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RESEARCH ARTICLE

Looming detection by identified visual interneurons during larval development of the locust *Locusta migratoria*

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SUMMARY

Insect larvae clearly react to visual stimuli, but the ability of any visual neuron in a newly hatched insect to respond selectively to particular stimuli has not been directly tested. We characterised a pair of neurons in locust larvae that have been extensively studied in adults, where they are known to respond selectively to objects approaching on a collision course: the lobula giant motion detector (LGMD) and its postsynaptic partner, the descending contralateral motion detector (DCMD). Our physiological recordings of DCMD axon spikes reveal that at the time of hatching, the neurons already respond selectively to objects approaching the locust and they discriminate between stimulus approach speeds with differences in spike frequency. For a particular approaching stimulus, both the number and peak frequency of spikes increase with instar. In contrast, the number of spikes in responses to receding stimuli decreases with instar, so performance in discriminating approaching from receding stimuli improves as the locust goes through successive moults. In all instars, visual movement over one part of the visual field suppresses a response to movement over another part. Electron microscopy demonstrates that the anatomical substrate for the selective response to approaching stimuli is present in all larval instars: small neuronal processes carrying information from the eye make synapses both onto LGMD dendrites and with each other, providing pathways for lateral inhibition that shape selectivity for approaching objects.

Key words: vision, looming, insect, larva, synapse, development.

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INTRODUCTION

As with other hemimetabolous insects, a newly hatched larval locust closely resembles the adult in form apart from the absence of wings, which grow gradually through the five larval instars (Sehnal, 1985). A first instar locust walks and hops, and can clearly use its eyes to detect and react to the approach of an experimenter's hand. The embryonic development of one identified visual neuron, the descending contralateral motion detector (DCMD), has been studied and by the time of hatching from the egg its morphology is similar to that of the adult (Bentley and Toroian-Raymond, 1981). However, it is not known exactly how well a newly hatched locust larva can see, to what extent the synaptic circuitry of its optic lobes has developed, or whether its visual neurons have the same selectivity for cues as in the adult.

The way responses of sensory interneurons develop through larval stages of hemimetabolous insects has been studied most in wind-sensitive and auditory pathways of orthopteroids. In *Locusta*, the threshold wind stimulus for an identified interneuron, A4I1, decreases during successive instars, but the first instar interneuron has the same directional selectivity as in the adult (Bucher and Pflüger, 2000). Similarly, identified auditory interneurons show increased sensitivity to sound stimuli with instar, and several response properties are established in early instars (Boyan, 1983), although the first instar auditory receptors are not yet functional so it is not known whether synaptic connections onto the interneurons are present before hatching. In wind-sensitive interneurons of both locusts and crickets, it is known that the pattern and relative strengths of connections from sensory neurons onto interneurons changes during larval development. These changes in connectivity provide a mechanism to maintain interneuron response properties through different instars, which is needed because the number of hair sensilla increases by as much as a factor of 10 from hatching to adulthood and because the mechanical properties alter as the size of individual sensilla increases (Anderson and Bacon, 1979; Newland et al., 1995; Pflüger et al., 1994). In contrast, the locust ear already has the adult complement of auditory receptors at hatching (Michel and Petersen, 1982).

As a locust's eye grows, new ommatidia are formed at its anterior margin (Anderson, 1978). In *Schistocerca*, the number of ommatidial facets increases fairly regularly through larval stages from ~2500 in the first instar to 9000–9500 in the adult, with the widths of individual facets increasing from just over 20 μm to ~40 μm (Bernard, 1937; Rafi and Burtt, 1974). An adult *Locusta* eye is slightly smaller than that of an adult *Schistocerca*, with ~8250 facets (Wilson et al., 1978) and an inter-ommatidial angle of 1 deg over most of the eye apart from an anterior flattened region (Horridge, 1978; Krapp and Gabbiani, 2005). We would expect that a young locust's visual capabilities will be inferior to those of the adult: a smaller number of ommatidia means that the image is sampled more coarsely, and the smaller diameter of lenses means that images are less sharply focused (Kirschfeld, 1976). Improved optical performance with increased eye size has been shown by comparing

behaviour within different individuals of the same species for two species of Hymenoptera (Spaethe and Chittka, 2003; Zollikofer et al., 1995) and during development of mantids (Kral and Poteser, 2009), but not by measuring responses from individual neurons.

Two large identified visual neurons in the adult locust's visual system, the lobula giant motion detector (LGMD) (O'Shea and Williams, 1974) and the DCMD (Rowell, 1971), are motion-sensitive neurons that respond selectively to approaching (or looming) objects (Rind and Simmons, 1992; Schlotterer, 1977). They play roles in triggering behavioural responses to approaching objects (Fotowat and Gabbiani, 2007; Santer et al., 2006; Santer et al., 2008). Individual neurons in other species have also been shown to respond to and trigger responses to approaching objects, but the way in which selectivity for approaching stimuli arises has been most thoroughly investigated in the locust LGMD (de Vries and Clandinin, 2012; Dewell and Gabbiani, 2012; Oliva et al., 2007; Preuss et al., 2006). The LGMD has an extensive dendritic arbour in the lobula, through which it collects inputs from ommatidia over a wide area of the visual field (Krapp and Gabbiani, 2005). It is connected with the DCMD in the protocerebrum by a synapse that conveys spikes one-for-one (O'Shea and Rowell, 1975b; Rind, 1984), so the LGMD's responses can be recorded as DCMD spikes. The DCMD's axon is the widest in the thoracic nerve cord and its relatively large spikes in extracellular recordings are readily distinguishable from those of other nerve cord interneurons. The LGMD and DCMD are briefly excited by many kinds of abrupt motion or luminance changes (Rowell, 1971), but generate their most vigorous and prolonged spike trains in response to images of approaching objects. Their spike rate tracks object approach in a way that depends on the size and approach speed of the stimulus (Hatsopoulos et al., 1995; Rind and Simmons, 1992).

The cues that enable the LGMD to discriminate approaching objects from those moving in other directions are increases in both the extent and the speed of movement of edges in the image (Simmons and Rind, 1992). An explanation for these characteristics is that LGMD excitation depends on a critical race in which the rate at which ommatidia are activated as the image grows over the eye surface must out-strip inhibition, which spreads between the visual afferent elements that excite the LGMD. Evidence for the existence of this lateral inhibition is that: stimulation of one part of the eye inhibits the LGMD's response to subsequent stimulation of another part of the eye (O'Shea and Rowell, 1975a; Pinter, 1977; Rind and Simmons, 1998); electron microscopy shows that the neuronal profiles which synapse onto LGMD dendrites also synapse with each other (Rind and Simmons, 1998); and model networks configured in this way respond selectively to approaching objects (Rind and Bramwell, 1996). Intrinsic membrane properties of the LGMD tune its responses to approaching stimuli (Peron and Gabbiani, 2009; Peron et al., 2007).

