Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.083360

LOOMING DETECTION BY IDENTIFIED VISUAL INTERNEURONS DURING LARVAL DEVELOPMENT OF THE LOCUST, *LOCUSTA MIGRATORIA*

Peter J. Simmons[1], Julieta Sztarker[1,2], F. Claire Rind[1],

1: Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK

2: Depto. Fisiología, Biología Molecular y Celular, FCEN, Universidad de Buenos Aires, IFIBYNE-CONICET, Pabellón 2 Ciudad Universitaria, Intendente Güiraldes 2160, Buenos Aires 1428, Argentina

Short title: Locust visual neuron larval development

Key Words: vision, looming, insect, larva, synapse, development

Email: p.j.simmons@ncl.ac.uk

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 shapes selectivity for approaching objects.

INTRODUCTION

As with other hemi-metabolous 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 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 hemi-metabolous 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 1st 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 1st 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 ten 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 about 2,500 in the 1st instar to 9,000 - 9,500 in the adult, with the widths of individual facets increasing from just over 20 μm to about 40 μm (Bernard, 1937; Rafi and Burtt, 1974). An adult *Locusta* eye is slightly smaller than *Schistocerca'*s, with about 8,250 facets (Wilson et al., 1978) and an inter-ommatidial angle of 1° 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 LGMD (lobula giant motion detector, O'Shea and Williams, 1974) and the DCMD (descending contralateral motion detector, 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 1-for-1 (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 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 approach (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 1st 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 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.

METHODS

Animals

We took individual *Locusta migratoria* (Linn.) from our gregarious breeding colony. Locusts were kept at 30 °C, with a 12:12 hour 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 four hours 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 6 instars, but concentrated on the 1st, 3rd, 4th and adult. 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 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 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), gain 1,000. 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). DCMD spikes were converted afterwards to events by 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) placed parallel to the locust's long axis with its centre aligned with the left eye and 80 mm from it, subtending 64° X 59°. Images were controlled by a microcomputer fitted with a visual stimulus card and raster generator (VSG2/1 and RG2, Cambridge Research Systems). 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 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 μWcm^{-2}, measured with a radiometer at the position of the eye (SL021 Photodetector, Ealing). 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 was plotted and statistical tests performed using SigmaPlot 11.0 (Systat Software Inc.). 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 0.1M 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 epoxy resin kit) at 60°C for 24 h. To locate the part of the optic lobe containing LGMD dendrites, 1 µm survey sections were cut, stained with 1% toluidine blue in 1% borax and examined with a light microscope. Ultrathin sections, approximately 80 nm thick, were cut using a diamond knife on a Reichert-Jung Ultracut E ultramicrotome, 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 fitted with an AMT CCD camera (Deben).

RESULTS

Eye development

The compound eye grows from 0.75 mm wide in a newly hatched 1st instar to 1.1 mm wide in a 4th instar and 2.0 mm wide in an adult (Figs. 1A-C). The width of the eye is about 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 4 live locusts of each instar, we viewed the eye through a dissecting microscope (Wild, with 20X eye-pieces). We counted the number of ommatidia along a light band that runs across the eye, which provides a consistent location in different instars (Figs. 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 2nd instar, rises more gradually to the 4th instar, and then more steeply again to the adult, which has 80 (range 76-84) facets (Fig 1D). By counting facets across the eye at different locations, we estimate that the total number in the adult is 8,134 (similar to a previous estimate, Wilson et al., 1978), compared with 2,200 in the 1st instar and 4,280 in the 4th 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 1st instar to 33 µm in the adult (Fig. 1D; measured in fresh exoskeleton with transmitted light; calculated from width across 10 facets in 3 locusts of each instar). Consequently

compared with an adult, a 1st instar locust views images with about a quarter the number of ommatidia and smaller facets so a poorer resolution.

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 discs approaching at various speeds between 0.5 and 10 ms^{-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. 2 A), 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 1st 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 about 40 ms before the end of stimulus movement. We found no significant difference between 1st 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, 6 repetitions in five individuals of each instar). DCMDs in 1st 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 5 different animals from 6 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 ms^{-1}; 2.5 s at 1 ms^{-1}; 1.25 s at 2 ms^{-1}; 0.5 s at 5 ms^{-1}; and 0.25 s at 10 ms^{-1}). Within each instar, the response to each stimulus speed differed from responses to other speeds (Fig. 3A). We showed within each instar that differences between responses to different stimulus speeds were significant by performing a Friedman repeated measures ANOVA, df 4 and $p<0.001$ in each case. The X^2 values from this analysis were : 1st instar 112.0; 3rd instar 111.1; 4th instar 113.0; adult 119.23; and each pairwise comparison within each instar was shown to be significantly different post-hoc by Student-Newman-Keuls Method, $p <0.05$. We also showed that, for each stimulus speed mean spike frequency varies

