

# Arousal Facilitates Collision Avoidance Mediated by a Looming Sensitive Visual Neuron in a Flying Locust

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**Rind FC, Santer RD, Wright GA.** Arousal facilitates collision avoidance mediated by a looming sensitive visual neuron in a flying locust. *J Neurophysiol* 100: 670–680, 2008. First published May 28, 2008; doi:10.1152/jn.01055.2007. Locusts have two large collision-detecting neurons, the descending contralateral movement detectors (DCMDs) that signal object approach and trigger evasive glides during flight. We sought to investigate whether vision for action, when the locust is in an aroused state rather than a passive viewer, significantly alters visual processing in this collision-detecting pathway. To do this we used two different approaches to determine how the arousal state of a locust affects the prolonged periods of high-frequency spikes typical of the DCMD response to approaching objects that trigger evasive glides. First, we manipulated arousal state in the locust by applying a brief mechanical stimulation to the hind leg; this type of change of state occurs when gregarious locusts accumulate in high-density swarms. Second, we examined DCMD responses during flight because flight produces a heightened physiological state of arousal in locusts. When arousal was induced by either method we found that the DCMD response recovered from a previously habituated state; that it followed object motion throughout approach; and—most important—that it was significantly more likely to generate the maintained spike frequencies capable of evoking gliding dives even with extremely short intervals (1.8 s) between approaches. Overall, tethered flying locusts responded to 41% of simulated approaching objects (sets of 6 with 1.8 s ISI). When we injected epinastine, the neuronal octopamine receptor antagonist, into the hemolymph responsiveness declined to 12%, suggesting that octopamine plays a significant role in maintaining responsiveness of the DCMD and the locust to visual stimuli during flight.

## INTRODUCTION

How is vision for action different from vision during passive observation? Attention and arousal are well known to influence processing of visual information either by sharpening response tuning of neurons in visual areas processing motion or by increasing sensitivity to a specific stimulus (Aston-Jones et al. 1992; Devauges and Sara 1990; Harley 1987; Womelsdorf et al. 2006). Without the change in state accompanying arousal, the responses of the visual system to a repeated stimulus often decline. This process of decline can be reversed either by the sudden presentation of a new stimulus or by a change in state caused by the release of neuromodulatory substances from the nervous system (Bacon et al. 1995; Berridge et al. 1993; Bicker and Menzel 1989; Cole and Robbins 1992; Evans 1985; Gray 2005; Gu 2002; Lapiz and Morilak 2006; Orchard et al. 1993; Ramirez and Pearson 1991; Rowell 1971b; Stern et al. 1995, 1999; Stevenson et al. 2005; Vankov et al. 1995; Weisel-Eichler and Liebersat 1996).

In insects, the neuromodulator octopamine is thought to perform functions similar to those of norepinephrine in mammals (Evans 1985). Rapid rises in octopamine level produce an increase in the general level of arousal, changing the insect from a resting state into an active state, ready to process incoming sensory stimuli and prepared for sustained activity or aggression (Bacon et al. 1995; Bicker and Menzel 1989; Evans 1985; Orchard et al. 1993; Ramirez and Pearson 1991; Rowell 1971b; Stevenson et al. 2005; Weisel-Eichler and Liebersat 1996). Octopamine is released both locally within the CNS, to modulate activity of target visual neurons (Bacon et al. 1995; Stern et al. 1995, 1999), and in a more widespread way into the blood, increasing the insect's preparedness either to escape a threatening stimulus by flight (Bicker and Menzel 1989; Orchard et al. 1993; Ramirez and Pearson 1991; Simpson et al. 2001; Weisel-Eichler and Liebersat 1996) or to maintain a fight (Stevenson et al. 2005). When locusts or crickets are forced to fly, or the mechanosensory pathways in their hind legs are stimulated, octopamine levels in the hemolymph and optic lobes increase (Bacon et al. 1995; Simpson et al. 2001). Octopamine elevation is particularly marked in the first 10 min of flight and at the time of flight initiation (David et al. 1985; Goosey and Candy 1980). Epinastine is a drug that specifically binds to neuronally located octopamine receptors in locusts and it can be used to block octopamine-induced behavior (Roeder et al. 1998; Stevenson et al. 2005).

Locusts are good subjects for investigating the role of arousal on identified neurons and behavior because they have some large identified visual neurons (O'Shea and Williams 1974; Rowell 1971a) and have evolved a high level of sensitivity to looming objects, probably as an adaptation to predation (Gabbiani et al. 1999; Hatsopoulos et al. 1995; Matheson et al. 2004; Paron et al. 2007; Rind and Simmons 1992, 1997; Schlotterer 1977). This sensitivity is mediated by two large identified neurons: the lobula giant movement detector (LGMD; O'Shea and Williams 1974) and its postsynaptic target, the descending contralateral movement detector (DCMD; Rowell 1971a). These neurons respond most strongly to rapidly approaching objects (Gabbiani et al. 1999, 2002, 2005; Hatsopoulos et al. 1995; Peron et al. 2007; Rind and Simmons 1992, 1997; Schlotterer 1977), with the DCMD following 1–1 spikes in the LGMD (Rind 1984). The DCMD excites some flight motoneurons (Simmons 1980) and can trigger predator avoidance during flight (Santer et al. 2005, 2006). In response to a looming stimulus that is indicative of a predator's approach, a tethered, flying locust will perform a

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“gliding dive,” in which it ceases to beat its wings for one or more wingbeat cycles; the wings are held elevated in stereotyped gliding posture (Santer et al. 2005). These gliding dives are evasive maneuvers and occur in response to approaching objects when a DCMD produces consecutive spikes at a frequency of  $\geq 150$  Hz during the elevation phase of a wingbeat cycle (Santer et al. 2006).

Previously, several studies have shown that the responses of the LGMD and DCMD neurons to both small moving objects and looming ones are subject to habituation (Bacon et al. 1995; Gray 2005; Matheson et al. 2004; Rogers et al. 2007; Rowell 1971b). The extent of the decline in the overall spike numbers, peak spike frequency, and variability in DCMD responses to repeated looming stimuli depends on the phase of the locust (Gray 2005; Matheson et al. 2004; Rogers et al. 2007). When Matheson et al. (2004) and Rogers et al. (2007) delivered 30 stimuli at 60-s intervals, to both solitary and gregarious locusts, they found a decline in spike numbers and peak spike frequency. The decline was more pronounced in solitary locusts where peak DCMD responses rapidly fell to  $< 100$  Hz (by the 15th and 7th stimulus in the respective studies).

A habituated DCMD still responds well to the presentation of a looming object of a novel size or approaching along a novel trajectory (Gray 2005). However, evidence for an arousal-based modulation of habituation comes from other accounts, which report that LGMD and DCMD neurons' responses can be restored by mechanical stimulation of the body, head, or ipsilateral antenna, or by application of octopamine (Bacon et al. 1995; Gauglitz and Stevenson 1993; Ramirez and Pearson 1991; Roeder et al. 1998; Rowell 1971b; Stern 1999). For example, a previously habituated DCMD response to a moving object can be partially restored by the intracellular depolarization of the identified octopaminergic PM4 neurons (Bacon et al. 1995). These cells are also excited by the same mechanical stimuli that have been shown to mediate dishabituation of the DCMD response (Rowell 1971b). Although such studies have correlated physiological parameters, such as constitutive octopamine levels, with the spiking activity of the LGMD–DCMD in response to various visual stimuli, none has measured DCMD responses to looming stimuli following changes in the state of arousal induced by natural behavior itself.

In this study we used two different behavioral manipulations to modify the level of arousal in locusts. First, we raised the level of arousal by stimulating the hind legs of locusts. This stimulation has already been shown to induce changes in the phase of a locust, eventually causing a solitary individual to become gregarious, with an altered response to visual stimuli and an accompanying change in behavior (Simpson et al. 2001). Second, we flew individual locusts in a wind tunnel to simulate the first 10 min of flight. Not only did these manipulations greatly reduce habituation of the spiking activity in an identified neuron in the visual system—the DCMD—but the change in arousal state induced in each case also increased the probability that this neuron produced the code that provoked evasive gliding behavior (Santer et al. 2006).

