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Olfactory system structure and function in newly hatched and adult locusts

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An important question in neuroscience is how sensory systems change as animals grow and interact with the environment. Exploring sensory systems in animals as they develop can reveal how networks of neurons process information as the neurons themselves grow and the needs of the animal change. Here we compared the structure and function of peripheral parts of the olfactory pathway in newly hatched and adult locusts. We found that populations of olfactory sensory neurons (OSNs) in hatchlings and adults responded with similar tunings to a panel of odors. The morphologies of local neurons (LNs) and projection neurons (PNs) in the antennal lobes (ALs) were very similar in both age groups, though they were smaller in hatchlings, they were proportional to overall brain size. The odor evoked responses of LNs and PNs were also very similar in both age groups, characterized by complex patterns of activity including oscillatory synchronization. Notably, in hatchlings, spontaneous and odor-evoked firing rates of PNs were lower, and LFP oscillations were lower in frequency, than in the adult. Hatchlings have smaller antennae with fewer OSNs; removing antennal segments from adults also reduced LFP oscillation frequency. Thus, consistent with earlier computational models, the developmental increase in frequency is due to increasing intensity of input to the oscillation circuitry. Overall, our results show that locusts hatch with a fully formed olfactory system that structurally and functionally matches that of the adult, despite its small size and lack of prior experience with olfactory stimuli.

The sense of smell, processed by the olfactory system, is essential for survival throughout the lifespan of most animals because it plays critical roles in behaviors such as foraging, avoiding predators, and finding mates[1–3]. During development an animal's relationship with the olfactory environment can profoundly transform as sources of, and preferences for, food frequently change[4], predation dangers can increase or decrease[5,6], and the onset of sexual maturity is often accompanied by increased sensitivity to pheromone cues involved in locating mates and courtship[7]. How the olfactory system is modified as an animal develops can reveal fundamental aspects of information processing by networks of neurons. The study of sensory processing in relatively simple animals such as insects has proved to be a useful strategy for answering basic questions about these phenomena[8–16].

Flies, moths, and other holometabolous insects undergo dramatic anatomical and physiological changes as they metamorphose from the larval to the adult stage[17]. In these animals, the diet and behavior of the larva can differ greatly from the adult[18]. These changes are reflected in the development of their nervous system[19,20]. For example, in the moth *Manduca sexta*, olfactory sensory neurons (OSNs) gradually begin to generate spontaneous and odor evoked activity[21,22], and glomeruli in the antennal lobes (ALs) start to form only in the 6th stage of development. In contrast, hemimetabolous insects like locusts and cockroaches hatch with bodies in many ways resembling the adult and later undergo incomplete metamorphosis[17]. Locust hatchlings, like adults, feed mainly on grass or other plants. Since locust hatchlings receive no parental care and may emerge at some distance from food sources, they need to forage independently to survive.

As locusts grow from hatchling to adulthood, they acquire experience with the olfactory environment, their body size increases by an order of magnitude[23], and their brain size doubles. We have shown recently that newly hatched locusts lacking any prior exposure to food are innately attracted by food odors[24]. Notably, earlier work in a related species, *Schistocerca gregaria*, established that newly hatched locusts have adult-like glomeruli in their ALs, and their OSNs and PNs respond by generating action potentials when odors are presented to the antennae[25].

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Here we provide an analysis of the structure and function of the first stages in the olfactory pathway in newly hatched and adult *Schistocerca americana*. Our quantitative results indicate that hatchling olfactory OSNs, local and projection neurons (LNs and PNs) function like those of adults: they respond with similar tunings to panels of odors with complex, coordinated, oscillatory activity patterns. Although smaller, LNs and PNs in hatchlings resemble those in adults. Notably, odor-elicited neural oscillations gradually increase in frequency as the locusts age, a change we found can be explained by increased excitatory drive as new OSNs are added to growing antennae. Overall, our results show that locusts hatch with a fully formed olfactory system that structurally and functionally matches that of the adult, despite its small size and lack of prior experience with olfactory stimuli.

Materials and methods

Animal subjects

The locust *Schistocerca americana* used in this study were reared in crowded indoor colonies in our lab. Mature females laid eggs inside cups filled with clean sand which were then incubated at 28.8 °C until the eggs hatched. We used locusts of either sex that were freshly hatched (1st instar), 10 days old (3rd instar), 20 days old (5th instar), and adult in our experiments.

Odor stimuli

We tested a large panel of different types of odorants including some associated with food sources and others that our locusts, raised in the laboratory, hadn't encountered before. Our goal was to test a range of different types of odor chemicals. Odorants were mixed in mineral oil and delivered by an olfactometer as square pulses within a constant stream of filtered and humidified air to the intact antennae[26]. The odor panel included 1-hexanol (hex), cis-3-hexen-1-ol (cis), 1-hexanal (hxa), 1-octen-3-ol (oct), cyclohexanone (cyc), furfuryl mercaptan (fur), ethyl mercaptan (eth), citral (cit), pentyl acetate (pen), camphor (cam), and wheat grass juice (wgj). The odorants were selected to include attractive food odors (wheat grass juice, 1-hexanol, cis-3-hexen-1-ol, pentylacetate), pheromone (hexanal), chemicals attractive to other insects (1-octen-3-ol is an attractant for mosquitoes), as well as chemicals that are known to be repellents (camphor, citral), or are not known to have relevance to locusts (cyclohexanone, furfuryl mercaptan, ethyl mercaptan). The concentrations of the odorants were adjusted in mineral oil to a partial vapor pressure of 0.024 (that of 1% hex); this procedure ensured similar numbers of molecules of each odorant were delivered. Wheat grass juice, which is a mixture of many volatiles, was not adjusted by vapor pressure. Grass juice was extracted from wheat grass (*Triticum aestivum*) grown in our laboratory as described[24]. All pure odorants were obtained from Sigma-Aldrich. In all experiments a 1 s pulse of the odor was delivered to an antenna of the locust.

Electrophysiology

Electroantennograms (EAG) were obtained by inserting a saline-filled sharp glass electrode (resistance ~ 10 MΩ) into the antenna and a silver-chloride ground electrode into one eye and were recorded by a DC amplifier (Model 440; Brown-Lee Precision, San Jose, CA). To make LFP and patch clamp recordings, a wax cup was built around the head to hold a bath of locust ringer solution, and a small section of the cuticle was then removed to expose the brain[26]. To record LFPs, a blunt glass, saline-filled electrode was placed above the calyx of the mushroom body. For whole-cell patch clamp recordings, patch electrodes were pulled from borosilicate glass capillaries by a pipette puller (Model P-97; Sutter Instruments), with the program parameters tuned to produce an electrode resistance around 6 MΩ when filled with locust internal solution[27] that was adjusted to osmolarity of about 350. Data were recorded through a MultiClamp 700A microelectrode amplifier and digitized via Digidata 1322A. Stimulus delivery and recordings were controlled by our custom LabView program running on a data acquisition PC.

