differences of escape performance to TS and LS

In order to further investigate the way crabs escape from TS and LS, we performed a quantitative analysis of some response parameters. Only those animals that passed the criteria of having performed an escape response were considered in this analysis. Fig. 3A shows the mean temporal course of the escape response to TS ($n = 31$) and LS ($n = 28$). A cursory inspection of this figure reveals several clear differences between responses to the two stimuli. First, the response to TS initiated significantly earlier than that to LS ($t = 13.5$, $P < 0.001$, Fig. 3B), thus confirming previous observations from field studies (Hemmi and Pfeil, 2010). Second, the escape intensity measured as the highest reached velocity

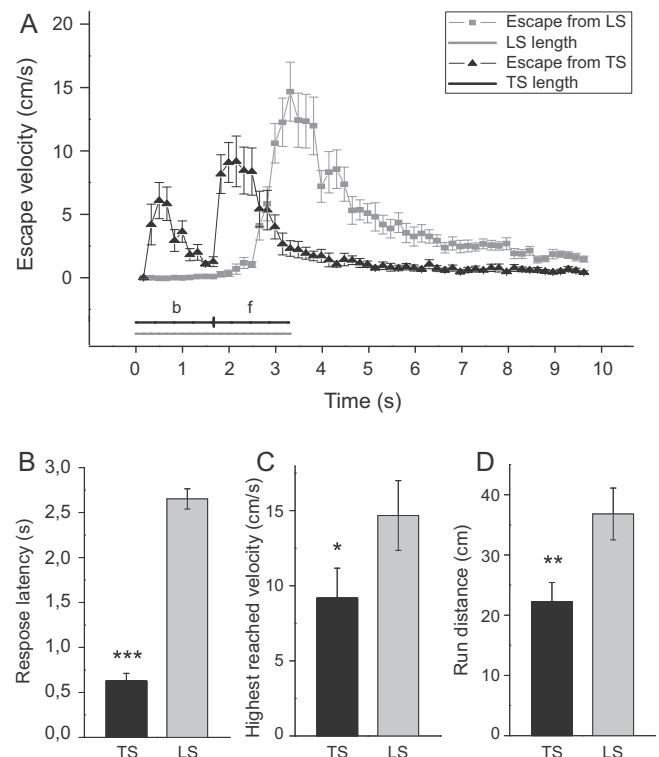


Fig. 3. Quantitative analysis of escape response parameters. (A) Mean temporal course of the escape response from TS ($n = 31$) and LS ($n = 28$). The black line represents the length of a TS trial including the backward (b) and forward (f) movement of the stimulus. The grey line represents the length of the LS trial (for further details see the method section). (B) Response latency. (C) Highest reached velocity. (D) Run distance. Values represent mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