significantly between instars (Fig. 3B). Friedman ANOVAs, df 3 and $p<0.001$, gave X^2 values for different approach speeds: 0.5 ms^{-1}, 46.2; 1 ms^{-1}, 46.2; 2 ms^{-1}, 58.3; 5 ms^{-1}, 71.1; 10 ms^{-1}, 47.4. At the slowest stimulus speeds (0.5 and 1 ms^{-1}) responses by 3rd, 4th and adult instars did not differ significantly from each other ('a' in Fig. 3B). For faster stimulus speeds (2 ms^{-1} and greater) responses by 1st, 3rd and adult instars did differ significantly from each other (Student-Newman-Keuls Method, $p<0.05$). We found no significant differences between 3rd and 4th 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). We showed that the effect of approach speed was significant by performing Friedman ANOVAs, df 4 and $p<0.001$, which gave X^2 values: 1st instar, 88.9; 3rd instar 102.0; 4th instar, 82.7; and adult 89.9. Pairwise comparison within each instar no significant differences in peak spike frequency for stimuli at 2 and 5 ms^{-1} in either 1st instars or adults ('a' in Fig. 4A), but in other cases peak spike frequency did differ significantly between stimulus speeds within each (Student-Newman-Keuls post-hoc analysis, $p<0.05$). For each stimulus speed, spike frequency differed between instars (Fig. 4B). We showed this was significant with Friedman ANOVAs, df 3 and $p<$ or $= 0.001$, which gave X^2 values for different approach speeds: 0.5 ms^{-1}, 16.2; 1 ms^{-1}, 33.7; 2 ms^{-1}, 35.0; 5 ms^{-1}, 38.3; 10 ms^{-1}, 38.7. For the slowest stimulus speed, 0.5 ms^{-1}, responses during the bin with the greatest number of spikes did not differ significantly between 1st and adult instars, but was less than those by 3rd and 4th instars (Fig. 4B, 0.5 ms^{-1}). This contrasts with the mean spike frequency throughout the stimulus at 0.5 ms^{-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 1st instar, but not significantly different from responses by 3rd or 4th instars (Fig. 4B, lower case letters at 2.0 and 10 ms^{-1}). Significances of differences between pairs of instars was shown by Student-Newman-Keuls Method, $p<0.05$.

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 ms^{-1}, and found that for each speed and instar there was a significant difference between responses to receding and approaching

rectangle (Mann-Whitney Test, $p<0.001$ in each case; six stimulus repetitions in each of five locusts). In the 1st and 4th instars, all 30 receding stimuli generated at least one spike, whereas in the adults 11 out of the 30 receding stimuli generated no spikes (Figs. 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, shown by a Friedman repeated measures ANOVA, df 2 and $p<0.001$, X^2 34.5, followed by the Student-Newman-Keuls Method 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 approaching stimuli. The latency to the first DCMD spike following start of rectangle recession decreased with instar (Fig. 5C; median ± 95% ci: 1st instar, 64.6 ±2.62 ms; 4th instar 55.5 ±2.38 ms; and adult 52.3 ±2.36 ms), a significant difference shown by a Kruskall-Wallis one-way ANOVA on ranks, df 2 and $p<0.001$, $H=28.4$, followed by Dunn's method, $p<0.05$, for pairwise comparison (a repeated measures test could not be used because sample sizes differed as several stimuli to adults generated no spikes).

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 1st instars, 4th instars and adult locusts (Mann-Whitney Test, $p<0.001$ in each case). Responses by the adult differed significantly from those by the 4th and 1st instars (Kruskal-Wallis one way ANOVA, df 2 H 26.6 and $p<0.001$; Dunn's method for pairwise comparison $p<0.05$ for adult compared with 1st or 4th instar but not for 1st compared with 4th 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° at the eye (Fig. 6). We used three different starting sizes; so the disk that started at the smallest size (3°) grew past the starting size of the two larger disks (which started at 15° or 30°).

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 1st instar locusts (Fig.6). When the disk started at either 15° or 30°, the response that followed was larger than the response to expansion of the disk past that size having started from a smaller size (Fig. 6). This effect was consistently found in both 1st 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 ms^{-1}, and found similar changes with stimulus repetition in 1st 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 4 repetitions the number of spikes in each response declined to 55-75 % of the number in the first response both in 1st instar and in adult locusts (6 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, first, they are considerably more wide than profiles of other neurons in the distal lobula, and, second, they are arranged along crescents that correspond with the fan-shaped arbours of the neurons near to 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 1st instar (Fig. 8A) and all later larval stages (Sztarker and Rind, in preparation), 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 one day-old 1st instars (Figs. 8B and D).