Physiological properties of these neurons can be altered by environmental conditions. In adult locusts that have been reared in darkness, responses by the DCMD are very greatly reduced and the responses habituate more rapidly and strongly compared with locusts reared under normal lighting conditions (Bloom and Atwood, 1980). However, in blow flies, it has been shown that at least two lobula plate large tangential neurons do not require visual experience, after the adult hatches from the pupa, to develop their normal selectivity for moving stimuli (Karmeier et al., 2001). Further indication of some plasticity in the input pathways to the LGMD and DCMD comes from observations of differences between solitarious and gregarious phase locusts (Rogers et al., 2007).

The major aims of this work were to determine whether a first instar locust's DCMD responds to visual stimuli in the same way as that of the adult, and whether there are changes in responses to visual stimuli through successive instars. In electrophysiological experiments, we found that the larval DCMD does respond selectively to the images of approaching objects, and it can discriminate between objects approaching at different speeds. However, its responses to approaching stimuli are neither as vigorous nor as selective as those in an adult DCMD. In both electrophysiological and ultrastructural studies, we found evidence for lateral interactions in all instars between optic lobe neurons that feed onto the LGMD.

MATERIALS AND METHODS

Animals

We took individual *Locusta migratoria* (Linnaeus 1758) from our gregarious breeding colony. Locusts were kept at 30°C under a 12 h:12 h light:dark cycle. Eggs were laid in sand-filled pots. Instar was determined by comparing the size of the body and wings and the shape of the pronotum with reference measurements we made in carefully staged locusts. Usually, first instar hoppers were taken within 4 h of hatching from the egg. We did not establish time from last moulting for other instars, other than that the adults were taken at least a week after the final moult.

Electrophysiology and visual stimulation

We made recordings from all six instars, but concentrated on the first, third and fourth instars and adults. A locust was first cooled to 4°C for 30 min. It was then placed upside down in a bed of plasticine shaped according to the locust's size and was secured using wire loops over the limbs. The head was gently pulled forwards and stabilised with a pin behind each gena. During an experiment, room temperature was 26–28°C.

The recording electrode was a sharpened minuten pin, 0.25 mm in width, held in a holder attached to a micromanipulator, and the indifferent electrode was a stainless steel wire inserted into the anterior of the abdomen. These electrodes were connected to an AC amplifier (Harvard Apparatus, Holliston, MA, USA) with a gain of 1000. The recording electrode was inserted through a small hole through the cuticle made just ventral and medial to the right meso-metathoracic connective nerve and was lowered until distinct spikes in response to movements of the experimenter's hand could be seen on an oscilloscope screen. The recorded waveform was sampled at 10 kHz and stored to disk using a 1401 interface and Spike2 Software (Cambridge Electronic Design, Cambridge, UK). DCMD spikes were converted afterwards to events using the time at which a horizontal cursor level was crossed. The locust was left for 10 min before any records of responses to visual stimuli were made.

The locust viewed the screen of a green electrostatic monitor (Kikusui COS1611, Yokohama, Japan) placed parallel to the locust's long axis with its centre aligned with the left eye and 80 mm from it, subtending 64×59 deg. Images were controlled by a microcomputer fitted with a visual stimulus card and raster generator (VSG2/1 and RG2, Cambridge Research Systems, Rochester, Kent, UK). The screen was refreshed at 200 Hz and had a resolution of 437 lines by 438 pixels. In most experiments with approaching stimuli, the image was of a 60 mm diameter dark disk approaching the locust. In experiments with receding stimuli or stimuli that changed in intensity, rectangles were used instead of the disk as the visual stimulator we used did not generate smoothly changing images of receding circular objects. The mean irradiances of the background and stimulus shape were 4.5 and 1.0 $\mu W\,cm^{-2}$,

respectively, measured with a radiometer at the position of the eye (SL021 Photodetector, Ealing, Holliston, MA, USA). A disk or rectangle started its simulated approach 2 m from the locust and stopped at the location of the screen, remaining stationary for 2 s. Two minutes separated one stimulus from the next, other than in studying habituation.

Data were plotted and statistical tests were performed using SigmaPlot 11.0 (Systat Software, Chicago, IL, USA). In most cases, non-parametric analysis was performed because in some tests variance was not sufficiently similar between samples or values were not distributed normally.

Electron microscopy

Opened locust heads were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer overnight. After washing and removal from the head capsule, the brain was placed in phosphate-buffered 1% osmium tetroxide for 1 h. It was dehydrated in an acetone series before impregnation with and embedding in Araldite (TAAB Laboratories, Aldermaston, Berkshire, UK) at 60°C for 24 h. To locate the part of the optic lobe containing LGMD dendrites, 1 mm survey sections were cut, stained with 1% Toluidine Blue in 1% borax and examined with a light microscope. Ultrathin sections, ~80 nm thick, were cut using a diamond knife on a Reichert-Jung Ultracut E ultramicrotome (Leica, Wetzlar, Germany), then stretched with chloroform and mounted on Pioloform film copper grids. Sections on grids were stained with 2% aqueous uranyl acetate and lead citrate (Leica). Grids were examined using a Philips CM 100 Compustage (FEI) Transmission Electron Microscope (Amsterdam, The Netherlands) fitted with an AMT CCD camera (Deben, Bury St Edmunds, Suffolk, UK).

RESULTS

Eye development

The compound eye grows from 0.75 mm wide in a newly hatched first instar to 1.1 mm wide in a fourth instar and 2.0 mm wide in an adult (Fig. 1A–C). The width of the eye is approximately 10% of the body length in a newly hatched locust, declining to half this proportion in the adult. Facet width is similar over most of the eye, except for a flat, forward-facing part that has larger facets than elsewhere (Horridge, 1978; Krapp and Gabbiani, 2005). In four live locusts of each instar, we viewed the eye through a dissecting microscope (Wild, with 20× eye-pieces; Heerbrugg, Switzerland). We counted the number of ommatidia along a light band that runs across the eye, which provides a consistent location in different instars (Fig. 1A–C). A newly hatched locust has 35 facets across this part of its eye (range 33–36), from the posterior to the border of the flattened anterior part. This number of facets increases steeply to the second instar, rises more gradually to the fourth instar, and then more steeply again to the adult, which has 80 facets (range 76–84) (Fig. 1D). By counting facets across the eye at different locations, we estimate that the total number in the adult is 8134 (similar to a previous estimate, Wilson et al., 1978), compared with 2200 in the first instar and 4280 in the fourth instar. This rate of increase is similar to previous estimates for *Schistocerca* (Bernard, 1937; Rafi and Burtt, 1974), which has slightly more facets and a larger eye than *Locusta*. Facet width also increases with age, from 17 μm in first instar to 33 μm in the adult (Fig. 1D; measured in fresh exoskeleton with transmitted light; calculated from the width across 10 facets in three locusts of each instar). Consequently, compared with an adult, a first instar locust views images with approximately one-fourth the number of ommatidia and smaller facets, and thus a poorer resolution.