EAG recordings and patch clamp recordings from PNs and LNs were obtained from 6 consecutive trials with each odor, and LFP responses were recorded in 10 trials with each odor. All experiments were conducted on awake animals.

Histology and imaging

In many experiments, either neurobiotin (Vector SP-1120) or lucifer yellow (Molecular Probes L453) was injected into recorded neurons at the conclusion of a session. Afterwards the brain was removed, fixed in 4% paraformaldehyde overnight, and then washed in phosphate buffered saline (PBS). When neurobiotin had been injected, the brain was transferred into PBS with 3% Triton X and kept for an hour to permeabilize the membrane, followed by conjugation of neurobiotin with streptavidin Alexa Fluor 488, 568, or 633 (Invitrogen S11223, S11226, or S21375, respectively). Because lucifer yellow is itself fluorescent, brains labeled with it did not require the conjugation step. All brains were then dehydrated by an ethanol series, cleared with methyl salicylate and imaged under a confocal microscope. Neuronal morphologies were traced from the confocal image stacks using NeuroLucida, Simple Neurite Tracer plugin in imagej, or neutube software.

Data analysis

Data analysis was carried out with custom written scripts in Matlab and Python. Signal processing and statistical tests were carried out using the functions from scipy and statsmodels packages in Python. All analysis scripts have been made available online.

Adult PNs have been shown to respond to odors with complex sequences of excitation and inhibition. As a measure of response complexity, we compared spiking epoch transitions in adult and young locusts. Spike rates in 400 ms wide bins were computed by simple histogram. The background firing rate (spontaneous activity before stimulus presentation) was averaged across trials to compute the baseline for each PN-odor pair. A PN was considered responsive in a given bin if its firing rate within 4 s from odor onset differed from the baseline

by more than one standard deviation. PN-odor pairs in which at least half the trials showed responses to odor puffs in the same bin were included for comparison of firing pattern complexity. We computed the firing rates in all 6 trials of these PN-odor pairs as the inverse of the inter-spike interval (ISI) and smoothed them with a Gaussian window with 33.33 ms standard deviation and 200 ms (6 standard deviations) width. A peak detection algorithm implemented in Python (https://gist.github.com/sixtenbe/1178136) was then used to identify local peaks in the firing rates. The peaks indicated excitatory epochs and the valleys between them indicated inhibitory epochs. Each valley counted for two transitions (excitatory-to-inhibitory and inhibitory-to-excitatory). If a peak appeared more than 200 ms after odor onset (or before the end of the response window, 4 s from odor onset), a spontaneous-to-excitatory (or excitatory-to-baseline) phase transition was counted. The distribution of transitions was compared between adult and hatchling locusts using Kolmogorov–Smirnov (KS) test, and showed no significant difference ($KS = 0.04$, $p = 0.84$).

To analyze LFP oscillations, signals were digitally bandpass filtered between 3 and 50 Hz. Spectrograms of the filtered signals were computed using a 0.5 s wide Hann window with 50% overlap between successive window positions. As successive segments of the antenna were removed, the spectrograms for trials 3–10 were averaged, and the frequency with the peak power within 1 s after odor onset was identified in this average spectrogram.

Results

Peripheral responses to odors are similarly tuned in hatchlings and adults

Antennae, the primary olfactory organs in insects, house OSNs in three different types of olfactory sensilla: basiconic, trichoidic, and coeloconic[28,29]. These OSNs initiate the olfaction process by generating spiking and inhibitory responses when odors enter their respective sensilla and bind to receptor proteins[30]. We characterized the tuning of the OSN population in hatchlings and adults with a panel of odorants by recording electroantennograms (EAGs), which assess the summed responses of OSNs across the antenna. The EAG signal reflects the superposition of the voltage generated by currents flowing through the cell membranes of the population of OSNs, and is widely used to assess the responsiveness of OSNs to odors[31–33]. We found that EAGs from naïve hatchlings and adults displayed similar patterns of tuning (Fig. 1). For example, hexanol, a prominent volatile released by wheat grass juice[34–36], evoked strong EAG responses in both adults and hatchlings, as did cis-3-hexen-1-ol, another wheat grass volatile known to evoke strong EAG response in other insects[36,37]. In contrast, we observed much weaker EAG signals in both hatchlings and adults to pentanol, octen-3-ol, citral, and cyclohexanone. Pairwise t-tests with multiple-comparison correction showed no significant differences in response to any given odor between the two age groups, and a linear mixed model fit, a statistic appropriate for analyzing samples of unequal size, confirmed this result. Pairwise p-value for t-tests: cyc 0.86, wgj 0.08, cit 0.02, oct 0.75, pen 0.37, hxa 0.90, cis 0.34, where alpha was 0.006 after Bonferroni's correction for multiple comparison (9 animals in each age-group; peak amplitude for hex, the normalizing value, was not compared; Table 1). For an ordinary least squares fit for the peak EAG amplitude as a function of age and stimulus using the formula "$amplitude = \beta_0 + \beta_1 \times age + \beta_2 \times stimulus$", where both age and stimulus were categorical variables, the coefficient

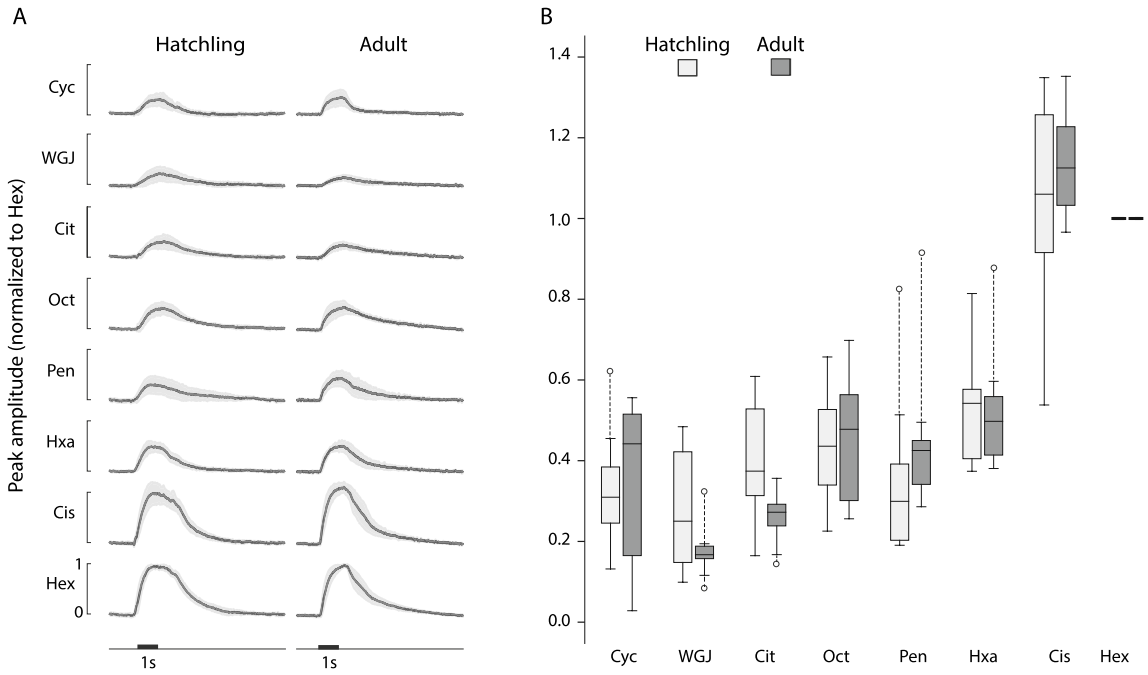

Figure 1. EAGs in adult (n = 9) and freshly hatched locusts (n = 8) show the ORN populations in hatchling and adults respond similarly to odors. (**A**) Average EAG responses to a panel of odors normalized by the maximum deflection elicited by hexanol in the same animal (black line). Gray band: 95% confidence level. Black horizontal bar: 1 s odor pulse. (**B**) Distribution of normalized peak EAG amplitude for each odor across animals. Horizontal lines in the middle: median; whiskers: 1.5 interquartile range; circles: values outside this range.