1st instar synapses already contain many spherical, electron-lucent vesicles with diameter about 38 nm clustered near to densely-staining presynaptic bars. Other features consistent with maturity are the absence of a double-barred presynaptic structure and of a postsynaptic bar density (Leitch et al., 1992). As in the adult, larval synapses show the presence of a few larger, electron-dense vesicles (Figs. 8D,E). Individual neurites of the LGMD are covered with many smaller profiles that make synapses with it (Figs. 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 (Figs. 8B-E). In the adult, this organisation provides anatomical pathways which 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 1st instar locust hatches, it has neurons that are capable of responding selectively to approaching stimuli and of discriminating different speeds of approach. In responding to approaching stimuli, the 1st instar DCMD behaves very much like the adult neuron by generating trains of spikes whose rate tracks object approach. As in the adult, the 1st instar DCMD generates different number of spikes in responses to different speeds of stimulus approach. 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 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, 1976; Rind et al., 2008). However, this explanation would not be consistent with the larger responses produced to receding objects by early instars compared with later, or to the relatively large responses by 1st 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 compared with 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, 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, 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 1st 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 four-fold between 1st instar to 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, but then also decay more rapidly than they do in earlier instars. 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 (1980) found that in responses by adults DCMDs to disks approaching at 0.2 ms^{-1}, numbers of spikes by locusts that had been reared in darkness were 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 (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 habituates 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 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 a 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 it responds to and the kinds of behavioural responses the LGMD and DCMD participate in alter as a locust develops from hatching to adult.

FUNDING: partly supported by a Marie Curie International Incoming Fellowship within the 7th European Community Framework Programme.

REFERENCES.

Anderson, H. (1978). Post-embryonic development of visual-system of locust, *Schistocerca gregaria*. 1. patterns of growth and developmental interactions in retina and optic lobe. *J. Embryol. Exp. Morph.* **45**, 55-83.

Anderson, H. and Bacon, J. (1979). Developmental determination of neuronal projection patterns from wind-sensitive hairs in the locust, *Schistocerca gregaria*. *Dev. Biol.* **72**, 364-373.

Bazazi, S., Buhl, J., Hale, J. J., Anstey, M. L., Sword, G. A., Simpson, S. J. and Couzin, I. D. (2008). Collective motion and cannibalism in locust migratory bands. *Current Biology* **18**, 735-739.

Bentley, D. and Toroian-Raymond, A. (1981). Embryonic and postembryonic morphogenesis of a grasshopper interneuron. *J. Comp. Neurol.* **201**, 507-518.

Bernard, F. (1937). Récherches sur la morphogenèse des yeux composés d'arthropodes. *Bull. biol. Fr. Belg. Suppl.* **23**, 1-162.

Bloom, J. W. and Atwood, H. L. (1980). Effects of altered sensory experience on the responsiveness of the locust descending contralateral movement detector neuron. *J. Comp. Physiol. A* **135**, 191-199.

Boyan, G. S. (1983). Postembryonic development in the auditory system of the locust. Anatomical and physiological characterisation of interneurones ascending to the brain. *J. Comp. Physiol. A* **151**, 499-513.

Bucher, D. and Pflüger, H. J. (2000). Directional sensitivity of an identified wind-sensitive interneuron during the postembryonic development of the locust. *J. Insect Physiol.* **46**, 1545-1556.

Dangles, O., Pierre, D., Christides, J. P. and Casas, J. (2007). Escape performance decreases during ontogeny in wild crickets. *J. Exp. Biol.* **210**, 3165-3170.

de Vries, S. E. J. and Clandinin, T. R. (2012). Loom-sensitive neurons link computation to action in the *Drosophila* visual system. *Curr. Biol.* **22**, 353-362.

Dewell, R. B. and Gabbiani, F. (2012). Escape behavior: linking neural computation to action. *Curr. Biol.* **22**, R153-R154.

Fotowat, H. and Gabbiani, F. (2007). Relationship between the phases of sensory and motor activity during a looming-evoked multistage escape behavior. *J. Neurosci.* **27**, 10047-10059.

Fotowat, H., Harrison, R. R. and Gabbiani, F. (2011). Multiplexing of motor information in the discharge of a collision detecting neuron during escape behaviors. *Neuron* **69**, 147-158.

Gray, J. R., Blincow, E. and Robertson, R. M. (2010). A pair of motion-sensitive neurons in the locust encode approaches of a looming object. *J. Comp. Physiol. A* **196**, 927-938.

Hatsopoulos, N., Gabbiani, F. and Laurent, G. (1995). Elementary computation of object approach by a wide-field visual neuron. *Science* **27**, 1000-1003.

Horridge, G. A. (1978). Separation of visual axes in apposition compound eyes. *Phil. Trans. R. Soc. Lond. B* **285**, 1-59.

Karmeier, K., Tabor, R., Egelhaaf, M. and Krapp, H. (2001). Early visual experience and the receptive-field organization of optic flow processing interneurons in the fly motion pathway. *Visual Neuroscience* **18**, 1-8.

Kirschfeld, K. (1976). The resolution of lens and compound eyes. In *Neural Principles of Vision*, (eds. F. Zettler and R. Weiler), pp. 356-370. New York: Springer.

Kral, K. and Poteser, M. (2009). Relationship between body size and spatial vision in the praying mantis – an ontogenetic study. *Journal of Orthoptera Research* **18**, 153-158.

Krapp, H. G. and Gabbiani, F. (2005). Spatial distribution of inputs and local receptive field properties of a wide-field, looming sensitive neuron. *J. Neurophysiol.* **93**, 2240-2253.

Leitch, B., Laurent, G. and Shepherd, D. (1992). Embryonic development of synapses on spiking local interneurones in locust. *J. Comp. Neurol.* **324**, 213-236.