## METHODS

### Visual stimuli

The computer program to calculate the geometry of object approach was written in Borland Delphi programming language. Graphic com-

mands were sent to a Cambridge Research Systems VSG2/3 image synthesizer and RG2 raster generator system. The resulting signals were displayed on a Kikusui COS1611 X-Y monitor using a green P31 phosphor. The RG2 raster generator produced a display with 438 dots  $\times$  437 lines, resulting in a pixel that subtended  $0.09^\circ$  at the eye. This is smaller than the acceptance angle of locust photoreceptors under either light- or dark-adapted conditions. The screen had a width of 110 mm ( $71.1^\circ$  at the eye) and a height of 80 mm ( $59.5^\circ$  at the eye). The components were chosen for the high speeds at which they could animate and display simple graphics.

The animation rate of approaching and receding objects was kept at 200 Hz (the maximum refresh rate of the RG2 unit). The front of the monitor was placed parallel to the long axis of the locust and stimuli were viewed monocularly, by the lateral surface of the compound eye. The object, a dark disc of 80-mm diameter, approached from 207 to 7 cm from the locust, at one of a range of constant velocities from 0.25 to 5  $\text{ms}^{-1}$ . Unless stated otherwise, each simulated object approach stopped when the disc subtended an angle of  $59^\circ$  at the eye. Consequently, collision would have occurred 280 ms after the end of object approach at 0.25  $\text{ms}^{-1}$ ; 140 ms after the end of object approach at 0.5  $\text{ms}^{-1}$ ; 70 ms after the end of object approach at 1  $\text{ms}^{-1}$ ; 35 ms after at 2  $\text{ms}^{-1}$ ; 23.3 ms after at 3  $\text{ms}^{-1}$ ; and 14 ms after at 5  $\text{ms}^{-1}$ .

## EXPERIMENTAL PROCEDURES

Experiments were performed on 152 adult male and female *Locusta migratoria*, obtained from a crowded colony kept at the University of Newcastle, or from an outside supplier (Blades Biological). Experiments were conducted at temperatures of 22–30°C. Locusts were either minimally dissected and restrained or tethered.

1) *Minimally dissected, restrained locusts.* The locust was mounted ventral side uppermost on a plasticine block and a fine minuten pin mounted on a piece of circuit board was manipulated into a 0.5-mm hole in the cervical sternite and lowered into contact with the nerve cord. An earth electrode was inserted into the thorax of the locust. DCMD spike trains were analyzed using Spike2 software (Cambridge Electronic Design). To manipulate levels of arousal, 10 s before each looming stimulus we delivered brush strokes to the hind leg, lasting 1 s. Stroking the leg is known to excite the octopaminergic brain neuron PM4, which increases DCMD responsiveness to brief translating visual stimuli (Bacon et al. 1995; Stern 1999).

2) *Tethered, wind-stimulated, or flying locusts.* A square window was cut in the ventral cuticle of the rostral thorax, just forward of the mesothoracic ganglion. Hook electrodes were implanted as in Santer et al. (2005). The square of removed cuticle was then waxed over the window as a lid and the locust mounted, dorsal side up by its pronotum to a brass rod. Locusts were tethered via the dorsal pronotum throughout the experiment. The locust grasped a paper ball and was relatively free to move. In trials where the locust was wind stimulated but not flying it continued to grasp the paper ball but was constantly stimulated with a 1.5  $\text{ms}^{-1}$  laminar air flow from a simple wind tunnel powered by a DC ball-bearing fan (Papst TYP 4212 NH; RS Components, Northants, UK). Air flow was made laminar by passing it through an array of aligned straws (Santer et al. 2005). Flying was induced by removing the paper ball and increasing the air to 3.0  $\text{ms}^{-1}$ . DCMD records were taken from four locusts during wind stimulation and in the first 5 min of flight. Stimuli, consisting of 80-mm-diameter objects approaching at 3  $\text{ms}^{-1}$ , were delivered in six blocks of six with a 2 min rest period between each block of six stimuli. The six stimuli were delivered at interstimulus intervals (ISIs) of 40, 20, 10, and 1.8 s. Each locust received 432 looming stimuli: 144 without wind stimulation or flight, 144 while wind stimulated, and 144 in flight.

Movement artifacts were removed from the extracellular DCMD records by digital filtering. Power spectra of nerve cord recordings with and without DCMD spikes were compared in Spike2. On the basis of this information, data were high-pass filtered in Spike2. In

most recordings, the DCMD was clearly the largest spike from the nerve cord.

When looms were presented during flight, locusts regularly performed gliding behaviors. Following these behaviors, flight normally resumed after one or two wingbeat cycles but occasionally locusts stopped flying briefly after they performed the glide. When this happened the locust was given a paper ball so tarsal contact was regained. Flight was then resumed after tarsal contact with the ball was lost and the experiment continued. These periods were usually brief compared with the stimulus delivery regime, with a mean time of  $2.18 \pm 0.37$  s (SE) as measured with an infrared sensor and occurred infrequently. For example, for one locust, during a delivery regime of 42 stimuli, at an ISI of 1.8 s, there were nine occasions when the locust stopped flying following object approach. Trials in which the locust was not flying were excluded from the analysis.

To investigate the role of the neuromodulator octopamine in the flight-induced arousal increase and predator avoidance we injected epinastine, an octopamine antagonist (Roeder et al. 1998) that prevents octopamine from binding to its neuronally located receptor, into the thorax of six locusts. Epinastine was applied by injecting 25  $\mu$ l of a 20 mM solution in saline into the thorax, through the arthroal membrane at the base of the prothoracic leg. This site was used because it does not disrupt vision or wing musculature and has recently been shown to be effective in raising octopamine levels in other insect brains: octopamine injected into the thorax of the bee was recovered from the brain after 15 min and elevation persisted for  $\geq 60$  min (Barron et al. 2007). After allowing 45 min for the antagonist to enter the optic lobes (Stevenson et al. 2005), we flew each locust in a laminar 3-m/s air flow and we used the infrared beam to monitor wingtip position during object approach. Stimuli were delivered in groups of six each separated by a 1.8-s interval. As a control, six locusts were injected with saline and treated as described earlier. In total, 540 stimuli were delivered to control animals and 462 to animals injected with epinastine.

### Statistical analysis of arousal effects

To statistically analyze the effect of arousal changes on the DCMD responses we used the statistical analysis software (SAS Institute) generalized linear modeling (Genmod) procedure, in which the data were plotted as a Poisson distribution on a logarithmic scale. A Type III analysis of the sum of means squared was made to assess the significance of each treatment either on DCMD activity or on behavior. This technique is good for analyzing experiments where repeated measures are made on individual subjects following various treatments so that interactions between the treatments can be analyzed simultaneously (Der and Everitt 2002). Additional analyses were used when simple comparisons were sufficient. Nonparametric tests were used if the data were found to have a nonnormal distribution.

## RESULTS

### Without arousal, DCMD spiking responses habituate

Previous studies using static, translating, and looming visual stimuli have shown that the DCMD response habituates to repeated stimulus presentations and that it can be dishabituated by various visual and mechanical stimuli (Bacon et al. 1995; Gray 2005; Rowell 1971a,b). In the first stage of our study, we wanted to build on these experiments to gather new information of direct relevance to a locust's performance of escape behavior during flight. For this reason we use looming visual stimuli that might represent the attacks of aerial predators and analyze aspects of the DCMD response previously shown to underlie the performance of an emergency gliding dive (Santer et al. 2006).