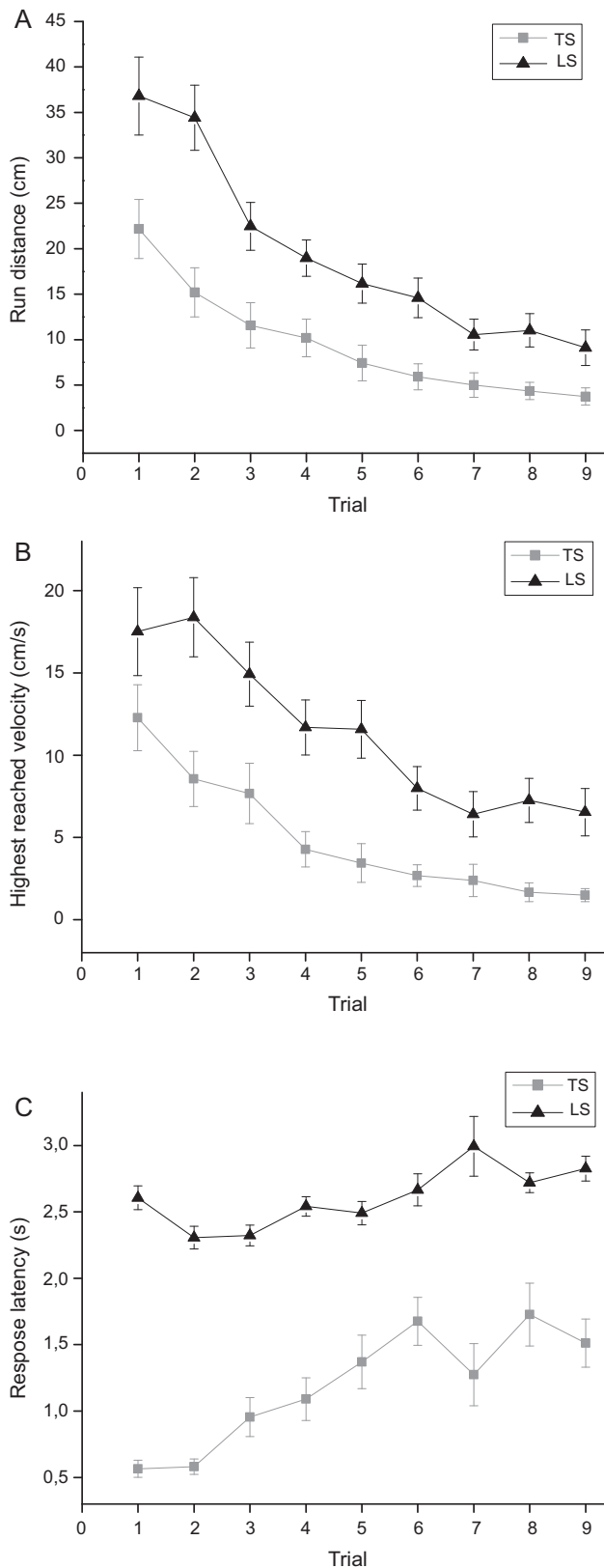


Fig. 4. Habituation of the escape response from TS and LS. Two groups of crabs were trained with 9 repeated presentations of the TS ($n = 27$) or the LS ($n = 29$), each stimulus separated by 1 min. Panels A–C respectively show the mean \pm s.e.m. distance, velocity and latency of escape per trial. Repeated measures ANOVAs performed on these data revealed habituation for TS and LS (statistical values are reported in the text).

and maximum running distance, was greater for LS than for TS ($t = 1.91$, $P = 0.04$, Fig. 3C; and $t = 3.5$, $P = 0.004$, Fig. 3D, respectively). Third, the escape velocity increased monotonically to LS until near the maximum image expansion point, and then slowly declined, while the escape velocity to TS had two peaks, corresponding to the backward and forward object motion. The peaks occurred at the moments when the stimulus was traveling near the center of the screen, i.e. when it was at the closest distance to the animal. Interestingly, the escape velocity reached during forward object motion was significantly higher than during backward motion.

Taken together, the analysis shows that the LS evoked a stronger escape response (higher velocity and greater running distance) than the TS, but the response started later (longer response latency).

3.4. Habituation of the escape response to TS and LS

The previous analyses have shown that crabs respond quite differently to TS or LS threats. We then decided to explore whether these differences extend to the way repeated stimulus presentation affects behavioral changes, i.e. the effect of habituation. We trained two groups of crabs with 9 presentations of either the TS ($n = 27$) or the LS ($n = 29$), each stimulus separated 1 min from each other. Fig. 4 shows the mean values of three escape parameters per trial. A: Run distance: A repeated measures ANOVA showed that LS evoked longer escape runs than TS (stimulus effect $P < 0.001$), and that both stimuli induced habituation (trial effect $P < 0.001$), with stimulus per trial interaction ($P < 0.001$). B: Highest reached velocity. The ANOVA showed that LS evoked higher escape velocities than TS (stimulus effect $P < 0.001$), and that both stimuli induced habituation (trial effect $P < 0.001$), without stimulus per trial interaction ($P = 3.78$). C: Response latency. The ANOVA revealed that LS evoked the escape response much later than TS (stimulus effect $P < 0.001$), and that both stimuli induced habituation (trial effect $P < 0.001$), with stimulus per trial interaction ($P < 0.001$).

4. Discussion

In a recent review on the escape response of crabs Hemmi and Tomsic (2012) discussed results obtained in the field and in the laboratory, which showed important similarities but also some differences. They pointed out the benefits and limitations of field and laboratory studies to understand animal behavior, and stressed the importance of combining the two approaches. Inspired by previous observations made in the field on the escape response of fiddler crabs to visual stimuli, here we performed experiments to evaluate whether the trajectory of simulated moving objects affects the behavioral responses measured in the laboratory. The experiments were performed with the grapsid crab *Neohelice*, in order to later investigate the characterized responses at the neurophysiological level.