Odor	T statistic	p-value
cyc	0.18	0.86
wgj	− 1.89	0.08
cit	− 2.53	0.02
oct	0.33	0.75
pen	0.92	0.37
hxa	− 0.13	0.9
cis	0.88	0.39

Table 1. Quantitative comparison of EAG response peaks for various odors in freshly hatched and adult. All odors were tested 9 times in each age group.

β_1 for age was 0.004, and not significantly different from zero (p = 0.87, n = 135 observations). Thus, we found that OSNs in the hatchling antennae can respond to a panel of odors with similar relative intensity profiles as those in the adult. This result suggests that age or experience with the olfactory environment does not substantially change the tuning of this population of neurons at the sensory periphery to general, non-pheromonal odorants.

Hatchling and adult AL neurons have similar morphologies

To compare olfactory antennal lobe neurons in hatchlings and adults, we made intracellular recordings from them, injected dye, imaged the neurons with a confocal microscope, and traced and measured the neurons (see "Materials and methods"; statistical comparisons between hatchling and adult neurons are given in Table 2). The anatomy of olfactory PNs in hatchlings resembled scaled-down versions of those in the adult, with spoke-like radiating dendrites ending in glomerular tufts in the AL, an axon projecting to the calyx of the mushroom body and forming a nest-like branching pattern, and a neurite continuing to and terminating with a thicket of branches in the lateral horn (LH) (Fig. 2). In adults and hatchlings, the numbers of radiating dendritic branches formed by PNs in the AL were about the same (14.6 ± 2.7 in hatchlings, 15.4 ± 2.5 in adults, mean ± SEM, t(5) = 0.22, p = 0.83), and similarly, the number of glomeruli per PN, defined as fiber bundles at the terminals of PN dendrites, were about the same, too (13.16 ± 0.79 in hatchlings and 14.68 ± 0.70 in adults, t(19) = 0.34, p = 0.74). Although resembling those in the adult, neurons in the hatchlings were smaller. For example, the lengths of PN axons were smaller in hatchlings, (401.0 ± 3.3 µm in hatchlings and 768.3 ± 28.6 µm in adults, t(5) = 12.75, p < < 0.001). Notably, though, PNs scaled by brain size; when an individual neuron's branch length was normalized by the diameter of the antennal lobe (134 ± 5.8 µm in hatchlings and 288.26 ± 9.0 µm in adults), normalized hatchling and adult values were about the same (4.92 ± 0.44 in hatchlings vs 4.78 ± 1.26 in adults, t(5) = 0.10, p = 0.92). PN branches in the calyx and the LH scaled similarly with age (Table 2A).

Quantitative analysis of LNs in hatchlings and adults was challenging because these neurons have diverse and dense morphologies including numerous very fine processes, making them difficult to completely trace and compare with one another. Nevertheless, visual inspection of many filled neurons indicated that hatchling LNs, like PNs, are smaller versions of the adult form. Figure 3 shows representative examples of LN morphologies in hatchlings and adults. Throughout development these neurons typically span the entire AL with dense branches radiating from the center. Summary information for a sampling of LNs that could be traced is provided in Table 3.

(A)	Hatchling	Adult	T statistic	p-value	Hatchling normalized	Adult normalized	T statistic	p-value
Total length of PN branches in AL	659.7 ± 89.5	1376.7 ± 353.3	1.97	0.08	4.92 ± 0.44	4.78 ± 1.26	0.10	0.92
Length of PN axon	401.0 ± 3.3	768.3 ± 28.6	12.75	< < 0.001	2.99 ± 0.13	2.67 ± 0.06	2.23	0.06
Total length of PN branches in MB	2986.7 ± 76.6	6753.1 ± 862.5	4.35	0.002	22.29 ± 0.82	23.43 ± 2.56	0.42	0.68
Total length of PN branches in LH	693.5 ± 101.7	1584 ± 168.1	4.53	0.002	5.18 ± 0.79	5.48 ± 0.52	0.31	0.76
(B)	**Hatchling**	**Adult**	**T statistic**	**p-value**				
Diameter of AL	134 ± 5.8	288.26 ± 9.0	14.41	<< 0.001				
No. of dendritic PN branches in AL	14.6 ± 2.7	15.4 ± 2.5	0.22	0.83				
No. of PN branches in MB calyx	88.4 ± 2.2	93.0 ± 14.6	0.31	0.76				
No. of spines in PN branches in MB	170.0 ± 10.9	206.4 ± 35.5	0.98	0.36				
No. of PN branches in LH	24.4 ± 5.0	27.2 ± 3.2	0.47	0.65				
No. of spines in PN branches in LH	42.4 ± 8.1	56.6 ± 7.7	1.27	0.24				
No. of glomeruli in AL	13.6 ± 2.2	14.6 ± 2.0	0.34	0.74				

Table 2. Quantitative comparison of PNs in locust hatchlings and adults. Numbers indicate mean ± SEM. All lengths are in µm.