As predicted by previous work on static, translating, or looming stimuli (Bacon et al. 1995; Gray 2005; Rogers et al. 2007; Rowell 1971a,b), we observed that when a locust passively viewed the stimulus without being aroused the DCMD response to a looming stimulus habituated when the stimulus was repeated (Fig. 1). Furthermore, the rate of DCMD habituation was greatest when the ISIs were shortest (Fig. 1A). In these experiments habituation showed as a significant effect of the stimulus number, from first to sixth, on the size of the DCMD response. For unaroused locusts a logistic regression analysis (see METHODS) showed a significant effect of stimulus number and ISI on DCMD spike number per stimulus ( $\chi^2_1 = 70.41$ ,  $P < 0.001$ ). The analysis also confirmed that the rate of DCMD response decline depended on the interval between the consecutive stimuli (ISIs), with significant decline in spike numbers between first and subsequent responses at ISIs of 20, 40, and 80 s but not at 300 s (statistical analysis for ISI = 20 s: stimulus 1 vs. stimulus 2  $\chi^2_1 = 16.64$ ,  $P < 0.0001$ ; 1 vs. 3  $\chi^2_1 = 39.32$ ,  $P < 0.0001$ ; 1 vs. 4  $\chi^2_1 = 57.19$ ,  $P < 0.0001$ ; 1 vs. 5  $\chi^2_1 = 71.15$ ,  $P < 0.0001$ ; for ISI = 40 s: stimulus 1 vs. stimulus 2  $\chi^2_1 = 6.55$ ,  $P = 0.0105$ ; 1 vs. 3  $\chi^2_1 = 14.88$ ,  $P = 0.0001$ ; 1 vs. 4  $\chi^2_1 = 21.67$ ,  $P < 0.0001$ ; 1 vs. 5  $\chi^2_1 = 23.96$ ,  $P < 0.0001$ ; for ISI = 80 s: stimulus 1 vs. stimulus 2  $\chi^2_1 = 0.65$ ,  $P = 0.4188$ ; 1 vs. 3  $\chi^2_1 = 2.99$ ,  $P = 0.0837$ ; 1 vs. 4  $\chi^2_1 = 9.01$ ,  $P = 0.0027$ ; 1 vs. 5  $\chi^2_1 = 12.27$ ,  $P = 0.0005$ ; for ISI = 300 there was no significant decline: stimulus 1 vs. stimulus 2  $\chi^2_1 = 0.92$ ,  $P = 0.3382$ ; 1 vs. 3  $\chi^2_1 = 3.25$ ,  $P = 0.0716$ ; 1 vs. 4  $\chi^2_1 = 0.07$ ,  $P = 0.7876$ ; 1 vs. 5  $\chi^2_1 = 0.84$ ,  $P = 0.3584$ ). DCMD habituation included a decline both in the number of spikes per stimulus (Fig. 1) and in the maximum spike rate during a stimulus (Fig. 2). In locusts where the DCMD was habituated, the DCMD response declined or ceased early during an approach (Fig. 2). The earliest decline in spike numbers occurred for locusts stimulated with the shortest intervals between looming stimuli, which was 20 s in these experiments (Fig. 2, A–C).

Previous reports show that a mechanical stimulus to a locust's head, body, or antennae can restore a habituated DCMD response to a static or translating visual stimulus (Bacon et al. 1995; Rowell 1971a,b). In the current study, we sought to determine whether mechanical stimulation of the hind leg could do the same to the DCMD response to a behaviorally important looming stimulus. When a locust was aroused between stimulus presentations with a 1-s-long mechanosensory stimulus to the hind-leg tibia, habituation was suppressed (Fig. 1B). The general effects of hind-leg stimulation on habituation were robust, repeatable, and reversible (Fig. 1B). Specifically, in habituated subjects, increasing arousal by hind-leg stimulation restored the overall DCMD spike numbers to a prehabituated level, as observed in experiments with nonlooming visual stimuli (Rowell 1971b). A logistic regression analysis confirmed that the negative effect of stimulus number on the DCMD response (habituation), which was significant in nonaroused locusts, was no longer significant in aroused ones (effect of stimulus number: with an approach speed of  $0.50 \text{ ms}^{-1}$  without arousal  $\chi^2_1 = 32.25$ ,  $P < 0.0001$ , with arousal  $\chi^2_1 = 3.38$ ,  $P = 0.6411$ ; with an approach speed of  $1.0 \text{ ms}^{-1}$  without arousal  $\chi^2_5 = 20.65$ ,  $P = 0.0009$ , with arousal  $\chi^2_1 = 6.66$ ,  $P = 0.2470$  and with an approach speed of  $5.0 \text{ ms}^{-1}$  without arousal  $\chi^2_1 = 1.20$ ,  $P = 0.9446$ , with arousal  $\chi^2_1 = 0.14$ ,  $P = 0.9996$ ). DCMD responses were elicited

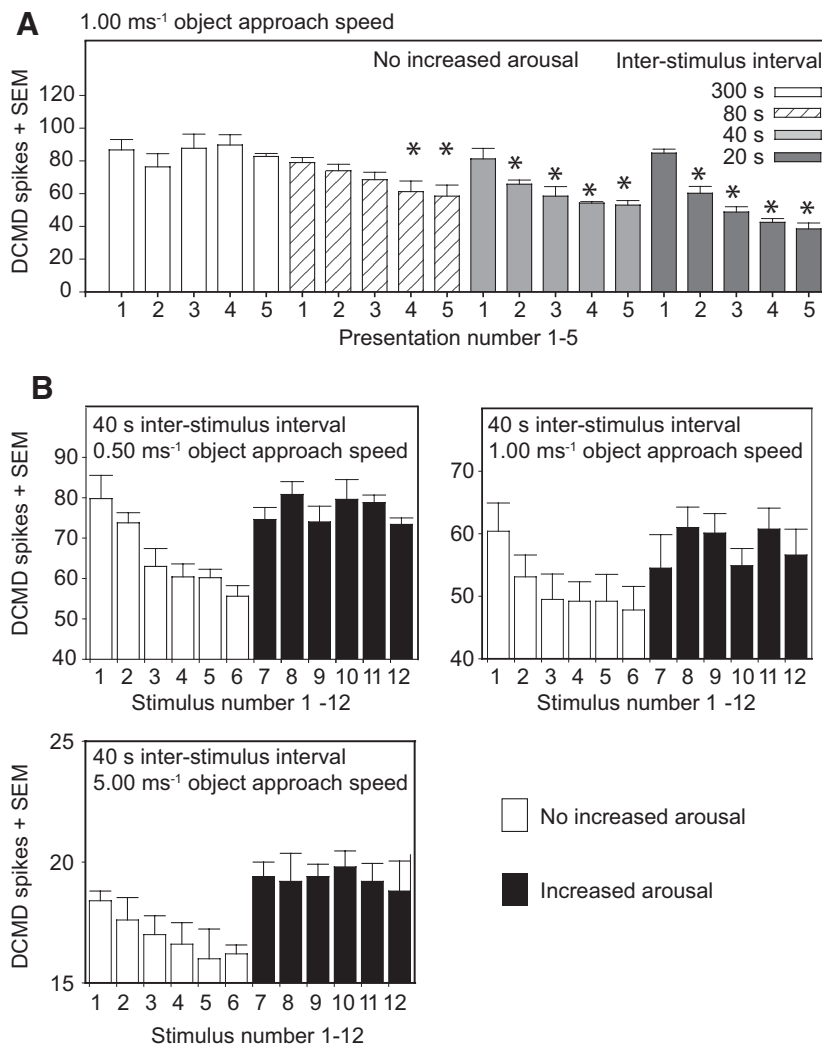


FIG. 1. Descending contralateral movement detector (DCMD) responses depended on the state of arousal. **A:** without an increase in arousal the DCMD response declined following repeated stimulation. Over the range tested, the rate of decline in the number of DCMD spikes generated increased as the interstimulus interval (ISI) was decreased. **B:** with approach speeds from 0.50 to 5.00 ms<sup>-1</sup>, this decline in total DCMD spike numbers could be reversed by increasing arousal by mechanical stimulation of the hind-leg (filled bars).

throughout the entire duration of the loom (Figs. 2 and 3) and the high-frequency DCMD spikes during the final stages of object approach were also restored. This effect of arousal on peak spike frequency is illustrated in Fig. 3, where the bars represent the DCMD mean spike frequency in 20 ms bins over 50 stimulus repetitions with 20-s intervals between each one. Object approach speeds were 0.25, 0.50, or 1.00 ms<sup>-1</sup>. The first 25 stimuli (gray bars) were delivered without any increase in arousal and the second 25 stimuli (dark bars) were each preceded by a stroke to the hind-leg tibia to increase the state of arousal. In the 60 ms before projected collision, the increase in arousal significantly increased the frequency of the DCMD response with approach speeds of 0.25, 0.50, and 1.00 ms<sup>-1</sup> (\**P* < 0.01, Mann-Whitney rank-sum test). The highest instantaneous spike rate was observed in aroused locusts immediately before object approach ceased (Fig. 3, A–D).