In nature *Neohelice* is preyed by different species of seabirds like gulls, oystercatchers and shorebirds, which approach the crab using several different strategies including walking, surface seizing and surface plunging (Bachmann and Martinez, 1999; Copello and Favero, 2001). Consequently, the visual features of a predatory attack widely vary for the crab. This makes unlikely the animal to recognize a predator relying on a single characteristic of the visual stimulus such as the retinal size or speed (Land and Layne, 1995a,b).

In previous studies we showed that computer simulations of directly approaching objects are highly effective to evoke the

escape response (Oliva et al., 2007; Oliva and Tomsic, 2012). Our present results show that crabs also escape from a large simulated object that moved parallel (TS) to them. When considering the escape response probability (Fig. 2), as well as the maximum velocity (Fig. 3C) and escape distance (Fig. 3D), the TS seemed less effective than the LS. However, responses to TS initiated much earlier than those to LS (Fig. 3A and B). This seems a paradoxical result because a predator approaching directly to its prey imposes more risk than one moving parallel to it. In our experiments, it could be argued that crabs responded later to the LS because of the time it takes for this stimulus to reach the size of the TS. However, experiments using a single object (i.e., without size difference), performed by Hemmi and Pfeil (2010) with fiddler crabs in the field, have shown the same result. The explanation given by these authors is that the crab escape response is initially triggered by the stimulus retinal speed, which in the case of LS is very low at the beginning of the expansion.

Contrasting with the response to LS, the escape response to TS quite often included another behavioral component, a defensive reaction that consisted in the animal raising the claws against the visual danger stimulus while escaping (Fig. 2B). This component became more frequently recruited as the TS moved at lower elevation. In nature, this type of behavior is often observed during male-male fight interactions. Thus, a TS moved at lower elevation might have an ambiguous meaning for the crab, being considered as a potential predator or as another male. In fact, an elevation based criteria has been proposed to distinguish predators from conspecifics in fiddler crabs (Land and Layne, 1995a; Smolka et al., 2011). Further experiments in *Neohelice* using different object sizes and elevations will be attempted to disentangle the stimulus parameters considered by the animal to decide whether to only escape or to add the defensive response component.

The response to TS showed two peaks of maximum velocity (Fig. 3A), related with backward and forward stimulus movement. The peak corresponding to forward movement being considerably higher than the peak corresponding to backward movement. In arthropods, neurons showing a preferred response direction are widely documented in the context of optic flow analysis (e.g. Borst and Egelhaaf, 1990; Egelhaaf and Borst, 1993; Borst et al., 2010), and in the context of object motion (e.g. Gonzalez-Bellido et al., 2013; Nordström, 2012). Moreover, a differential sensitivity to forward and backward direction of translational objects has been shown in the lobula giant movement detector neuron of the locust (Dick and Gray, 2014; McMillan and Gray, 2012), a neuron that shares many characteristics with the monostriated lobula giant neurons of the crab (Oliva et al., 2007; Oliva and Tomsic, 2014). Therefore, it will be interesting to look at the sensitivity to backward and forward motion in these crab's neurons. Yet, the higher velocity developed during forward motion (second part of the TS motion cycle) could be due to a sensitization produced by the first part of the motion cycle. Because in our experiments the stimulus always moved backward first, and then forward, further experiments are needed to disclose whether the factor that prompted the high escape velocity during the second half of the TS trial was the motion direction or a sensitization effect. In any case, a preferred motion direction or a sensitization effect should be reflected, and deserves to be investigated, at the level of the lobula giant neurons (Sztarker and Tomsic, 2008, 2011).

Habituation of the escape response of crabs to the sight of moving objects has been studied in the field (Hemmi and Merkle, 2009; Fathala et al., 2010a,b; Raderschall et al., 2011) and even more intensively in the laboratory (reviewed in Tomsic et al., 2009). Our present result shows that habituation occurs also with computer simulated objects, and it can be induced with both TS and LS. We have previously shown that habituation to visual danger stimuli is accounted by the activity of lobula giant neurons (e.g.

Tomsic et al., 2003; Sztarker and Tomsic, 2011). These studies were performed by using a real moving object, which somehow limits the possibilities of electrophysiological investigations. Knowing that habituation can be driven by computer generated stimulation, which easily allows changing the position, direction, velocity, and many other stimulus parameters, we can now investigate how these changes affect habituation at both behavioral and neuronal level more deeply.

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