Michel, K. and Petersen, M. (1982). Development of the tympanal organ in larvae of the migratory locust (*Locusta migratoria*). *Cell Tissue Res.* **222**, 662-676.

Newland, P. L., Watkins, B., Emptage, N. J. and Nagayama, T. (1995). The structure, response properties and development of a hair plate on the mesothoracic leg of the locust. *J. Exp. Biol.* **198**, 2397-2404.

O'Shea, M. and Rowell, C. (1975a). Protection from habituation by lateral inhibition. *Nature* **254**, 53-55.

O'Shea, M. and Rowell, C. (1975b). A spike-transmitting electrical synapse between visual interneurons in the locust movement detector system. *J. Comp. Physiol.* **97** 143-158.

O'Shea, M. and Rowell, C. (1976). The neuronal basis of a sensory analyser, the acridid movement detector system. II. response decrement, convergence, and the nature of the excitatory afferents to the fan-like dendrites of the LGMD. *J. Exp. Biol* **65** 289-308.

O'Shea, M. and Williams, J. L. D. (1974). The anatomy and output connections of a locust visual interneurone: the lobula giant movement detector (LGMD) neurone. *J. Comp. Physiol.* **91**, 257-266.

Oliva, D., Medan, V. and Tomsic, D. (2007). Escape behavior and neuronal responses to looming stimuli in the crab *Chasmagnathus granulatus* (Decapoda: Grapsidae). *J. Exp. Biol.* **210**, 865-880.

Peron, S. and Gabbiani, F. (2009). Spike frequency adaptation mediates looming stimulus selectivity in a collision-detecting neuron. *Nature Neurosci.* **12**, 318 – 326.

Peron, S. P., Krapp, H. G. and Gabbiani, F. (2007). Influence of electrotonic structure and synaptic mapping on the receptive field properties of a collision-detecting neuron. *J. Neurophysiol.* **97**, 159-177.

Pflüger, H. J., Hurdelbrink, S., Czjzek, A. and Burrows, M. (1994). Activity-dependent structural dynamics of insect sensory fibers. *J. Neurosci.* **14**, 6946-6955.

Pinter, R. (1977). Visual discrimination between small objects and large textured backgrounds. *Nature* **270,** 429-431.

Preuss, T., Osei-Bonsu, P. E., Weiss, S. A., Wang, C. and Faber, D. S. (2006). Neural representation of object approach in a decision-making motor circuit. *J. Neurosci.* **26**, 3454-3464.

Rafi, F. and Burtt, E. (1974). Visual acuity in larval instars of *Schistocerca gregaria* and adults of two other Orthopterans, Acridoidea. *Zool. Anz.* **193**, 305-313.

Rind, F. C. (1984). A chemical synapse between two motion detecting neurones in the locust brain. *J. Exp. Biol.* **110**, 143-167.

Rind, F. C. and Bramwell, D. I. (1996). A neural network based on the input organisation of an identified neuron signalling impending collision *J. Neurophysiol.* **75** 967-985.

Rind, F. C. and Leitinger, G. (2000). Immunocytochemical evidence that collision sensing neurons in the locust visual system contain acetylcholine. *J. Comp. Neurol.* **423**, 389-401.

Rind, F. C., Santer, R. D. and Wright, G. A. (2008). Arousal facilitates collision avoidance mediated by a looming sensitive visual neuron in a flying locust. *J. Neurophysiol.* **100**, 670-680.

Rind, F. C. and Simmons, P. J. (1992). Orthopteran DCMD neuron - a reevaluation of responses to moving-objects. I. Selective responses to approaching objects. *J. Neurophysiol.* **68**, 1654-1666.

Rind, F. C. and Simmons, P. J. (1998). Local circuit for the computation of object approach by an identified visual neuron in the locust. *J. Comp. Neurol.* **395**, 405-415.

Rogers, S. M., Krapp, H. G., Burrows, M. and Matheson, T. (2007). Compensatory plasticity at an identified synapse tunes a visuomotor pathway. *J. Neurosci.* **27**, 4621-4633.

Rowell, C. H. F. (1971). The orthopteran descending movement detector (DMD) neurones: a characterisation and review. *J. Comp. Physiol.* **73**, 167-194.

Rowell, C. H. F., O'Shea, M. and Williams, J. D. (1977). Neuronal basis of a sensory analyzer, the acridid movement detector system. IV. The preference for small field stimuli. *J. Exp. Biol.* **68**, 157-185.

Santer, R. D., Rind, F. C. and Simmons, P. J. (2012). Predator versus prey: locust looming-detector neuron and behavioural responses to stimuli representative of attacking bird predators. *PlosOne* **7**(11), e50146.

Santer, R. D., Rind, F. C., Stafford, R. and Simmons, P. J. (2006). Role of an identified looming-sensitive neuron in triggering a flying locust's escape. *J. Neurophysiol.* **95**, 3391-3400.

Santer, R. D., Yamawaki, Y., Rind, F. C. and Simmons, P. J. (2008). Preparing for escape: an examination of the role of the DCMD neuron in locust escape jumps. *J. Comp. Physiol. A* **194**, 69-77.