High-frequency spikes during the final period of a DCMD's response to a looming stimulus have been implicated in triggering emergency predator-avoidance glides (Santer et al. 2006). These high-frequency spikes are therefore a behaviorally important parameter of the DCMD response. We concentrated on these last-minute DCMD spikes in a further five locusts (locusts 1–5, Fig. 4), where a comparison of the DCMD spike numbers in aroused and nonaroused locusts in the final

milliseconds before collision confirmed our earlier observations that DCMD spike numbers were always significantly greater for aroused subjects, irrespective of loom speed over the range 0.25–5.00 ms<sup>-1</sup> ( $\chi^2_1 = 39.2$ , *P* < 0.001 for loom speeds in the range 0.25–1.00 ms<sup>-1</sup> and  $\chi^2_1 = 12.8$ , *P* < 0.001 for loom speeds of 5 ms<sup>-1</sup>). Spike numbers obtained from aroused locusts were 26–50% (95% confidence limits) greater than the equivalent values recorded from nonaroused subjects with looming speeds in the range 0.25–1.00 ms<sup>-1</sup>. For looming speeds of 5 ms<sup>-1</sup>, the spike numbers were 7–26% (95% confidence limits) greater for aroused locusts than in the nonaroused (Fig. 4). In these experiments, maximum instantaneous frequencies of 500–600 Hz were generated in aroused locusts (Fig. 4, inset, bottom right graph). To permit a direct comparison of the DCMD responses at various looming speeds, spike numbers were counted over time intervals that coincided with a growth of 35° in the angular subtense of the stimulus, at each speed (see Fig. 4 legend for exact intervals). When we looked at the DCMD responses in these experiments, particularly at spike frequencies around 200 Hz as collision was imminent (Fig. 5A), we found that, in nonaroused locusts, the numbers of DCMD spikes occurring at frequencies >200 Hz was low and that when the arousal of the locust was increased, the number of spikes at frequencies >200 Hz

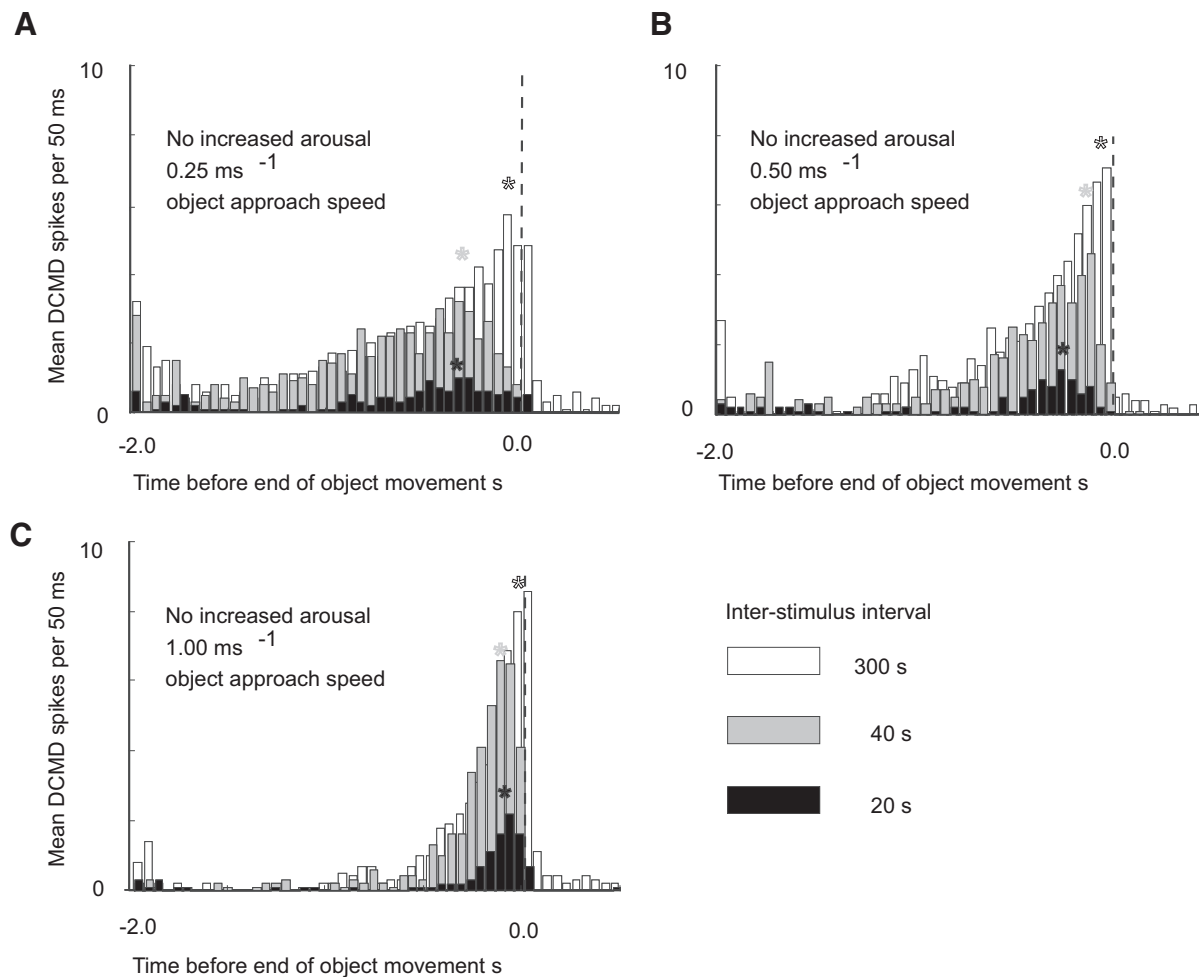


FIG. 2. Without arousal, the shape of the DCMD response envelope to looming stimuli, as well as DCMD spike numbers, depended on the interval between stimuli. Each graph shows DCMD responses to object approach at a particular speed but with different ISIs (20, 40, or 300 s). Approach speeds were 0.25, 0.50, and  $1.00 \text{ ms}^{-1}$  in A, B, and C, respectively. Bars show the mean DCMD spike frequency in 50-ms bins, for 5 stimulus repetitions with a single locust. Ten spikes per bin would generate a maintained spike frequency of  $\geq 200 \text{ Hz}$ . With shorter ISIs, the number of spikes in the DCMD decreased and the maximum spike frequency occurred earlier in the approach (marked by \*). The shift in the timing of the maximum response was greater for the slower stimulus approach speeds. Data from this experiment were also used in Fig. 5B.

increased significantly (Fig. 5B; paired Student's *t*-test,  $*P < 0.002$ ). The DCMD responses where arousal had been increased contained six or more consecutive DCMD spikes at frequencies  $> 200 \text{ Hz}$  (example circled in Fig. 5A) in 56 of 148 occasions (38%) in aroused locusts, but in only 2 of 122 occasions (0.016%) in nonaroused subjects.