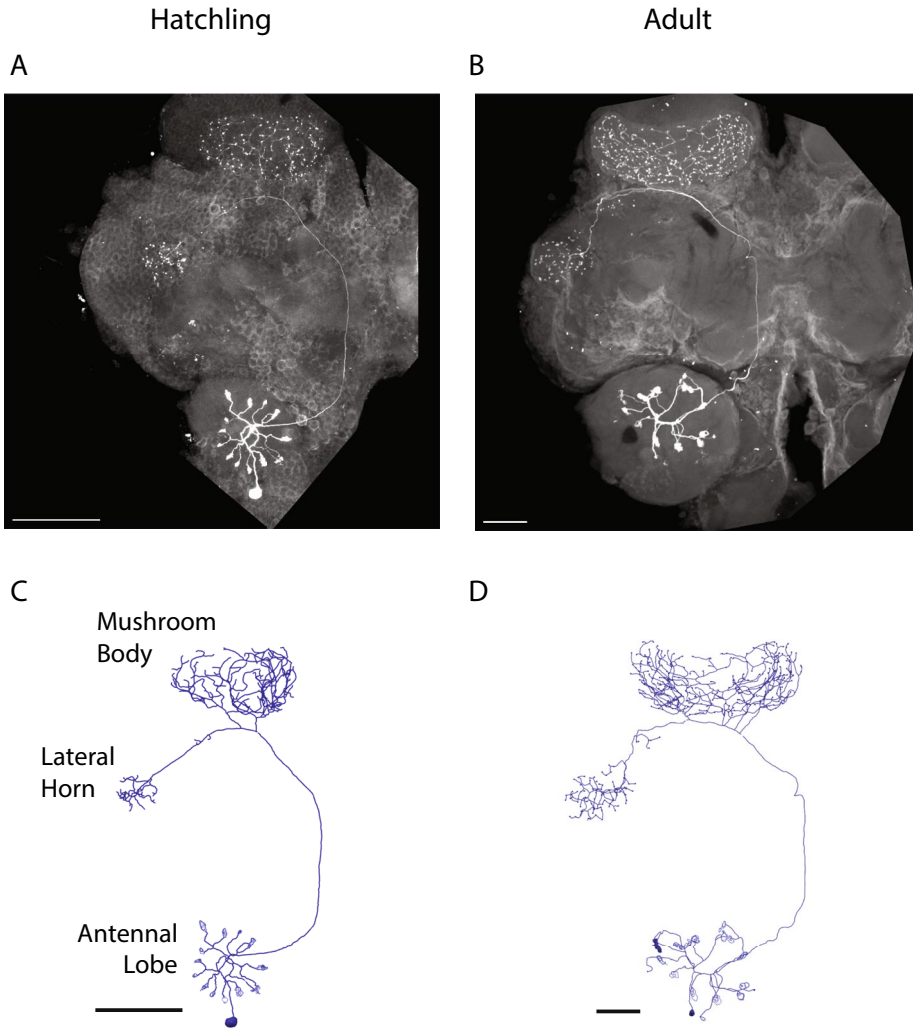

Figure 2. Morphologies of PNs in the locust AL are similar between hatchlings and adults. Shown are representative examples; see Table 2 for detailed comparisons. (**A**) Maximum intensity projections of hatchling and (**B**) adult hemibrains with dye-filled PNs. (**C**) Traced and reconstructed morphologies of the same hatchling and (**D**) adult PNs. Scale bar: 100 μm.

Hatchling and adult AL neurons respond similarly to olfactory stimuli

We next used patch clamp electrophysiology to systematically compare odor-elicited responses in AL neurons in newly hatched and adult locusts. We first recorded from 15 PNs each in hatchling and adult locusts as they responded to a panel of odors, each puffed into a constant airstream directed at the antennae (see "Materials and methods").

Neurons in hatchling and adult locusts responded to odors with comparable assortments of firing patterns including complex sequences of excitation and inhibition defined by peaks and troughs in the stimulus evoked firing rate. Figure 4A shows examples of these firing patterns as voltage traces obtained by intracellular recording; Fig. 4B shows the reliability of these responses with additional examples shown as spike rasters from 15 PNs each in adult and freshly hatched locusts responding to 8 odorants presented 6 times each. Notably, the spontaneous and odor-elicited firing rates of hatchling PNs were significantly lower than those of the adult (Fig. 4C); a 2-way ANOVA revealed a significant effect of age ($F = 35.87$, $df = 1$, $p << 0.001$) with no significant effect of odor or the interaction term, $p = 0.38$ and 0.80 respectively).

We compared the complexity of odor-elicited spiking patterns in PNs by computing the number of transitions between high and low firing rates within a 4 s time window beginning with odor onset and compared the distribution of the number of transitions across trials ($n = 414$ in adults and 398 in hatchlings; Fig. 5)[38]. A Kolmogorov–Smirnov test comparing two empirical distributions revealed no significant differences between the two age groups (KS statistic = 0.04, $p = 0.84$). This analysis suggests olfactory circuitry in the hatchling brain, though smaller in scale, can generate complex odor-elicited sequences of spiking responses like those observed in adult PNs.

We then made patch clamp recordings from LNs. In the locust, LNs do respond to odors, but unlike PNs, they do not generate action potentials. Instead, they generate patterns of membrane potential depolarization