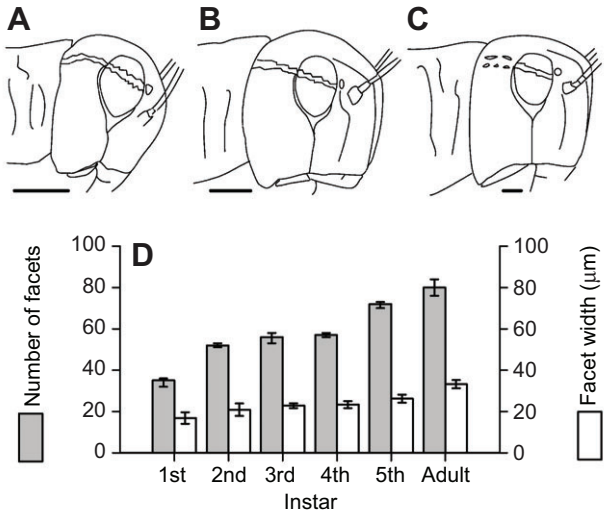

Fig. 1. Compound eye morphology in different instars. Drawings of the heads of (A) first instar, (B) fourth instar and (C) adult *Locusta migratoria* from the side. Scale bars, 1 mm. The horizontal band of light facets that runs across the eye is shown. (D) Plots of number of facets along the ventral border of the light band (means from three locusts) and facet width (average width determined from the width across 10 facets; means from three locusts). Error bars are ranges of values.

Responses by the DCMD to images of approaching objects

To investigate whether the DCMD in different instars responds to images of approaching objects, we recorded responses to images of 60 mm diameter dark disks approaching at various speeds between 0.5 and 10 m s^{-1}, or $l/|v|$ values (radius divided by approach velocity) between 3 and 60 ms (Fig. 2). The amplitude of DCMD spikes in our extracellular nerve cord recordings generally increased with instar number (Fig. 2A), and we analysed results only when we could clearly distinguish DCMD spikes from those of other axons by their large amplitude (almost all recordings from adults and just under half from first instar locusts). Raw recordings (Fig. 2A), raster plots (Fig. 2B) and spike rate histograms (Fig. 2C) show that, in all larval instars as well as the adult, DCMD spike rate consistently increases during an approaching stimulus. For approach speeds of 1 m s^{-1} or more, the 20 ms bin with the highest mean spike frequency was consistently the bin immediately following the end of stimulus movement (Fig. 2C). For an approach speed of 0.5 m s^{-1}, in all instars spike frequency started to decline approximately 40 ms before the end of stimulus movement. We found no significant difference between first instars and adults in the time of the peak instantaneous spike frequency for any of the approach speeds we used (Mann–Whitney test, $P<0.02$ in all cases, six repetitions in five individuals of each instar). DCMDs in first instars responded less vigorously than in older instars, and the adult DCMD response started earlier during each approach. Differences between instars were more marked as stimulus speeds increased.

From soon after hatching, the DCMD can distinguish different speeds of approach. To show this discrimination within a particular instar, results were collected in five different animals from six repetitions of each stimulus, giving 30 stimulus repetitions in total (Figs 3, 4). The mean rate of DCMD spikes during each stimulus is plotted in Fig. 3, calculated as number of spikes during the stimulus divided by its duration (stimulus duration at each speed was: 5 s at 0.5 m s^{-1}, 2.5 s at 1 m s^{-1}, 1.25 s at 2 m s^{-1}, 0.5 s at 5 m s^{-1} and 0.25 s at 10 m s^{-1}). Within each instar, the response to each stimulus speed differed from responses to other speeds (Fig. 3A). We showed within

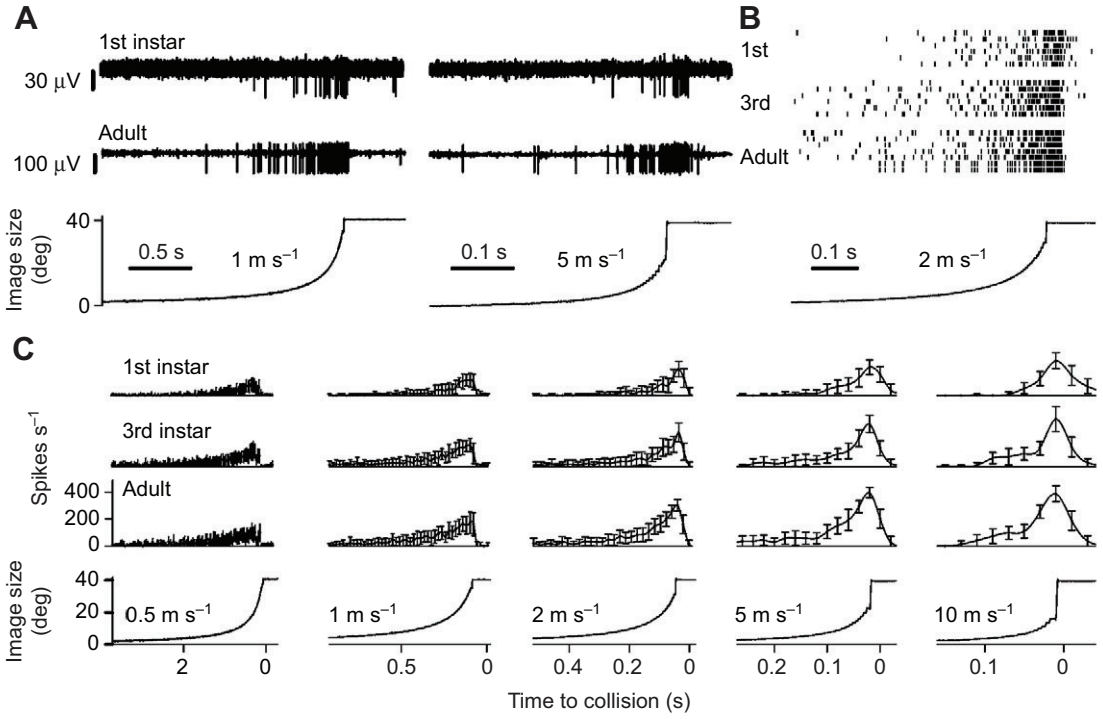

Fig. 2. Responses by the descending contralateral motion detector (DCMD) in different *Locusta migratoria* instars to approaching stimuli. (A) DCMD spikes in extracellular nerve cord recordings from a first instar and an adult locust to images of disks approaching at 1 and 5 m s^{-1}. Image size, as angular subtense at the eye, is shown in the lower panels. (B) Raster plots of DCMD spike times in six responses to a 2 m s^{-1} approaching stimulus in individuals of a first instar, third instar and adult. (C) Spike frequency histograms of first instar, third instar and adult DCMDs responding to images of disks approaching at different speeds, as indicated on the stimulus monitor traces (mean ± s.d. for each 20 ms bin, *N*=30).