Schlotterer, G. (1977). Response of the locust descending movement detector neuron to rapidly approaching and withdrawing visual stimuli *Can. J. Zool.* **55**, 1372-1376.

Sehnal, F. (1985). Morphology of insect development. *Ann. Rev. Entomology* **30**, 89-109.

Simmons, P. J. and Rind, F. C. (1992). Orthopteran DCMD neuron - a reevaluation of responses to moving-objects. II. Critical cues for detecting approaching objects. *J. Neurophysiol.* **68**, 1667-1682.

Simmons, P. J. and Rind, F. C. (1997). Responses to object approach by a wide field visual neurone, the LGMD2 of the locust: characterization and image cues. *J. Comp. Physiol. A* **180**, 203-214.

Spaethe, J. and Chittka, L. (2003). Interindividual variation of eye optics and single object resolution in bumblebees. *J. Exp. Biol.* **206**, 3447-3453.

Wilson, M., Garrard, P. and Mcginness, S. (1978). Unit structure of the locust compound eye. *Cell Tissue Res.* **195**, 205-226.

Zollikofer, C. P. E., Wehner, R. and Fukushi, T. (1995). Optical scaling in conspecific *Cataglyphis* ants. *J. Exp. Biol.* **198**, 1637-1646.

598

599 FIGURE LEGENDS

600 Fig. 1. Compound eye morphology in different instars. Drawings of the heads of (A) 1st, (B)
601 4th and (C) adult instar *Locusta* from the side. Scale bars: 1 mm. The horizontal band of light
602 facets that runs across the eye is shown. (D) plots of number of facets along the ventral
603 border of the light band (means from 3 locusts) and facet width (average width determined
604 from the width across 10 facets; means from 3 locusts). Error bars are ranges of values.

605 Fig. 2. Responses by the DCMD in different instars to approaching stimuli. (A) DCMD
606 spikes in extracellular nerve cord recordings from a 1st instar and an adult locust to images of
607 disks approaching at 1 and 5 ms^{-1}. Image size, as angular subtense at the eye, is monitored in
608 the lower panels. (B) Raster plots of DCMD spike times in six responses to a 2 ms^{-1}
609 approaching stimulus in individuals of a 1st, 3rd and adult instar. (C) Spike frequency
610 histograms of 1st, 3rd, and adult instar DCMDs responding to images of disks approaching at
611 different speeds, as indicated on the stimulus monitor traces (mean ±s.d. for each 20 ms bin,
612 n=30).

613 Fig. 3. Mean spike rate by DMCDs of larval and adult locusts during responses to disks
614 approaching at different speeds. The mean spike rate was obtained by dividing the number of
615 spikes in each response by stimulus duration. (A) Each of the three graphs shows mean spike
616 rate at different approach speeds for individuals of the instar shown on the graph; graphs for
617 the 4th instar, not shown here, were similar to those for the 3rd instar. (B) Each of the three
618 graphs shows mean spike rate in individuals of different instars for the approach speed
619 shown on the graph. Figs. 3A and B include the same data. In each graph, each box shows
620 median, interquartile range, and error bars indicating 5% - 95% range; six different responses
621 in five individual locusts. In B, 'a' indicates responses that were not significantly different at
622 a particular stimulus speed (Student-Newman-Keuls Method, $p<0.05$, following Friedman
623 repeated measures analysis of variance on ranks).

624 Fig. 4. DCMD spike rate during the 20 ms bin with the greatest number of spikes during
625 responses to disks approaching at different speeds. (A) Each of the three graphs shows spike
626 rate at different approach speeds for individuals of the instar shown on the graph; graphs for
627 the 4th instar, not shown here, were similar to those for the 3rd instar. (B) Each of the three
628 graphs shows spike rate in individuals of different instars for the approach speed shown on

the graph. Lower case letters indicate responses to a particular stimulus speed that were not distinguishable in a particular graph (Student-Newman-Keuls Method, $p<0.05$, following Friedman repeated measures analysis of variance on ranks). Other details as in Fig. 3.