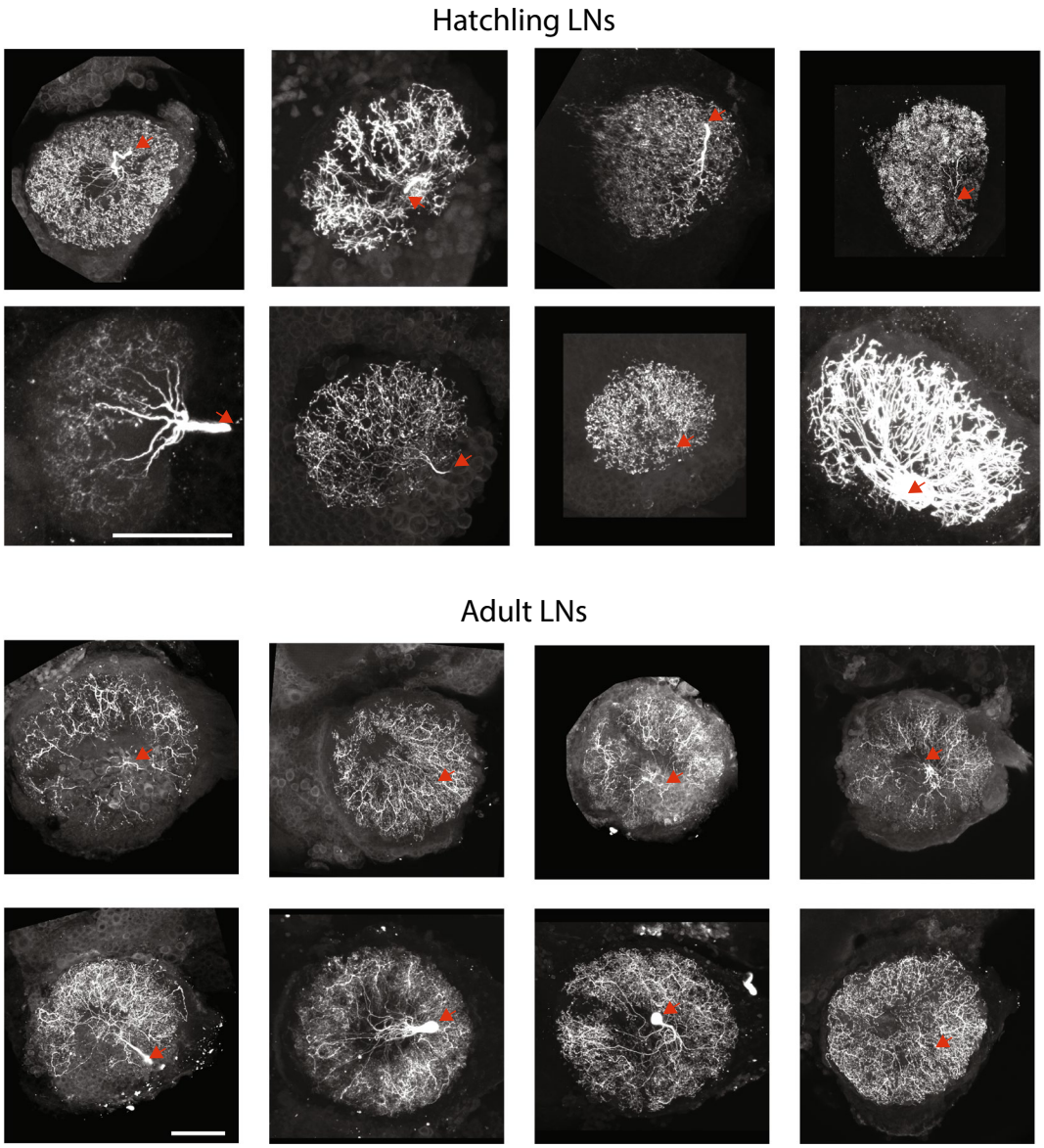

Figure 3. Representative examples of LNs in locust hatchlings (top) and adults (bottom). The images show the maximum intensity projections of the AL containing the dye-filled cells. Red arrow: location of the cell body which is often detached when the patch electrode is removed. Scale bar: 100 µm.

Hatchling				Adult			
Cell ID	No. of branches	Total length of branches	Normalized total branch length	Cell ID	No. of branches	Total length of branches	Normalized total branch length
1.11.2012	2158	24,791.00	185.01	2.6.2019	2239.00	60,726.00	210.66
3.13.2008	427	8127.00	60.65	2.9.2012	1304.00	41,684.00	144.61
12.28.2011	739	10,165.00	75.86	3.10.2011	1832.00	45,058.00	156.31
11.30.2011	2679	29,897.00	223.11	3.15.2011	1543.00	29,444.00	102.14
3.30.2011	766	12,452.00	92.93	3.24.2011	727.00	20,479.00	71.04
5.16.2011	1590	22,009.00	164.25				
AL diameter	134 ± 5.8				288.26 ± 9.0		

Table 3. Comparison of the morphologies of traced LNs. All lengths are given in µm. Where indicated, lengths were normalized by the average diameter of the antennal lobes.

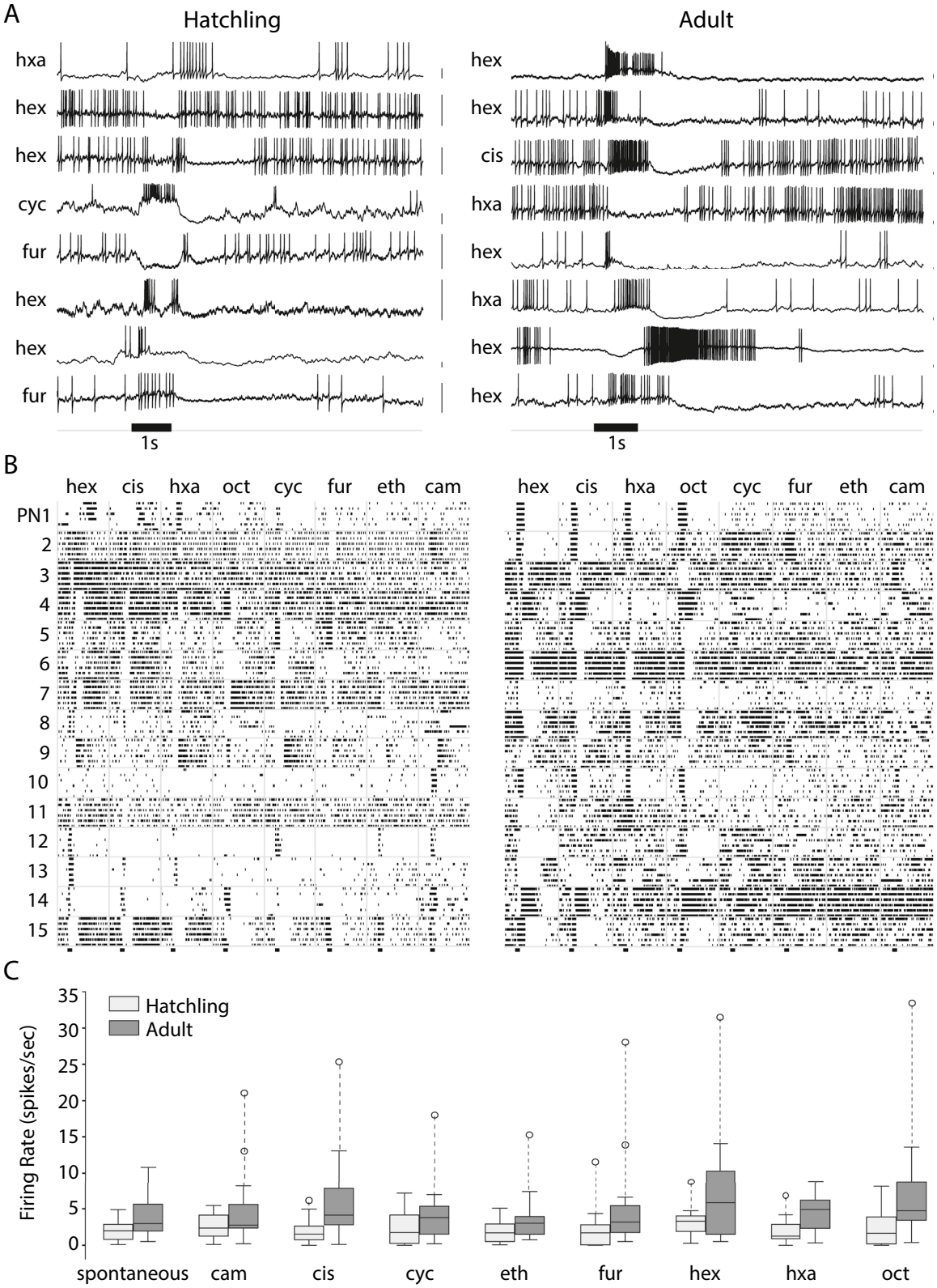

Figure 4. Odors elicit similar patterns of spikes in hatchling and adult PNs, although hatchling PN responses contain fewer spikes. (**A**) Representative example membrane potential records from PNs in the AL of both hatchling and adult locusts show combinations of excitation and inhibition that vary with the odor and the cell. Black horizontal bar: 1 s odor pulse. Odors: hex, hexanol; cis: cis-3-hexen-1-ol; hxa: hexanal; oct: octen-3-ol; cyc: cyclohexanone; fur: furfuryl mercaptan; eth: ethyl mercaptan; cam: camphor. Scale bars: 5 mV. (**B**) Additional examples: raster plots show spike trains in 15 PNs each in adults and hatchlings tested with 8 different odors. (**C**) Hatchling PNs generated fewer spikes spontaneously and in response to odors than adult PNs; see text for statistical analysis.