#### *Flying boosts arousal and stops significant DCMD habituation*

Our experiments on quiescent locusts showed that the rate of DCMD habituation was greatest when the intervals between stimuli were shortest and included both a reduction in the numbers of spikes per stimulus and a decline in the peak spike rate (Gray 2005) with the response ceasing early, before the loom had stopped. We wanted to see the effect of a specific behavioral action on these aspects of DCMD habituation and we chose flight as the behavioral manipulation. This behavior is also a relevant one since evasive glides can occur only when a locust is in flight. Since evasive glides occur in flight in response to imminent collision and are triggered by high-

frequency spikes in the final stage of the DCMD response, the observed effects of flight and arousal on these spikes are crucial in understanding the triggering of antipredator behavior. Just as we observed in subjects aroused by hind-leg tibial stimulation, we expected that the act of flying would produce an aroused state in locusts that would concomitantly reduce our ability to habituate the DCMD to the repeated looming stimuli. To examine this, we compared the DCMD responses of flying locusts with those of wind-stimulated nonflying locusts and nonflying locusts without wind stimulation (Fig. 6, A and B). Wind stimulation was used as a control in this experiment because wind stimulates hairs in the same manner a flying locust would experience during air movement, although the subjects do not undergo the change in physiological state that accompanies the activation of flight muscles and sustained activity. For these experiments we chose ISIs equal to or less than those that had caused strong DCMD habituation in our previous experiments. We examined the three measures of DCMD response strength that we had shown earlier to be affected by habituation: the number of spikes during a stimulus, the peak instantaneous spike frequency, and the persistence

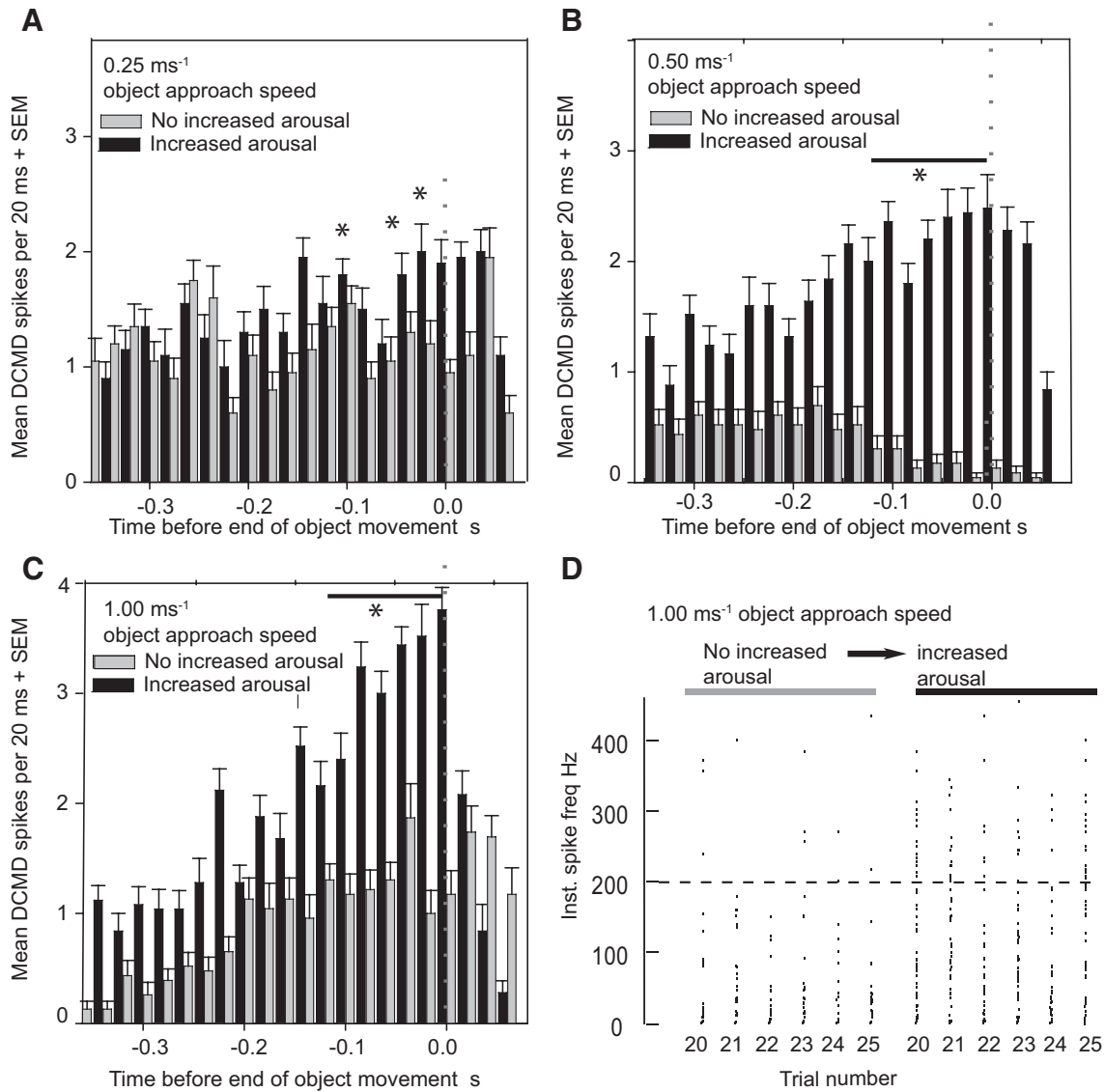


FIG. 3. Arousal restored the peak firing frequency and the ability of the DCMD to follow the increase in image size throughout object approach. With arousal, responses reach mean spike rates per 20-ms bin of almost 200 Hz. A–C: bars represent the DCMD mean spike frequency, over 50 stimulus repetitions at 20-s intervals, with approach speeds of 0.25, 0.50, and 1.00 ms<sup>-1</sup>. The first 25 stimuli (gray bars) were delivered without any increase in arousal; then a further 25 stimuli (colored bars) were each preceded by a stroke to the hind leg tibia, raising the state of arousal. At 60 ms before projected collision, arousal significantly increases the DCMD response with approach speeds of 0.50 and 1.00 ms<sup>-1</sup> ( $P < 0.01$ ; Mann–Whitney rank-sum test). D: instantaneous DCMD spike frequencies during the last 6 approaches are shown for the aroused and nonaroused trials.

of the response throughout the loom. As we predicted, all three measures of response strength showed the effects of habituation in wind-stimulated nonflying locusts (Fig. 6A). We could also see that flight had not increased the overall DCMD response compared with wind stimulation because the responses to the first stimulus were either the same in both cases or higher during wind stimulation (stimulus 1 in Fig. 6A, *i* and *ii*). Least-squares multiple comparisons of spike number: for ISI = 1.8 s  $\chi^2_1 = 2.56$ ,  $P = 0.1094$ ; for ISI = 10 s  $\chi^2_1 = 1.43$ ,  $P = 0.2314$ ; for ISI = 20 s  $\chi^2_1 = 3.21$ ,  $P = 0.0732$ ; least-squares multiple comparisons of peak instantaneous spike frequency: for ISI = 1.8 s  $\chi^2_1 = 397.99$ ,  $P < 0.0001$ ; for ISI = 10 s  $\chi^2_1 = 7.53$ ,  $P < 0.0061$ ; for ISI = 20 s  $\chi^2_1 = 17.45$ ,  $P < 0.0001$ . When we statistically compared DCMD responses to approaching objects in flying and nonflying locusts, across three ISIs (1.8, 10, and 20 s), we found that both spike number

and peak instantaneous frequency were significantly greater in aroused flying locusts compared with wind-stimulated ones (logistic regression analysis gave  $\chi^2_1 = 6.56$ ,  $P < 0.0104$  for spike number and  $\chi^2_1 = 277.75$ ,  $P < 0.0001$  for peak instantaneous frequency). Looking at the DCMD responses to each ISI separately revealed the greatest boost induced by flying occurred at the shortest interval. With 1.8 s between approaches, the number of DCMD spikes was 33% higher (95% confidence level; range = 13–51%) and peak instantaneous spike frequency was 56% higher (95% confidence level; range = 51–61%) in flying subjects.