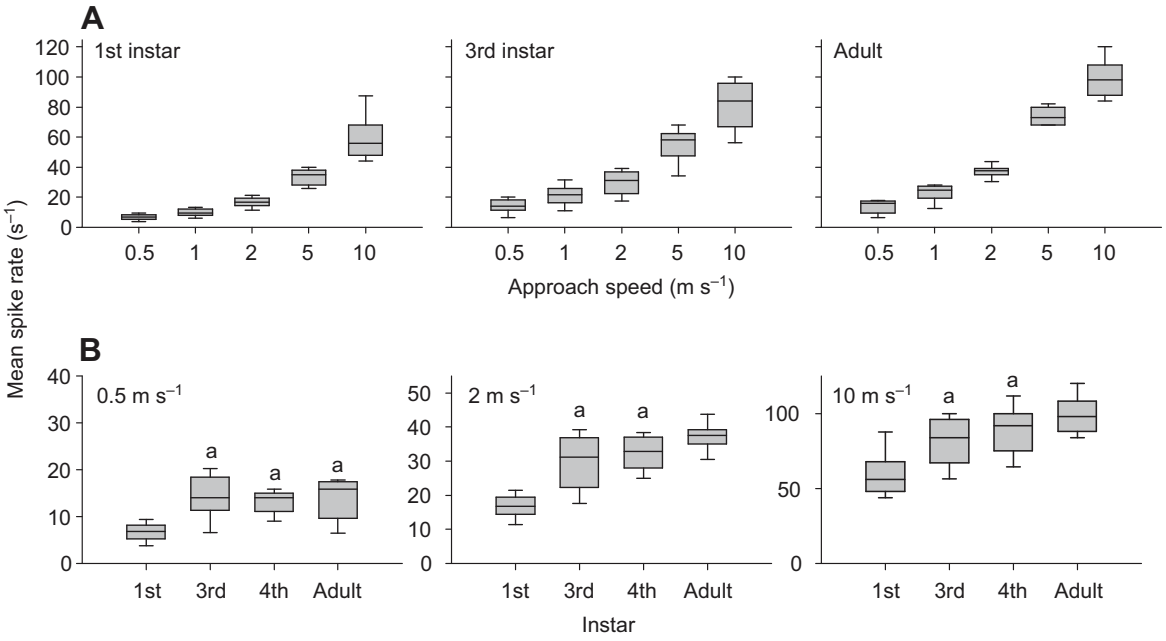

Fig. 3. Mean spike rate by DMCD of larval and adult *Locusta migratoria* during responses to disks approaching at different speeds. The mean spike rate was obtained by dividing the number of spikes in each response by stimulus duration. (A) Mean spike rate at different approach speeds for individuals of the instar shown on the graph; graphs for the fourth instar, not shown here, were similar to those for the third instar. (B) Mean spike rate in individuals of different instars for the approach speed shown on the graph. A and B include the same data. In each graph, each box shows the median, interquartile range and error bars indicating 5–95% range from six different responses in five individual locusts. In B, 'a' indicates responses that were not significantly different at a particular stimulus speed (Student–Newman–Keuls test, $P<0.05$, following Friedman repeated-measures ANOVA on ranks).

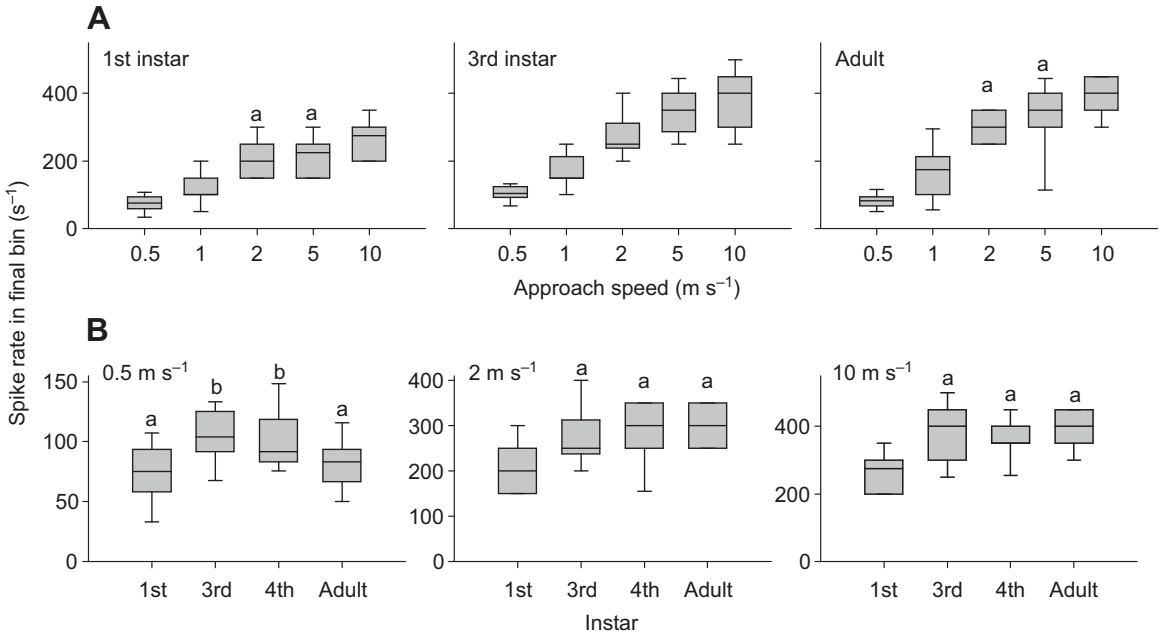

Fig. 4. DCMD spike rate in *Locusta migratoria* during the 20 ms bin with the greatest number of spikes during responses to disks approaching at different speeds. (A) Spike rate at different approach speeds for individuals of the instar shown on the graph; graphs for the fourth instar, not shown here, were similar to those for the third instar. (B) Spike rate in individuals of different instars for the approach speed shown on the graph. Lowercase letters indicate responses to a particular stimulus speed that were not distinguishable in a particular graph (Student–Newman–Keuls test, $P<0.05$, following Friedman repeated-measures ANOVA on ranks). Other details as in Fig. 3.