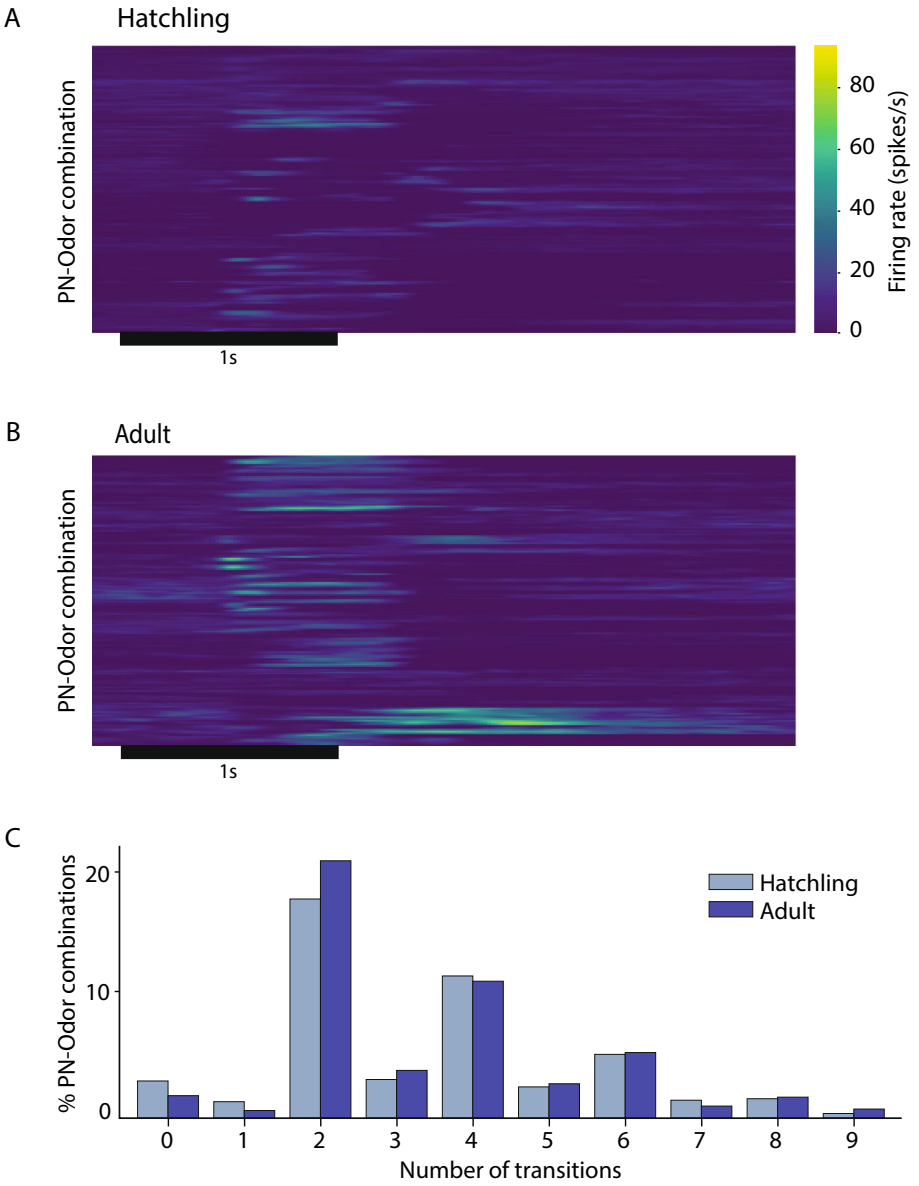

Figure 5. PNs in the hatchling antennal lobe produce complex multiphasic patterns of firing like those in the adult. (**A**) and (**B**) Transitions between excitatory and inhibitory epochs in odor-elicited responses of hatchling and adult PNs. Color indicates firing rate in spikes/s; black horizontal bar: 1 s odor pulse. (**C**) PNs in adults and in hatchlings did not differ significantly in response complexity as defined by the number of epoch transitions; see text for statistical analysis.

or hyperpolarization and small calcium spikelets[8,10]. Therefore, we characterized LN activity patterns based on membrane potential. The representative examples shown in Fig. 6 reveal LNs in newly hatched and in adult locusts display a similar array of response types to puffs of odorants.

Odor-evoked LFP oscillations are slower in hatchlings

In adult locusts, odor presentations are known to elicit the synchronized and oscillatory spiking of populations of PNs centered around 20 Hz, a phenomenon that can be assessed by local field potential (LFP) recordings made by extracellular electrodes in the AL and the MB[14,39]. To compare the coordination of AL neurons at different ages, we recorded odor elicited LFPs in the MBs of freshly hatched, 10 days old, 20 day old, and 35 day old animals, corresponding to first, third, and fifth instar larval and adult locusts. LFP oscillations were evident in the hatchlings, indicating that even at this early developmental stage the reciprocal circuitry between LNs and PNs in the AL necessary for the generation of oscillations was already present and appropriately tuned[40]. However, we found, over the course of development, that oscillations changed in four ways: the frequency, amplitude, and duration of the oscillations gradually increased; and the delay between odor delivery and oscillation onset increased (Fig. 7).