The rate of decline in both spike number and peak instantaneous frequency with successive approaches was significantly reduced by flight compared with wind stimulation over all ISIs and at each ISI (logistic regression analysis gave:  $\chi^2_1 = 572.78$ ,  $P < 0.0001$  for spike number and  $\chi^2_1 = 4,774.71$ ,  $P <$

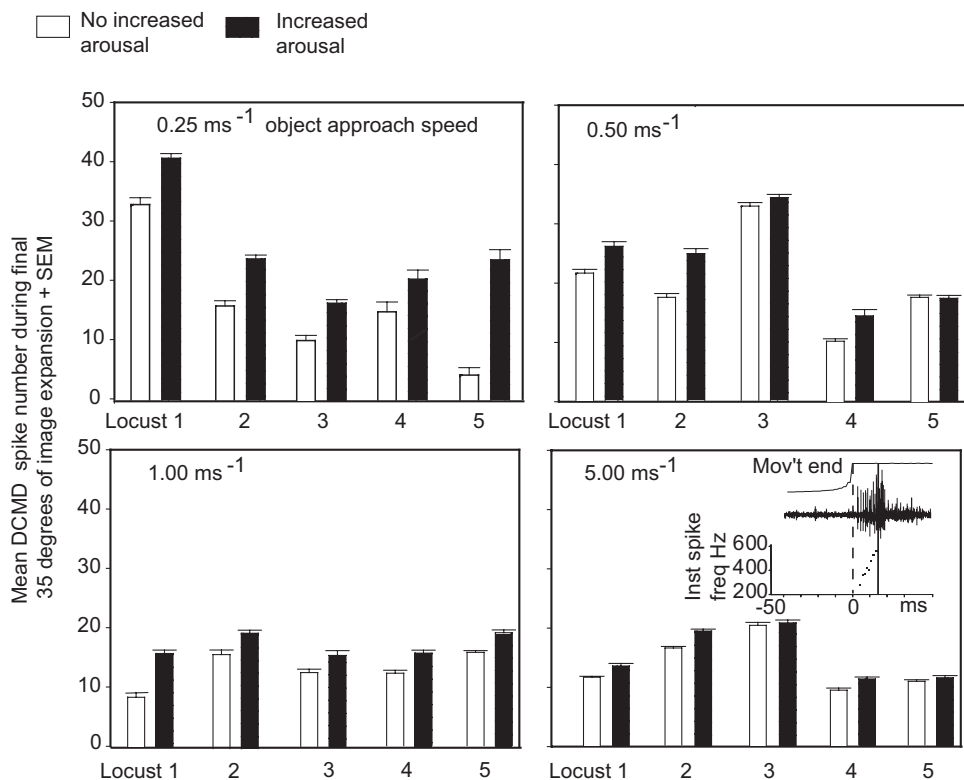


FIG. 4. Arousal increased the number of spikes in the DCMD in response to approaching objects during the final stages of object approach. When the arousal of the locust was increased by stroking the hind leg tibia (dark bars), the number of spikes increased significantly. The inset in the bottom right graph shows the increasing DCMD spike frequency throughout the response to a  $5 \text{ ms}^{-1}$  object approach. Projected collision (solid line) would have occurred 14 ms after motion ceased (dotted line). The duration of the DCMD response and section of the response analyzed for each object approach velocity depended on the velocity and was the final 400 ms for approach speeds of  $0.25 \text{ ms}^{-1}$  ( $-400$  to  $0 \text{ ms}$ ), the final 200 ms for approach speeds of  $0.50 \text{ ms}^{-1}$  ( $-220$  to  $0 \text{ ms}$ ), and the final 100 ms for approach speeds of  $1 \text{ ms}^{-1}$  ( $-100$  to  $0 \text{ ms}$ ). The final 25 ms were used for approaches at  $5 \text{ ms}^{-1}$ . These intervals allowed a comparison of the DCMD responses to the same change in angular subtense (from  $25$  to  $59^\circ$ ) with different loom speeds.

$0.0001$  for maximum spike frequency taking all ISIs together; for  $\text{ISI} = 1.8 \text{ s}$   $\chi_1^2 = 295.22$ ,  $P < 0.0001$  for spike number and  $\chi_1^2 = 4,068.68$ ,  $P < 0.0001$  for peak instantaneous frequency;

for  $\text{ISI} = 10 \text{ s}$   $\chi_1^2 = 38.4$ ,  $P < 0.0001$  for spike number and  $\chi_1^2 = 134.83$ ,  $P < 0.0001$  for peak instantaneous frequency; finally, for  $\text{ISI} = 20 \text{ s}$   $\chi_1^2 = 17.12$ ,  $P < 0.0001$  for spike number and

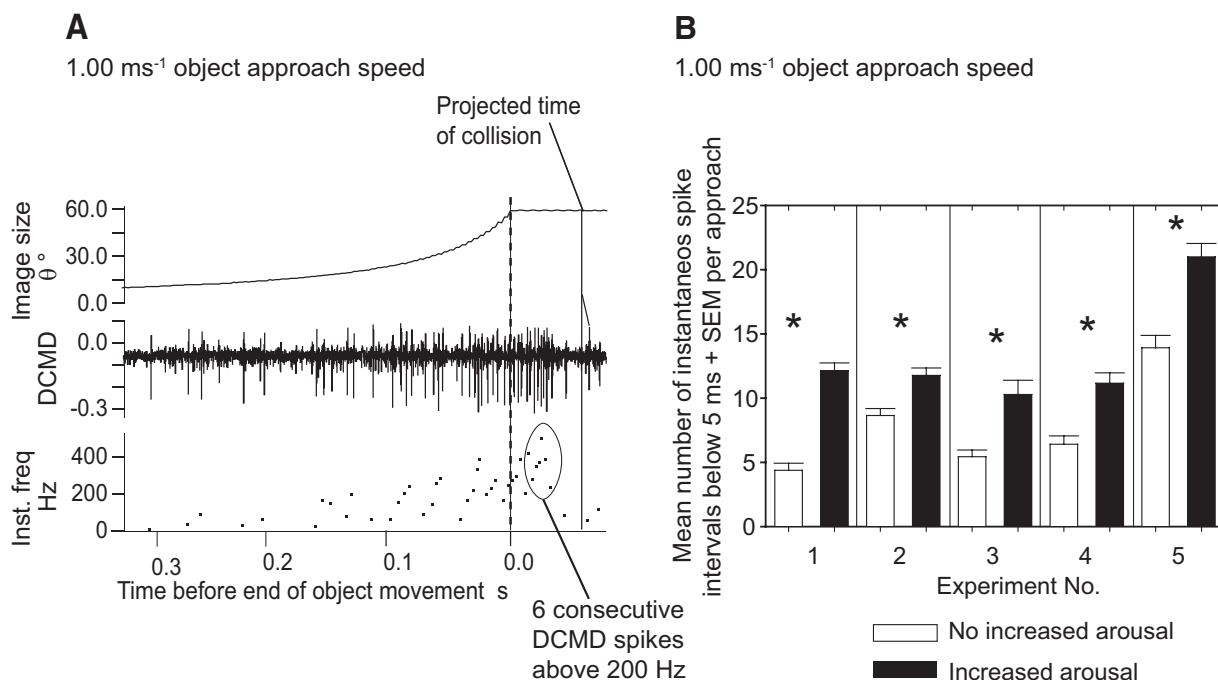
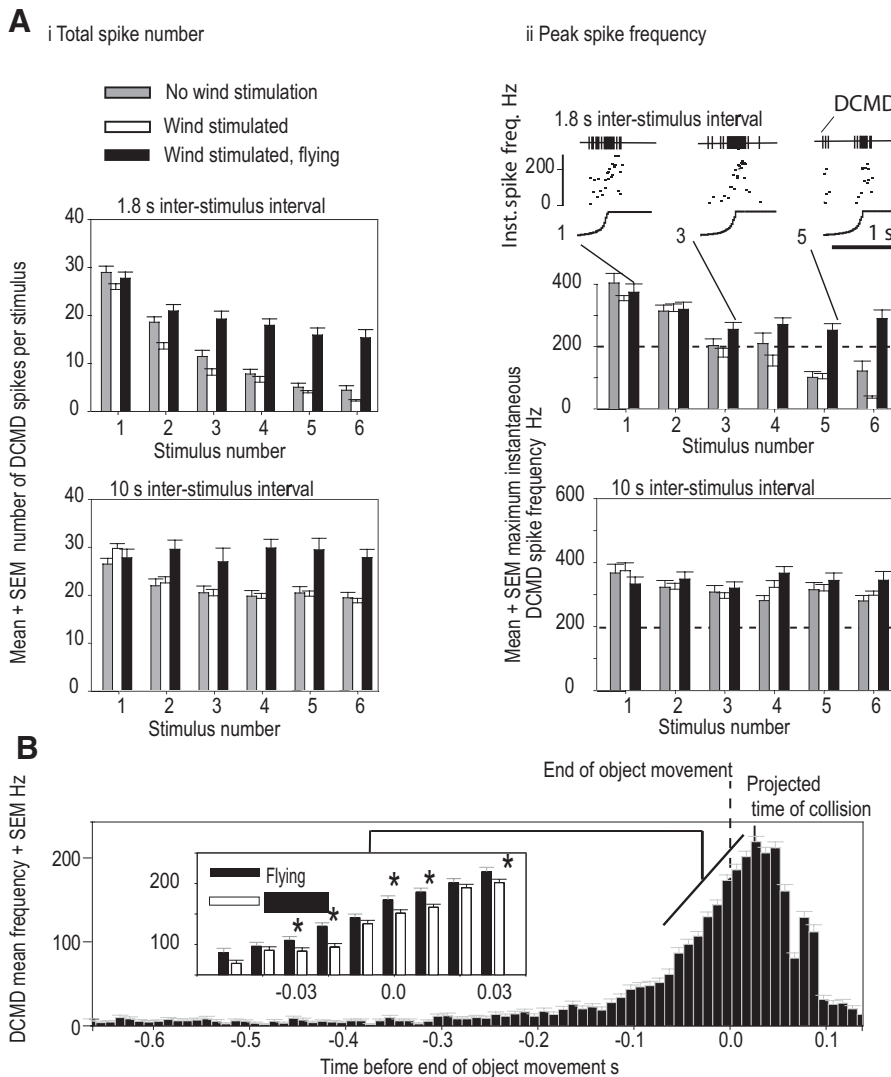