each instar that differences between responses to different stimulus speeds were significant (Friedman repeated-measures ANOVA, d.f.=4 and $P<0.001$ in each case). The χ^2 values from this analysis were: first instar 112.0, third instar 111.1, fourth instar 113.0 and adult 119.23; and each pairwise comparison within each instar was shown to be significantly different by *post hoc* Student–Newman–Keuls tests ($P<0.05$). We also showed that, for each stimulus speed, mean spike frequency varies significantly between instars (Fig. 3B). Friedman ANOVAs, d.f.=3 and $P<0.001$, gave χ^2 values for different approach speeds: 0.5 m s^{-1}, 46.2; 1 m s^{-1}, 46.2; 2 m s^{-1}, 58.3; 5 m s^{-1}, 71.1; 10 m s^{-1}, 47.4. At the slowest stimulus speeds (0.5 and 1 m s^{-1}), responses by third instars, fourth instars and adults did not differ significantly from each other ('a' in Fig. 3B). For faster stimulus speeds (2 m s^{-1} and greater), responses by first instars, third instars and adults did differ significantly from each other (Student–Newman–Keuls test, $P<0.05$). We found no significant differences between third and fourth instar locusts for any stimulus speed

Within each instar, there were significant differences between peak spike frequency at different stimulus speeds, measured as mean spike frequency during the 20 ms bin with the greatest number of spikes (Fig. 4A). The effect of approach speed was significant (Friedman ANOVAs, d.f.=4, $P<0.001$; χ^2 values: first instar 88.9; third instar 102.0; fourth instar 82.7; and adult 89.9). Pairwise comparison within each instar showed no significant differences in peak spike frequency for stimuli at 2 and 5 m s^{-1} in either first instars or adults ('a' in Fig. 4A), but in other cases peak spike frequency did differ significantly between stimulus speeds within each instar (Student–Newman–Keuls *post hoc* analysis, $P<0.05$). For each stimulus speed, spike frequency differed between instars (Friedman ANOVAs, d.f.=3, $P\leq0.001$; χ^2 values for different approach speeds: 0.5 m s^{-1}, 16.2; m s^{-1}, 33.7; 2 m s^{-1}, 35.0; 5 m s^{-1}, 38.3; 10 m s^{-1}, 38.7; Fig. 4B). For the slowest stimulus speed, 0.5 m s^{-1}, responses during the bin with the greatest number of spikes did not differ significantly between first instars and adults, but was less than those by third and fourth instars (0.5 m s^{-1}; Fig. 4B). This contrasts with the mean spike frequency throughout the stimulus at 0.5 m s^{-1} (Fig. 3B), reflecting a slower rate of spike frequency increase at slow stimulus speeds in adults compared with younger instars. For faster approach speeds, the relative vigour of the peak response by adults increased, so it was significantly greater than the response by the first instar, but not significantly different from responses by third or fourth instars (Fig. 4B, lowercase letters at 2.0 and 10 m s^{-1}). The significance of differences between pairs of instars was shown by the Student–Newman–Keuls test ($P<0.05$).

Stimulus cues

The DCMD responds to many types of movement, including objects receding from the locust. In all instars, the number of spikes in response to a receding rectangle (Fig. 5A) was less than the number in response to the same rectangle approaching at the same speed (Fig. 5B). We tested two different speeds of movement, 2 and 5 m s^{-1}, and found that for each speed and instar there was a significant difference between responses to receding and approaching rectangles (Mann–Whitney test, $P<0.001$ in each case; six stimulus repetitions in each of five locusts). In the first and fourth instars, all 30 receding stimuli generated at least one spike, whereas in the adults 11 out of the 30 receding stimuli generated no spikes (Fig. 5A,C). Although responses to receding stimuli were often initially vigorous in larval locusts, they never lasted longer than 15–20 ms. Differences in numbers of spikes produced by receding objects differed significantly between instars (Friedman repeated-measures ANOVA, d.f.=2, $P<0.001$, $\chi^2=34.5$, followed by the Student–Newman–Keuls test with $P<0.05$ for each pairwise comparison). The number of DCMD spikes in response to receding objects decreased with instar, the opposite effect to that found for

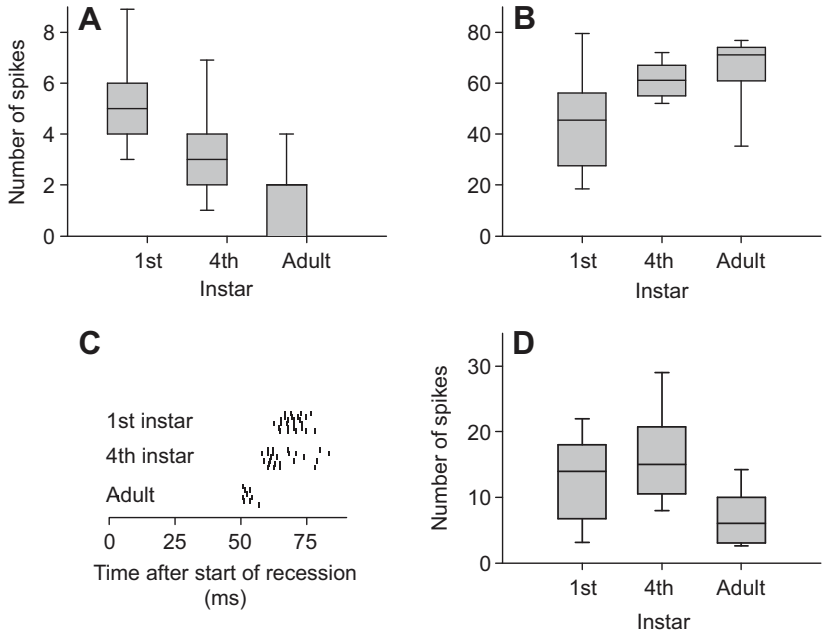

Fig. 5. Responses to receding stimuli and to luminance changes compared with responses to approaching stimuli. (A) Numbers of spikes by first instar, fourth instar and adult *Locusta migratoria* in response to a rectangle receding at 2 m s^{-1}. (B) Numbers of spikes by first instars, fourth instars and adults in response to a rectangle approaching at 2 m s^{-1}. (C) DCMD spike times in six stimulus repetitions in locusts of three instars to the image of a rectangle receding at 2 m s^{-1}. (D) Numbers of spikes by first instars, fourth instars and adults in response to a rectangular area of screen darkening with the same time course as that of the rectangle approaching at 2 m s^{-1}. Data for A–D are from the same set of locusts. In A, B and D each box shows the median, interquartile range and 5–95% range from 30 repetitions.

approaching stimuli. The latency to the first DCMD spike following start of rectangle recession decreased significantly with instar (mean ± sd: first instar 65.3±6.5 ms, N=22; fourth instar 58.3±5.7 ms, N=22; adult 53.7±3.6 ms, N=18; one-way ANOVA, d.f.=2, F=22.0, P<0.001, followed by the Holm-Sidak method for pairwise comparison, P<0.05; Fig. 5C).