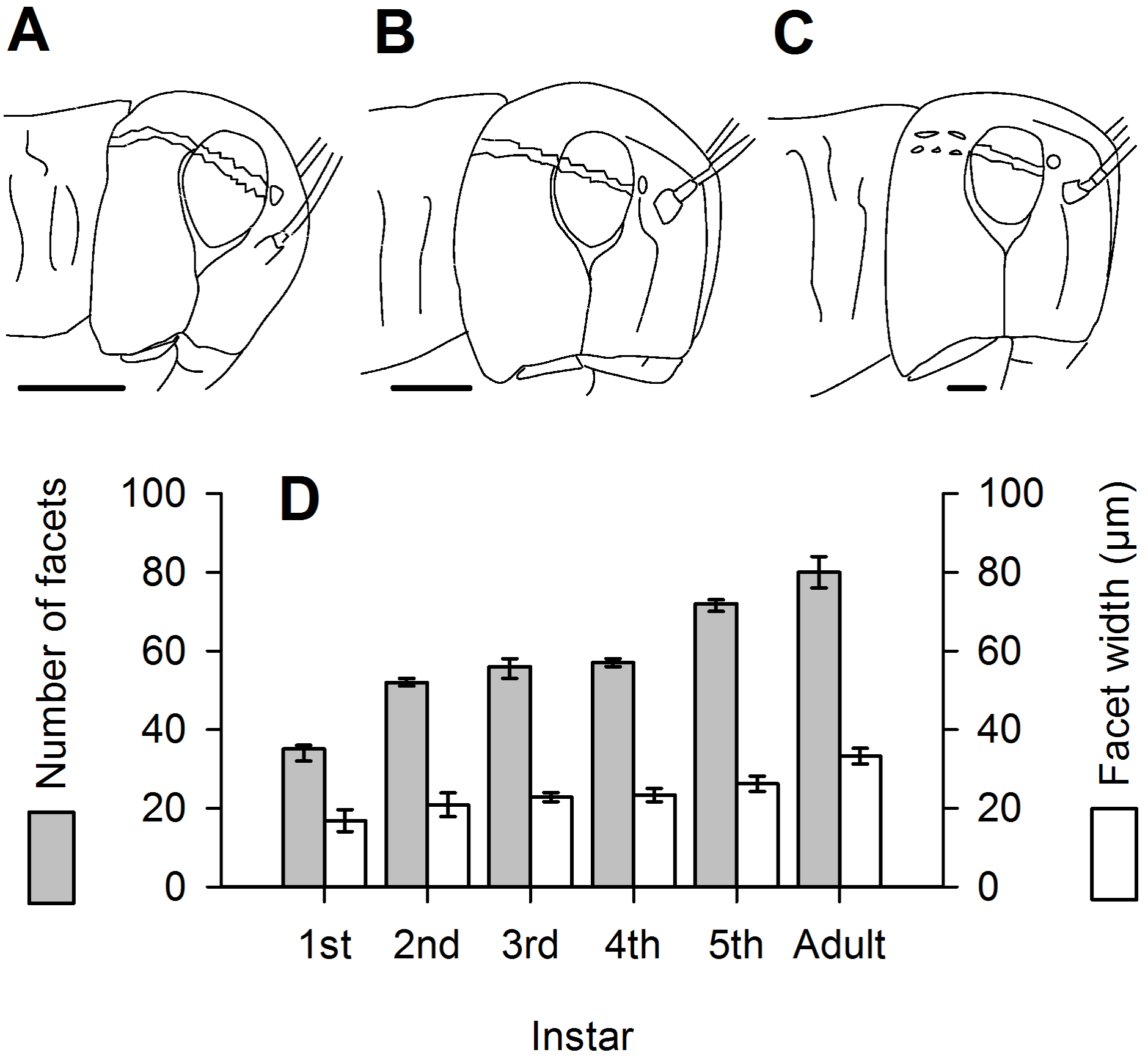
Fig. 5. Responses to receding stimuli and to luminance changes compared with responses to approaching stimuli. (A) Histogram of numbers of spikes by 1st, 4th and adult instars in response to a rectangle receding at 2 m/s. (B) Histogram of numbers of spikes by 1st, 4th and adult instars in response to a rectangle approaching at 2 ms^{-1}. (C) DCMD spike times in six stimulus repetitions in locusts of three instars to the image of a rectangle receding at 2 ms^{-1}. (D) Histogram of numbers of spikes by 1st, 4th and adult instars in response to a rectangular area of screen darkening with a the same time-course as that of the rectangle approaching at 2 ms^{-1}. Data for A-D comes from the same set of locusts. In A, B, and D each box shows median, interquartile range and 5%-95% range from 30 repetitions.

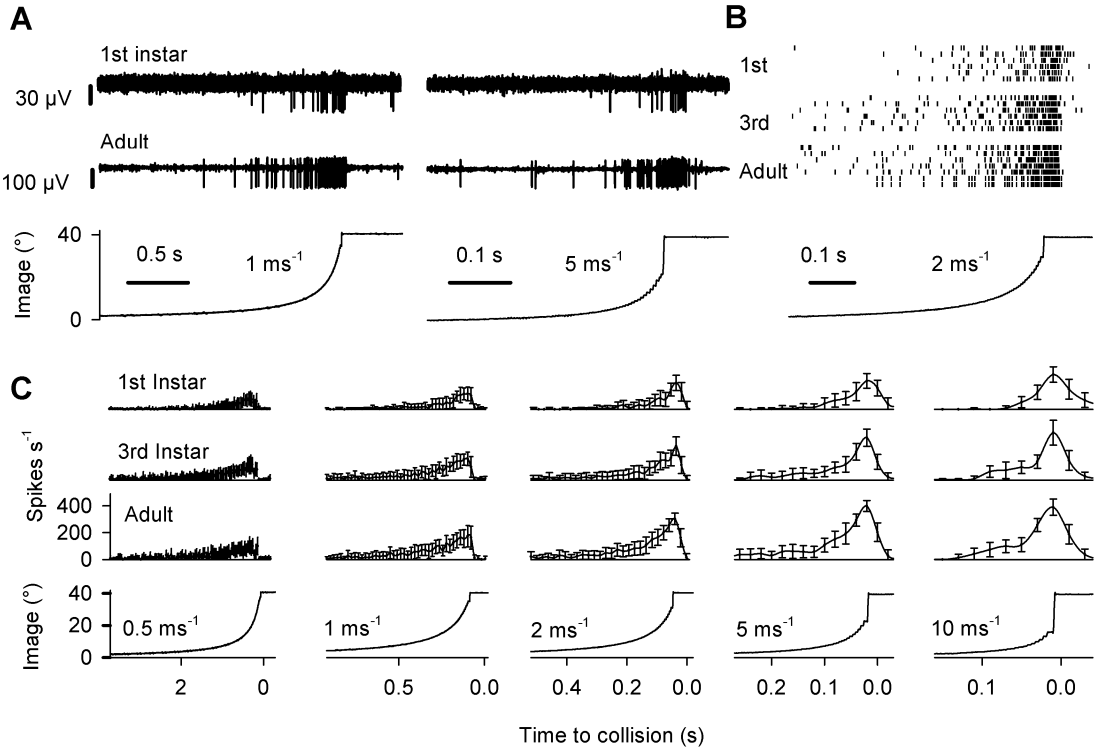
Fig. 6. Responses to images of dark disks that expanded at a constant rate across the screen, with three different initial sizes. The disk expanded to a final subtense at the eye of 41°, starting from 3°, 15° or 25°. On the monitor of angular image size, symbols indicate times of the start of movement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