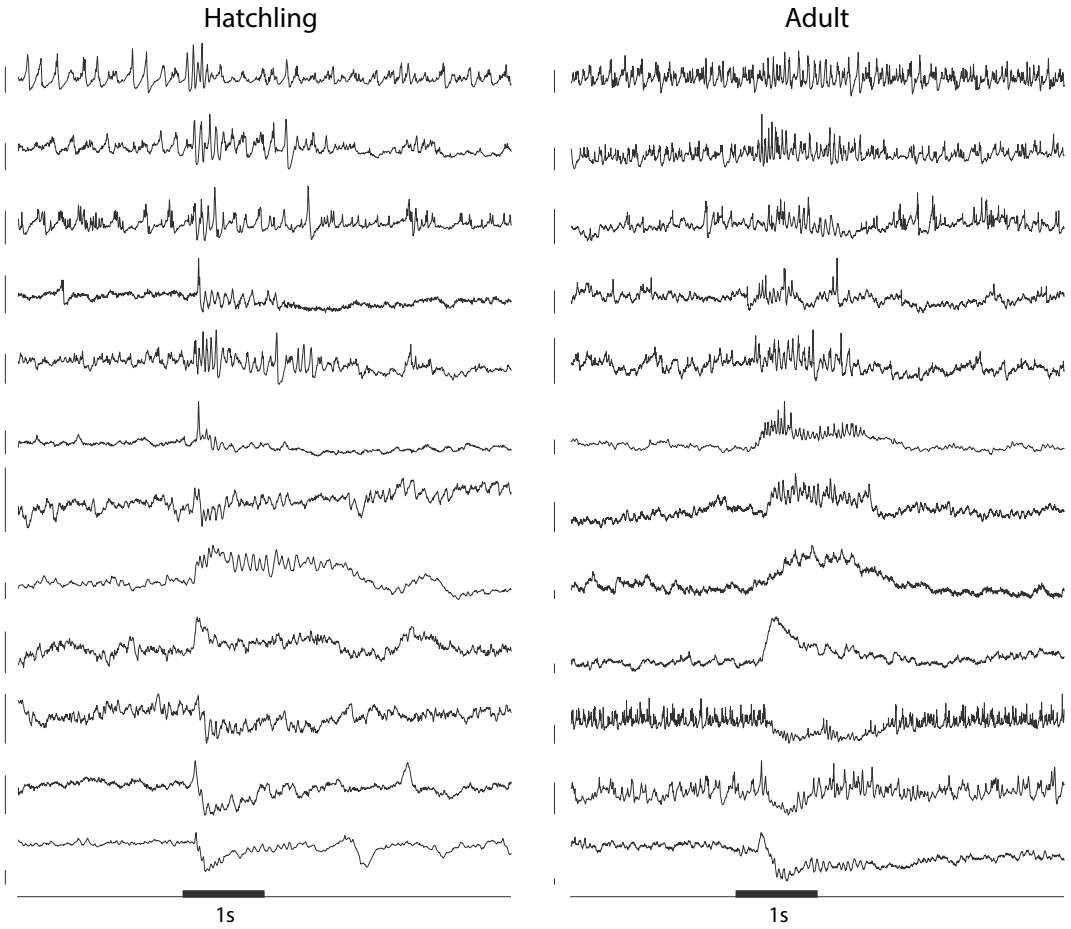

Figure 6. LNs in hatchlings and adults respond similarly to odors. Representative examples of membrane potentials of LNs in hatchling and adult locusts responding to 1 s odor pulses (black bar). Each trace is from a different LN. Locust LNs do not produce action potentials, but rather calcium spikelets, the rate of which may change upon odor presentation. LNs membrane potential can also oscillate, depolarize, hyperpolarize, or undergo a mixture of these. LNs in locusts of both age groups show similar responses. Scale bars: 10 mV.

Antenna length correlates with LFP oscillation frequency

What mechanism underlies these age-dependent shifts in odor-elicited oscillatory activity? Earlier work established that, in the AL, oscillation frequency is determined by the intensity of odor-elicited excitation from the afferent layer driving the oscillator circuitry[32]. Locusts add OSN-containing segments to their antenna as they develop, beginning with approximately 8,000 OSNs spread over 11–13 segments in hatchlings, and increasing to over 80,000 OSNs spread over 24–26 segments in adults[28,29,41–44]. The evident relationship between the developmental increase in the number of OSNs and the developmental increase in oscillation frequency led us to hypothesize that oscillation properties might be determined by the number of OSNs driving the oscillator circuit. To test this, in adult locusts, we trimmed successive antennal segments while recording odor-elicited LFPs in the MB ipsilateral to the antenna. As a within-animal control, we left the contralateral antenna intact while also recording odor-elicited LFPs from that side of the brain (Fig. 8A). We found that the LFP oscillation frequency measured in the ipsilateral MB significantly decreased as antennal segments were removed, whereas the frequency remained unchanged on the intact side (Fig. 8B, C). A linear regression (ordinary least squares fit) of the number of cuts to the peak LFP frequency during odor presentation showed significant negative correlation with the number of cuts for ipsilateral side (coefficient = -0.52 ± 0.11, $R^2 = 0.258$, $p << 0.01$), but not for the contralateral side (coefficient = 0.06 ± 0.079, $R^2 = 0.023$, $p = 0.445$). To confirm that this reduction of LFP frequency was due to the progressive reduction in number of OSNs and not by gradual systemic degradation following injury, we also performed quicker experiments where we directly cut the antenna after the 7th segment (leaving 19 segments intact), and then after the 10th segment (leaving 16 segments intact, Supplementary Fig. 1); this approach yielded the same result. As antennal segments were removed, the amplitude of the LFP oscillations measured in the ipsilateral (but not contralateral) MB gradually decreased, but this change in intensity would not affect the analysis of frequency.