The adult DCMD is known to respond much more vigorously to edge movements than to changes in luminance (Simmons and Rind, 1992). We showed that the same is true for larval locusts. Responses to an approaching rectangle (Fig. 5D) were significantly greater than those to a darkening rectangular area in first instars, fourth instars and adult locusts (Mann–Whitney test, P<0.001 in each case). Responses by the adult differed significantly from those by the fourth and first instars (Kruskal–Wallis one-way ANOVA, d.f.=2, H=26.6, P<0.001; Dunn's method for pairwise comparison, P<0.05 for adults *versus* first or fourth instars but not first *versus* fourth instars).

We found that, in all instars, the DCMD generated greater responses to an approaching compared with a receding object irrespective of whether the object was darker or lighter than the background (data not shown).

In order to generate a response that is sustained and vigorous, the adult DCMD needs image edges to move with increasing speed as they grow (Simmons and Rind, 1992). This is because stimulation of one part of the retina suppresses, after a delay, the response by the DCMD to stimulation of another part. In all instars we showed that stimulation of one part of the eye suppresses the response to stimulation of another part of the eye soon afterwards. We did this by presenting locusts with dark disks that grew at a constant rate to subtend 41 deg at the eye (Fig. 6). We used three different starting sizes, so the disk that started at the smallest size (3 deg) grew past the starting size of the two larger disks (which started at 15 or 30 deg). The response to any one of these movements was initially brisk and then declined, unlike responses to approaching disks (Fig. 2). Both the increase and the decline in response were more rapid in the adult than the first instar locusts (Fig. 6). When the disk started at either 15 or 30 deg, the response was larger than the response to a disk that expanded past either of those sizes after having started its expansion from a smaller size (Fig. 6). This effect was consistently found in both first instar and adult locusts. The initial spike frequency following the start of movement increased with the starting size of the disk and so with the extent of moving edge in the initial movement.

Habituation of responses to approaching stimuli

In each instar, the response to an approaching disk habituated with stimulus repetition, and it recovered over time without stimulation or else if the locust's leg or abdomen was brushed. We counted the number of spikes in responses to 60 mm diameter dark disks approaching at 2 m s^{-1}, and found similar changes with stimulus repetition in first instar and adult locusts (Fig. 7). In examining habituation, we experimented with locusts that had not previously experienced visual stimuli during an experiment, and left each for 10 min before delivering a series of identical approaching stimuli. We found considerable variability between individual locusts. However, when a stimulus was repeated every 30 s, after four repetitions the number of spikes in each response declined to 55–75% of the number in the first response both in first instar and in adult locusts (six individuals of each instar). The response in both instars recovered to its initial value following a recovery period of 10 min after 10 successive stimuli, and then the response declined with stimulus repetition more quickly than during the initial series of stimuli (Fig. 7).

Ultrastructural organisation of synapses onto the LGMD

In the adult, profiles of neurites of the LGMD and LGMD2 are readily recognisable in sagittal sections through the distal lobula because: (1) they are considerably wider than profiles of other neurons in the distal lobula; and (2) they are arranged along crescents that correspond with the fan-shaped arbours of the neurons near the posterior face of the lobula (Rind and Simmons, 1998). Intracellular staining demonstrated that the profiles of the LGMD2 are nearer to the brain surface than those of the LGMD. Two crescents of wide neuronal profiles are found in sections through the distal lobula in the first instar (Fig. 8A) and all later larval stages (J.S. and F.C.R., unpublished), which means we can identify large profiles of the LGMD and LGMD2 with reasonable certainty in all larval instars. Analysing LGMD profiles, we found synapses that were indistinguishable from those of adults in all larval samples, including

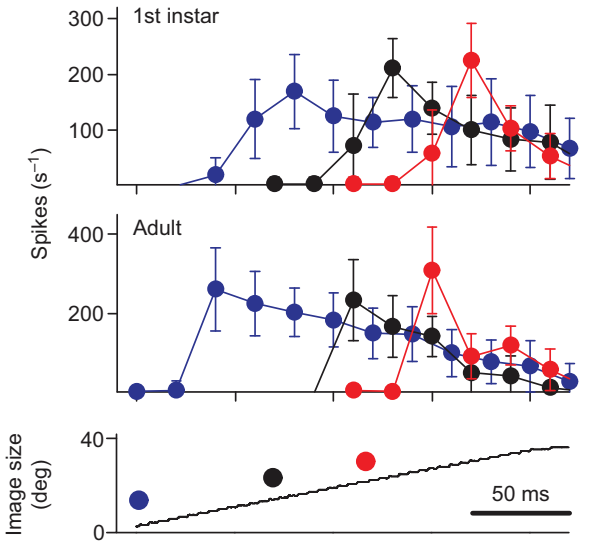

Fig. 6. Responses by *Locusta migratoria* DCMD to images of dark disks that expanded at a constant rate across the screen, with three different initial sizes. The disk expanded to a final subtense at the eye of 41 deg, starting from 3, 15 or 25 deg. On the monitor of angular image size, symbols indicate times of the start of movement for the three starting sizes and correspond with the symbols in the top two panels that plot mean ± s.d. DCMD spike rate (20 ms bins from six repetitions in each of three animals).