FIG. 5. Arousal increased the number of high-frequency DCMD spikes. *A*: during object approach in an aroused locust, instantaneous spike frequency in the DCMD reached  $>200 \text{ Hz}$ , particularly when collision was imminent. In this example, spike frequencies exceeded  $200 \text{ Hz}$  and 6 consecutive spikes occurred at  $\geq 200 \text{ Hz}$  (circled). *B*: approaches were presented at intervals of  $40 \text{ s}$  to either nonaroused (open bars) or aroused (filled bars) locusts. For nonaroused locusts, the numbers of DCMD spikes occurring at frequencies  $>200 \text{ Hz}$  was low. When the arousal state of the locust was increased, the number of spikes at frequencies  $>200 \text{ Hz}$  increased significantly ( $*P < 0.002$ , paired Student's *t*-test). In particular, increased levels of arousal led to increases in the number of approaches generating  $>6$  consecutive spikes at  $200 \text{ Hz}$ : 56 of 148 approaches with increased levels of arousal and only 2 of 122 without arousal. Trials were in blocks of 6, with a total of 18 to 42 approaches per block. The analysis shown in Fig. 6*B* excluded the first 3 responses of the experiment in nonaroused locusts.



**FIG. 6.** Flying protects the DCMD from habituation, enabling the locust to react to impending collisions even with very short intervals between successive stimuli. DCMD responses to looming objects in tethered flying locusts were very robust. Stimuli consisted of a dark disc of diameter 80 mm approaching the eye at 3 ms<sup>-1</sup>. The end of disc movement is shown by a dashed vertical line. Experiments were performed on 4 locusts with 144 stimuli delivered when the locust was not flying, 144 stimuli during wind stimulation, and 144 during flight, each separated by an ISI of either 1.8, 10, 20, or 40 s (data from 20 and 40 s separation between stimuli are not shown). **A:** comparison of habituation in the DCMD neuron during repeated presentation of a stimulus looming at a velocity of 3 ms<sup>-1</sup> in 2 different behavioral states: wind stimulated and flying. **i:** total spike number per stimulus response. **ii:** peak instantaneous spike frequency per response. In flying locusts the peak instantaneous spike frequency remained >200 Hz at all ISIs. **Top right:** DCMD activity is shown for 3 looming stimuli (numbers 1, 3, and 5 of a sequence of 6), separated by an ISI of 1.8 s. **B:** the time course of the mean DCMD response to looming stimuli in the flying locusts is shown, divided into 10 ms bins. The responses persisted beyond the end of object approach (dashed line), with the maximum response occurring at the projected time of collision (solid line). The *inset* then compares DCMD responses in flying and nonflying wind-stimulated locusts in the 90 ms immediately prior to collision. Intervals during which flight had a statistically significant effect are marked with an asterisk ( $P < 0.02$ , Mann-Whitney rank-sum test).

$\chi^2_1 = 31.68$ ,  $P < 0.0001$  for peak instantaneous frequency). The wind stimulation provided during flight was twice the velocity of that for nonflying locusts, although this factor was unlikely to underlie the effect of flight on habituation because rather than preventing habituation, wind stimulation of a quiescent locust was either not significant or was associated with an increase in habituation (Fig. 6, gray bars compared with unfilled bars).

In flying locusts, we consistently found that the DCMD response to a looming stimulus persisted beyond the end of object motion and that high rates of spiking were produced at the end of object approach (Fig. 6B). The DCMD spike rates in an aroused flying locust in these experiments were >150 Hz for 40 ms (Fig. 6B), which is close to the duration of a whole wingbeat cycle (Santer et al. 2005; Wilson and Weis-Fogh 1962), and would be sufficient to evoke gliding dives (Santer et al. 2006). Short evasive glides were regularly observed in response to looming stimuli delivered during flight but not without a looming stimulus.

*Boost to predator-avoidance responses during flight can be prevented by epinastine*

We hypothesize that the boost in the LGMD response during flight is mediated by octopamine released in the optic lobe. To

test this hypothesis, we measured the occurrence of evasive gliding in the presence of epinastine, a blocker of neuronally located octopamine receptors. In the presence of epinastine we would expect strong habituation in the evasive gliding behavior because the response of the DCMD in producing high spike frequencies (which is necessary for the gliding behavior) would no longer be protected from habituation during flight. Also if there were any additional preflight role for octopamine on arousal we may find that the response to the first stimulus may be reduced. For these experiments, we chose ISIs equal to or less than those that had caused strong DCMD habituation in our previous experiments (shown in Fig. 6). Six stimuli were delivered at 1.8-s intervals, a total of 540 to control locusts and 462 to locusts injected with epinastine. We categorized responses as a “pause” or “glide” if there was an interruption in flight >25% of the mean duration of the preceding 10 wingbeats (Santer et al. 2005); otherwise, the response was classified as “no glide.” The number of responses when flight was not resumed during the 1.8 s between stimuli was also recorded. The results of these experiments are shown in Fig. 7.

In total, out of these 1,002 stimulus presentations, control locusts showed 221/540 pauses or glides compared with 57/462 in epinastine-treated ones; and control locusts showed



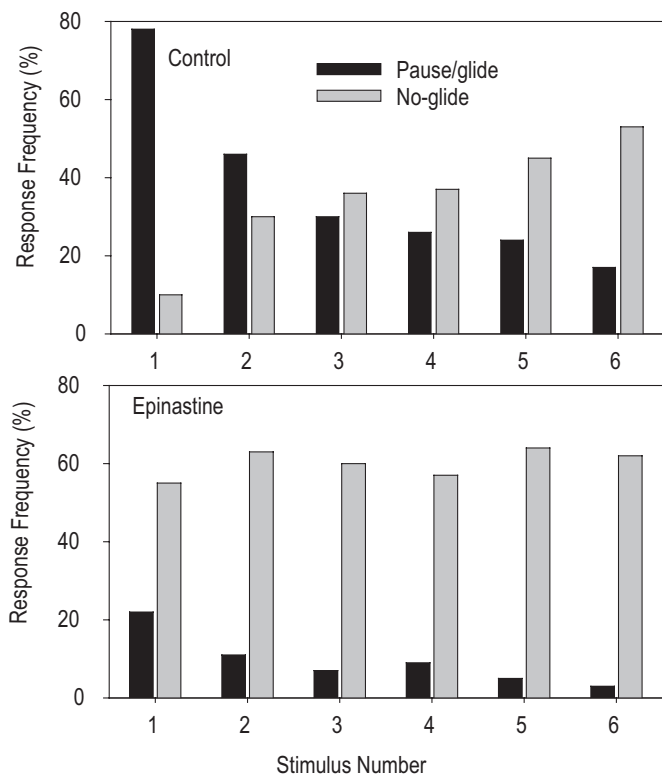


FIG. 7. Injection of epinastine, a specific blocker of neuronal octopamine receptors, significantly reduces the protective effect of flight on the evasive gliding behavior. Stimuli consisted of a dark disc of diameter 80 mm approaching the eye at  $3 \text{ ms}^{-1}$  and were given in groups of 6, each stimulus separated by an ISI of 1.8 s. Experiments were performed on 12 locusts; 45 min prior to flight 6 had been injected with epinastine and 6 with saline as controls. An infrared beam was used to monitor wing-tip position and responses by flying locusts to imminent collision are plotted as the percentage of stimuli resulting in either 1) a pause or glide during flight lasting  $<1.8 \text{ s}$  (dark bars); or 2) no-glide (gray bars). Stimuli delivered to nonflying locusts have not been plotted.