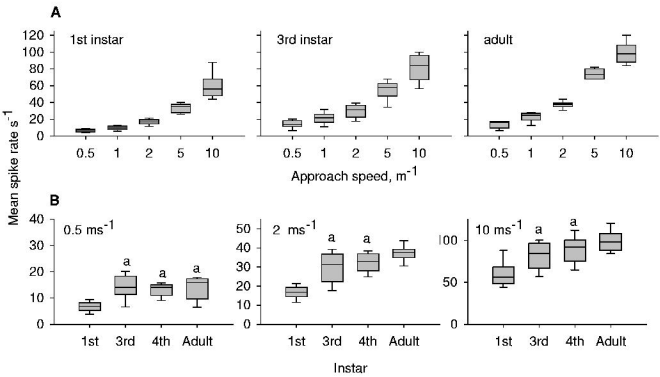
Fig. 7. Habituation and dis-habituation of DCMD response in 1st instars and in adults. The stimulus was a 60 mm diameter dark disk approaching at 2 ms^{-1}, repeated every 30 s. After the first 10 stimulus repetitions was a 10 minute interval (gap in graphs) before the 11th stimulus. The number of spikes 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 6 individuals are plotted in each graph.

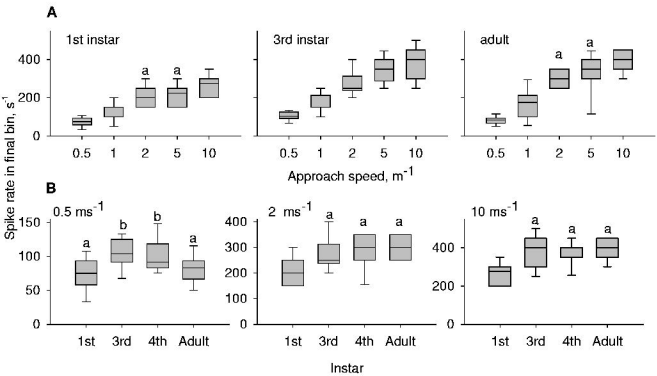
Fig. 8. Electron micrographs of synapses onto the LGMD in different instars. (A) Low power micrograph of a sagittal section of the lobula in a 1st instar locust showing the two crescents of processes belonging to the LGMD (black arrows) and LGMD2 dendritic trees (white arrows). (B-E) Details of synapses 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) 1st instar and (C) 4th instar. (D, E) Detail of dyadic synapse involving 2 profiles that are

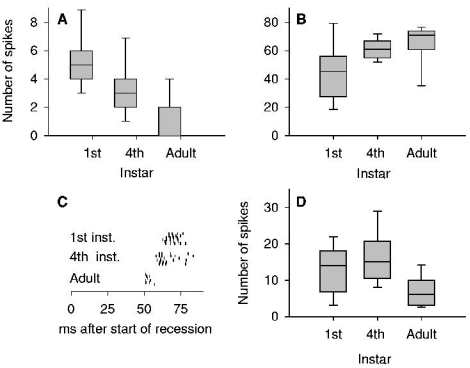
presynaptic to the LGMD and to each other in (D) 1st instar and (E) adult. Examples of large, electron-dense vesicles are enclosed in squares. Scale bars: (a), 10 µm; (b-d), 500 nm.