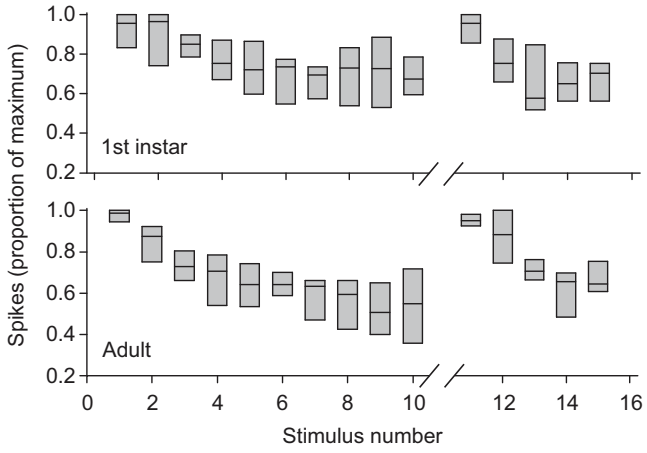

Fig. 7. Habituation and dis-habituation of DCMD response in first instar and adult *Locusta migratoria*. The stimulus was a 60 mm diameter dark disk approaching at 2 m s^{-1}, repeated every 30 s. After the first 10 stimulus repetitions there was a 10 min interval (gap in graphs) before the 11th stimulus. The number of spikes in the final 2 s of each stimulus was expressed as the proportion of the maximum number for all 15 stimulus repetitions to each animal. The median and quartiles from six individuals are plotted in each graph.

1-day-old first instars (Fig. 8B,D). First instar synapses already contain many spherical, electron-lucent vesicles with a diameter of ~38 nm clustered near to densely staining presynaptic bars. Other features consistent with maturity are the absence of both a double-barred presynaptic structure and a postsynaptic bar density (Leitch et al., 1992). As in the adult, larval synapses show the presence of a few larger, electron-dense vesicles (Fig. 8D,E). Individual neurites of the LGMD are covered with many smaller profiles that make synapses with the LGMD (Fig. 8B,C). The organisation of these synapses is the same in larvae as in adults: synapses are always dyadic, with the LGMD as one postsynaptic element and a similar, neighbouring profile that synapses with the LGMD as the other (Fig. 8B–E). In the adult, this organisation provides anatomical pathways that might allow columnar elements that excite the LGMD to inhibit each other, and the same organisation is clearly present in all instars.

DISCUSSION

We have shown that as soon as a first instar locust hatches, it has neurons that are capable of responding selectively to approaching stimuli and discriminating different speeds of approach. In responding to approaching stimuli, the first instar DCMD behaves very much like the adult neuron by generating trains of spikes whose rate tracks the approach of the object. As in the adult, the first instar DCMD generates different numbers of spikes in response to different stimulus approach speeds. The main changes that happen through the six instars are: an increase in the rate and number of spikes generated in response to an approaching object; a decrease in response latency; and a decrease in the number of spikes during brief responses to receding objects or changes in luminance. There is a gradual improvement in a selective approach to approaching over receding stimuli through instars, judged from the numbers of spikes generated in response to approaching compared with receding stimuli.

A possible explanation for the lower spike frequency in larval neurons is that the animals are more susceptible to the effects of handling, which might depress the responsiveness of their sensory neurons by habituation. It is well established that habituation occurs in the synaptic pathways that converge onto the LGMD's main dendritic fan and excite it (O'Shea and Rowell, 1975a; O'Shea and Rowell, 1976; Rind et al., 2008). However, this explanation would not be consistent with the larger responses to receding objects produced by early instars compared with later instars, or to the relatively large responses by first instar locusts compared with adult locusts to darkening stimuli. In addition, our finding that responses to repeated, approaching stimuli in different instars habituate and dis-habituate in similar ways supports the notion that our experimental treatment did not suppress responses in larvae any more than in adults. A second possible reason why earlier instars generate lower numbers of spikes compared with later instars in response to approaching stimuli might be that the chemical synapse that connects the LGMD with the DCMD (Rind, 1984) is not as strong and reliable in early instars compared with the adult, so some LGMD spikes might not trigger spikes in the DCMD. The relatively vigorous responses by DCMDs in young instars to receding objects or luminance changes again argue against this. It seems to be a general property of larval locust interneurons that they generate lower spike rates than in the adult (Boyan, 1983; Bucher and Pflüger, 2000), which might limit the ability of young animals, relative to older animals, to discriminate stimuli.

Our results indicate that the neuronal pathways and synaptic interactions needed for selectivity for approaching stimuli develop before the locust has emerged from its egg and so before it starts to experience moving visual stimuli. As in the adult (Simmons and Rind, 1992), the LGMD and DCMD in larvae generate smaller responses to changes in luminance than they do to movements. This means that in all instars the LGMD integrates inputs from stimuli that travel over different parts of the eye; it does not simply react to shadows. We showed that in all larval stages, the responses by the LGMD to edges moving at constant speeds adapt,