53/540 no-glides compared with 361/462 in treated ones. In the remaining 320 trials the locust was not flying. The probability that a locust would glide in response to a looming stimulus on any repetition was significantly reduced in animals in which octopamine receptor activity had been blocked (108 compared with 44;  $\chi^2_1 = 27.2$ ,  $P < 0.001$ ). However, we also found that the probability a locust would glide decreased as a function of the number of repetitions in the same manner for both the control and epinastine-treated locusts ( $\chi^2_1 = 0.04$ ,  $P = 0.849$ ). Conversely, epinastine injection significantly increased the probability that a locust would show a no-glide regardless of stimulus repetition number (1–6) ( $\chi^2_1 = 97.7$ ,  $P < 0.001$ ). Whereas epinastine-treated locusts exhibited a high probability of no-glides in response to all stimuli regardless of the repetition number, the control animals exhibited a repetition-dependent increase in no-glides ( $\chi^2_1 = 31.1$ ,  $P < 0.001$ ).

## DISCUSSION

In this study we have investigated the link between arousal, sensory responses, and behavioral action in a locust's preparedness for the performance of a DCMD-triggered gliding dive. The evasive glide of a flying locust is probably a last-chance maneuver to avoid an attacking bird and a critical event

in the decision to perform a glide is a sustained spike rate of  $\geq 150 \text{ Hz}$  in the DCMD neuron during wing elevation (Santer et al. 2005, 2006). Strong arousal is required for the DCMD neuron to reach this level of response, particularly with multiple approaches separated by only a few seconds. This strong arousal can be mediated either by flight itself or by mechanosensory stimulation of the locust's hind leg.

When we increased arousal, using either a tactile stimulus to the locust's hind leg or inducing a locust to fly (David et al. 1985; Rowell 1971b; Stern et al. 1995), we found three effects on the way the DCMD responded to approaching stimuli. First, the maximum frequency of spikes was high, often reaching a rate of  $\geq 200 \text{ Hz}$  for 40 ms, the level found to trigger an evasive glide if occurring at the correct phase of the wingbeat cycle (Santer et al. 2006). Second, the DCMD's response increased throughout image growth until the end of stimulus movement. An increase in arousal could restore high rates of discharge and the relationship of peak firing to the end of approach in a previously habituated DCMD neuron. Third, the DCMD responded to approaches separated by as little as 1.8 s with a spike rate that could trigger a collision-avoidance glide.

In crickets, flying has already been shown to alter behavioral thresholds and to promote aggression in previously defeated individuals (Hofmann and Stevenson 2000; Stevenson et al. 2005). In crickets the increase in aggression due to flight is thought to be mediated by a neuronal octopamine receptor because the effects of a period of flight on aggression are abolished by octopamine depletion and by injection of epinastine, a specific and selective neuronal octopamine receptor antagonist (Hofmann and Stevenson 2000; Stevenson et al. 2005). Flight had specific effects on aggressive behavior without enhancing the cricket's general level of excitability (Stevenson et al. 2005). In the locust, flight increased the likelihood of high-frequency discharge in the DCMD. The effect was stronger than that of wind stimulation applied to a nonflying locust, which suggests that flight specifically increases DCMD responsiveness to a level that would trigger collision-avoidance behaviors. A role for neuronal octopamine receptors in preventing DCMD habituation has been suggested by Roeder et al. (1998) who injected epinastine into the locust prothorax and found that DCMD activity in response to repeated moving objects was significantly reduced in a dose-dependent way.

When we injected epinastine into the locust, the effect of flight in protecting collision-avoidance behavior from habituation was significantly reduced. Even the response to the first stimulus was reduced compared with controls following epinastine injection, confirming a role for octopamine in the flight-induced increase in the response of DCMD and in collision-avoidance behavior. Antibodies against the neuronal octopamine receptors have recently been developed (Degen et al. 2000; Farooqui et al. 2004) and may allow these neuronal octopamine receptors to be localized to specific neurons on the LGMD–DCMD pathway.

At what stage in the LGMD–DCMD pathway could protection from habituation occur? One likely site is at the synapses made by the afferents onto the main LGMD dendritic tree in the distal lobula, in that these synapses are thought to be prone to decrement (O'Shea and Rowell 1976). In the crayfish, transmission at sensory afferent synapses onto the lateral giant (LG) interneuron, which mediates escape tail flips, also decre-

ments with repeated stimulation (Wine and Krasne 1972; Yeh et al. 1996, 1997). There is clear evidence that this synaptic decrement is subject to modulation so that the synapses are protected from habituation in dominant individuals by neuro-modulators such as octopamine and 5-hydroxytryptamine (5-HT) (Araki and Nagayama 2003; Yeh et al. 1996, 1997). 5-HT has recently been found to facilitate synaptic input onto the LG by interacting directly with a transmembrane receptor on the LG itself (Araki et al. 2005). In the locust, where the DCMD and the LGMD responses to approaching objects are protected from habituation, it may be that the excitatory postsynaptic potentials evoked in the LGMD by visual afferents from the medulla are maintained in strength during repeated stimulation by arousal and by flight. The octopaminergic PM4 neurons are candidates for this protective role because they have arborizations in the same area of the lobula as the dendritic fan of the LGMD (Bacon et al. 1995). Their excitation can partially restore a habituated DCMD response to a small moving object, although it is not known whether they make direct or indirect contact with the afferents or the LGMD itself (Bacon et al. 1995).

Adult locusts in a swarm occur at high densities, averaging  $\leq 50$  million individuals per  $\text{km}^2$  (Rainey 1954). In early 1954, air reconnaissance observations registered 50 swarms that invaded Kenya, covering an area of about 1,000  $\text{km}^2$  and rising to 1,000 to 1,500 m above the ground (Rainey 1954). The largest of these 50 swarms covered 200  $\text{km}^2$  and consisted of about 10 billion locusts. Typically, in a locust swarm, daily flight activity begins with short rolling flights with frequent landings, followed by more sustained flights by midmorning (Symmons and Cressman 2001). During these take-offs, adult migratory locusts need persistent collision-avoidance responses. Not only could locusts collide with other locusts, but also predatory birds gather around swarms, and many of these bird species have been seen attacking flying locusts (Smith and Popov 1953). Prior to take-off, locusts descend from vegetation to bask on the ground (Symmons and Cressman 2001). As the densities of adult locusts on the ground increase, frequent mechanical stimulation occurs between individuals. These same mechanical stimuli to the hind legs by neighboring locusts are implicated in triggering swarm formation, causing locusts to become gregarious and aggregate together (Buhl et al. 2006; Rainey 1954; Simpson et al. 2001). In this overcrowded state, as mimicked by our mechanical stimulus to the hind leg, the DCMD in a swarming locust would be primed for its action in triggering collision avoidance during flight. After take-off, arousal mediated by increased octopamine levels would then increase again for the first 10 min of flight (David et al. 1985; Goosey and Candy 1980). The result would be that DCMD-mediated collision-avoidance glides are triggered reliably even with so many potential predator attacks or collisions occurring in a short space of time.

In summary, the DCMD response to approaching objects is protected by flight and mechanical stimulation of the hind leg, both of which are known to increase octopamine levels and are now shown to increase arousal. This effect is not seen in response to other stimuli such as wind stimulation. Arousal increases the response of an identified collision-detecting visual neuron to enhance collision avoidance during normal behavior of the locust; the increase in arousal is mediated via neuronal receptors for octopamine. The precise way in which

this is done is as yet unknown but the pathways and the mechanism involved may shed light on attention and vigilance in a wide range of animals, less amenable to detailed scrutiny.

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#### GRANTS

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