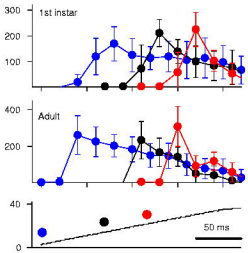

A
B
C
D
Number of facets
Facet width (µm)
100
80
60
40
20
0
1st
2nd
3rd
4th
5th
Adult
Instar

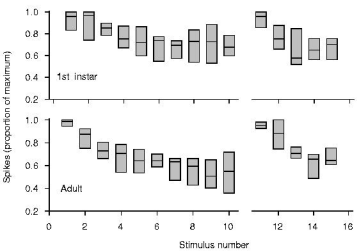

A
1st instar
30 µV
Adult
100 µV
Image (°)
40
0
0.5 s
1 ms-1
0.1 s
5 ms-1
B
1st
3rd
Adult
0.1 s
2 ms-1
C
1st Instar
3rd Instar
Adult
Spikes s-1
400
200
0
Image (°)
40
20
0
0.5 ms-1
1 ms-1
2 ms-1
5 ms-1
10 ms-1
2
0
0.5
0.0
0.4
0.2
0.0
0.2
0.1
0.0
0.1
0.0
Time to collision (s)

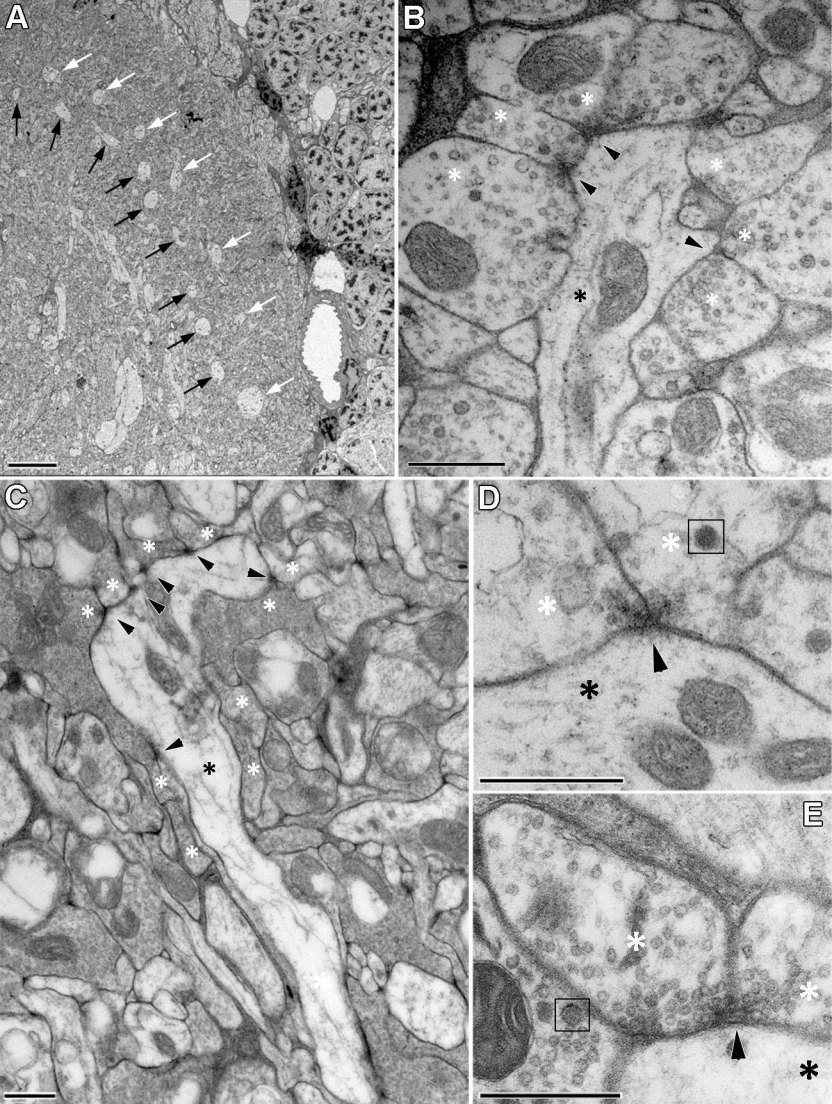

A
1st instar
3rd instar
adult
Mean spike rate s-1
Approach speed, m-1
0.5
1
2
5
10
B
0.5 ms-1
2 ms-1
10 ms-1
a
1st
3rd
4th
Adult
Instar


A
1st instar
3rd instar
adult
Spike rate in final bin, s-1
0
200
400
a
a
a
a
0.5
1
2
5
10
Approach speed, m-1
B
0.5 ms-1
2 ms-1
10 ms-1
0
50
100
150
200
300
400
a
b
b
a
a
a
a
a
a
1st
3rd
4th
Adult
Instar


A
Number of spikes
0
2
4
6
8
1st
4th
Adult
Instar
B
0
20
40
60
80
1st
4th
Adult
Instar
C
1st inst.
4th inst.
Adult
0
25
50
75
ms after start of recession
D
Number of spikes
0
10
20
30
1st
4th
Adult
Instar


1st instar
300
200
100
Adult
400
200
Spikes / s
40
0
Image size (°)
50 ms


Spikes (proportion of maximum)
1.0
0.8
0.6
0.4
0.2
1st instar
Adult
0
2
4
6
8
10
12
14
16
Stimulus number


A
B
C
D
E