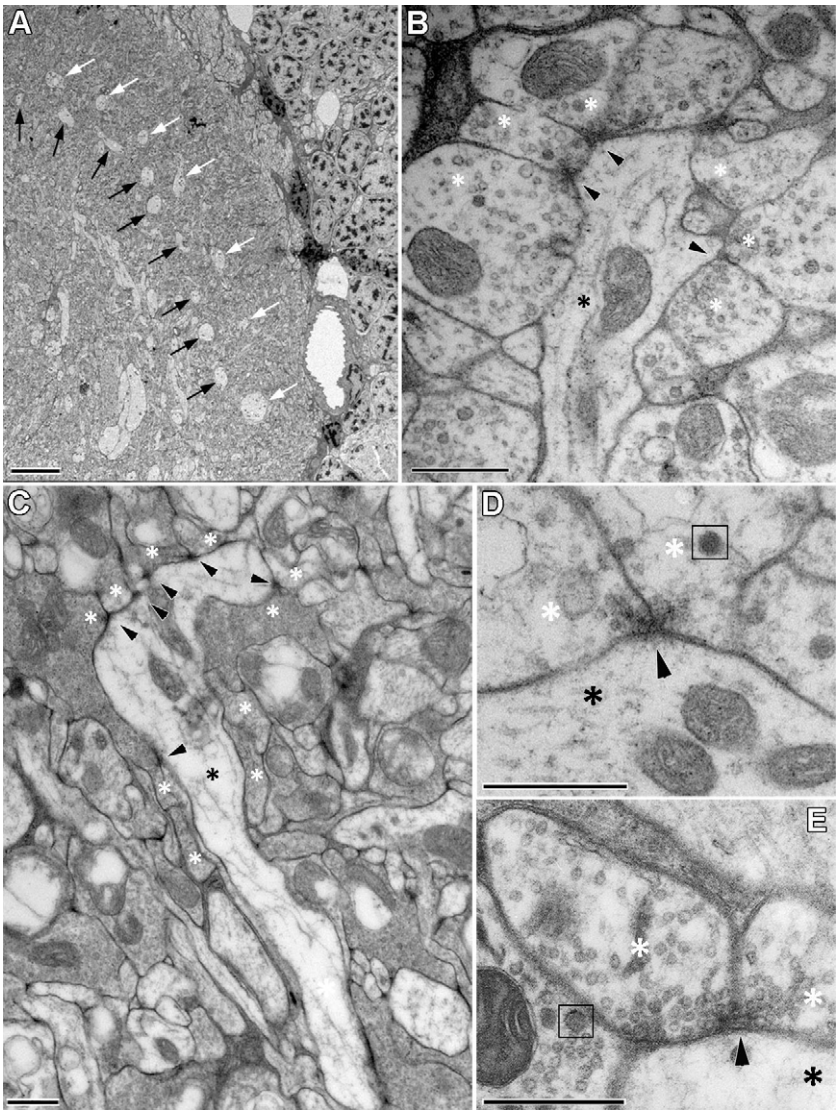

Fig. 8. Electron micrographs of synapses onto the lobula giant motion detector (LGMD) in different instars of *Locusta migratoria*. (A) Low power micrograph of a sagittal section of the lobula in a first instar locust showing the two crescents of processes belonging to the LGMD (black arrows) and LGMD2 dendritic trees (white arrows). (B–E) Details of synapses in different instars. LGMD profiles are marked with black asterisks, profiles presynaptic to the LGMD with white asterisks and synapses with black arrowheads. (B,C) LGMD profiles surrounded by several presynaptic profiles in (B) first instar and (C) fourth instar locusts. (D,E) Detail of dyadic synapse involving two profiles that are presynaptic to the LGMD and to each other in (D) first instar and (E) adult locusts. Examples of large, electron-dense vesicles are enclosed in squares. Scale bars: (A) 10 µm; (B–D) 500 nm.

which suggests that before emerging from the egg the pathways that suppress responses by one part of the eye to responses by another part are established. A mechanism for this is presynaptic inhibition between neurons in columns in the medulla that correspond with ommatidia (O'Shea and Rowell, 1975a; O'Shea and Rowell, 1976; Rind and Bramwell, 1996), and a likely anatomical substrate for this inhibition is provided by the reciprocal arrangement of synapses from small profiles that synapse both with the LGMD and with each other (Rind and Leitinger, 2000; Rind and Simmons, 1998). By using electron microscopy we have shown that this synaptic arrangement is already present in the first instar. The synapses in the larvae are indistinguishable in structure from those in the adult, and do not show any of the characteristics of immature synapses in the embryonic nervous system (Leitch et al., 1992).

One likely explanation for the increase with instar in DCMD response to approaching objects is that the number of ommatidial units that excite the LGMD increases, although the effect this has will depend on how the LGMD's electrical properties change with growth. The number of ommatidia in a locust's eye increases almost fourfold between the first instar and the adult. An increase in strength of excitation to the LGMD due to this increase in ommatidium number is consistent with the decrease in response latency, which is most easy to gauge from responses to receding stimuli or to circles expanding at constant velocity, but is also apparent in responses to approaching objects. It is likely that, as the strength of excitation to the LGMD increases, so too will the strength of lateral inhibition presynaptic to it. In adults, responses to constantly expanding stimuli are larger and build up more rapidly than in earlier instars, but then also decay more rapidly. This is consistent with stronger initial excitation to the LGMD and DCMD in adults compared with younger locusts, followed by stronger lateral inhibition reducing the later responses. Strong initial excitation followed by lateral inhibition can also explain why peak responses by adults compared with younger locusts are relatively large for rapidly approaching stimuli, but not for the slowest stimuli we used. During the slowest stimuli, in adults the lateral inhibition may develop rapidly enough to reduce excitation during the final stages of an approaching stimulus. Differences between instars in the strength of lateral inhibition might also explain why responses to receding stimuli or to luminance changes are relatively large in the younger larvae, although we have not studied the smaller LGMD dendritic subfields that have been implicated in suppressing responses to wide-field stimuli (Rowell et al., 1977).

Although our experiments indicate that visual experience is not needed for the LGMD and DCMD to develop their ability to respond selectively to visual stimuli, it is possible that their responses can be influenced by visual stimuli they experience during larval life. Bloom and Atwood (Bloom and Atwood, 1980) found that in responses by adult DCMDs to disks approaching at $0.2\,m\,s^{-1}$, numbers of spikes by locusts that had been reared in darkness were reduced by nearly 90% compared with locusts that had experienced normal light:dark cycles as larvae. The difference in responses between dark- and light-reared locusts was much greater than the differences we found between adult and first instar locusts, and an explanation for this large reduction in responsiveness is that responses by photoreceptors are very much reduced in locusts that have been reared in darkness compared with locusts that have been reared normally (Bloom and Atwood, 1980). However, Bloom and Atwood (Bloom and Atwood, 1980) also provided evidence that sensory experience can affect the properties of synaptic connections in the optic lobes. They found that the rate and extent of habituation in responses by the DCMD is much greater than normal in dark-reared locusts. Because the site of habituation is the synaptic connections from afferent neurons onto the LGMD (O'Shea and Rowell, 1976), properties of this connection must be sensitive to lighting levels during development. However, we cannot yet judge whether experience of moving stimuli in early larval life affects the functional development of synaptic connections in the insect optic lobe.

A number of characteristics implicate the DCMD in a role in the behavioural responses that a locust makes to avoid imminent collisions, including capture by predators. The DCMD responds well to approaching images, and its axon is the widest in the nerve cord and has a rapid conduction velocity. In adults, one function for the DCMD is to trigger a diving glide during flight, which might enable evasion from capture by predatory birds or collision with other locusts in a swarm (Santer et al., 2012; Santer et al., 2006). It also plays a role in triggering jumps (Fotowat and Gabbiani, 2007; Santer et al., 2008), and recent evidence suggests that it plays distinct roles during different phases in preparing for and performing a jump (Fotowat et al., 2011). The DCMD acts in concert with other interneurons in the control of jumps, some of which respond to approaching stimuli (Gray et al., 2010; Simmons and Rind, 1997). The natural predators that chase locusts might differ according to the age and size of locusts, as has been shown for *Nemobius* crickets, in which early instars are particularly vulnerable to predation by wolf spiders (Dangles et al., 2007). In a dense swarm, locust hoppers attack each other (Bazazi et al., 2008). Observation of locusts in our colony shows that larvae of all ages orient away from approaching stimuli, and often jump in response to attempts to catch them. More work is needed to establish whether the kinds of natural stimuli the larvae respond to and the kinds of behavioural responses the LGMD and DCMD participate in alter as a locust develops from hatching to adult.

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AUTHOR CONTRIBUTIONS

P.J.S conducted the electrophysiology and analysed the data; J.S. and F.C.R. conducted the ultrastructural investigation; all authors contributed to preparing the manuscript.

COMPETING INTERESTS

No competing interests declared.

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