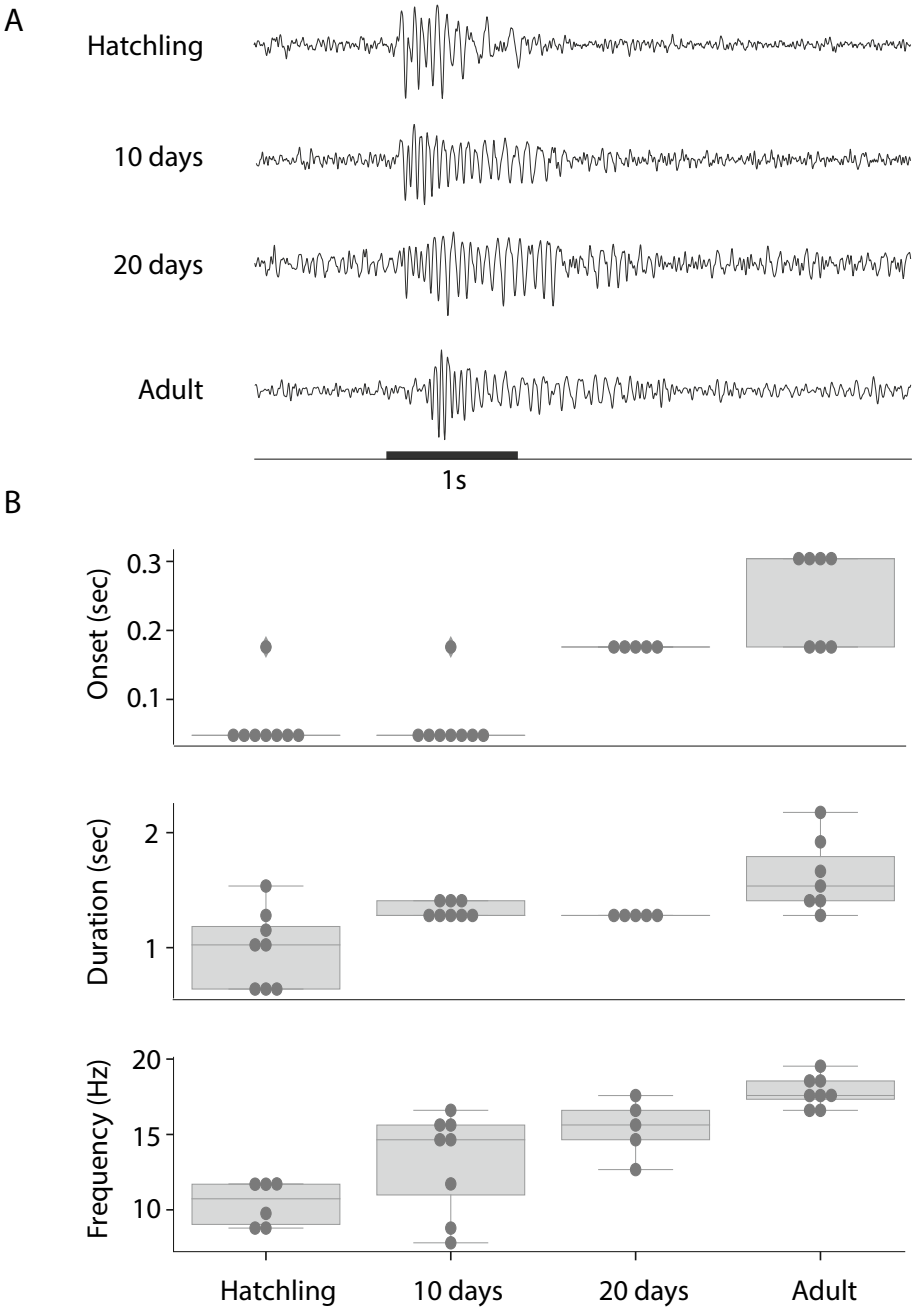

Figure 7. In response to odor puffs, hatchlings generate lower-frequency LFP oscillations than adults. (**A**) Representative LFP oscillations recorded in the MBs of hatchling, 10 day old, 20 day old, and adult locusts; Black horizontal bar: 1 s pulse of 100% hexanol. (**B**) properties of LFP oscillation across these age groups. Boxes cover first to third quartile, interior line: median, whiskers: 1.5× inter-quartile range, circles: individual datapoints, and diamonds: outliers.

Discussion

Locusts have served as useful models to understand how olfactory stimuli are encoded by neural circuits[8–16,45]. Here we investigated the development of peripheral stages of the olfactory pathway by comparing naïve, freshly hatched, and adult locusts. One might hypothesize that developmental growth or experience with the olfactory environment would substantially alter the structures and functions of the locust's olfactory circuitry. However, at the level of confocal microscopy, hatchling olfactory neurons resembled those of the adult, just smaller, scaled by overall brain size. EAG recordings from hatchling and adult antennae revealed OSNs responded with similar sensitivities to a panel of odors across development. A larger odorant panel including pheromones, and recordings from individual OSNs might reveal more specific experience- or age-dependent changes in tuning.

Earlier work has shown that in hemimetabolous insects like locusts, the main structures of the brain, including the antennal lobes and their full complement of glomeruli, are well formed upon hatching[46–50], although more

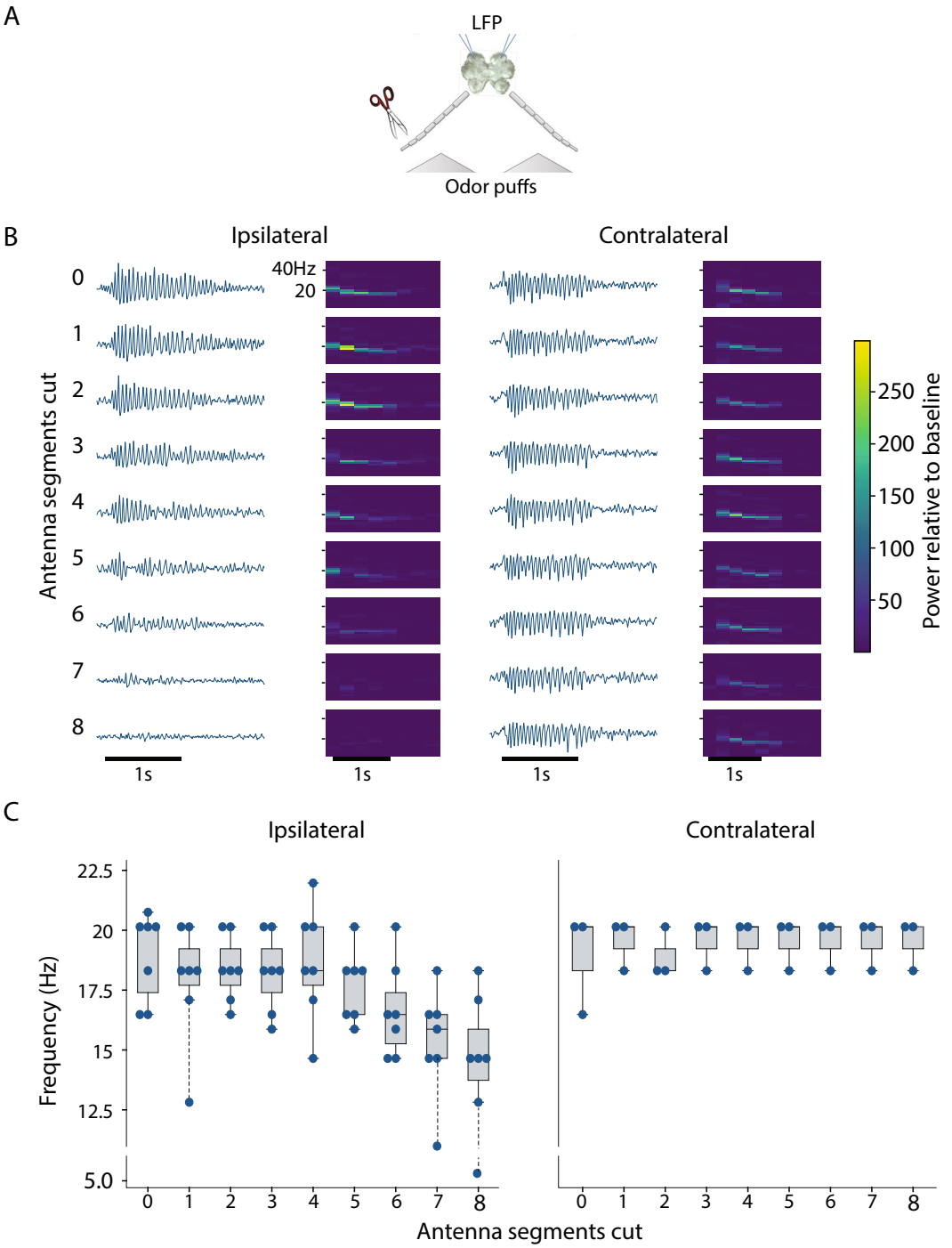

Figure 8. LFP oscillation frequency correlates with the number of ORN-containing antennal segments; removing segments from adults reduced oscillation frequency. (**A**) Experiment scheme for trimming antennal segments from one side of an adult animal while recording LFPs from the ipsilateral MB; the contralateral antenna and MB served as a within-animal control. (**B**) Odor-evoked LFPs and corresponding spectrogram in the MB ipsilateral (left) to the trimmed antenna (left half) and the same in the contralateral AL (right) of a single animal. Black horizontal bars: 1 s odor presentation. (**C**) As antennal segments were trimmed, LFP frequency in the ipsilateral MB decreased (left), but the frequency in the contralateral MB remained unchanged (right) (n = 7 antennae in 4 animals). Blue dots: individual data points; boxes cover first to third quartile, interior line: median, whiskers: 1.5× inter-quartile range; dotted line: outliers. Each data point shows the frequency with the peak power sampled 2–3 s in the averaged spectrogram of trials 3–10. First two trials were excluded because oscillations usually develop after 2 odor presentations. Odorant: hexanol.

subtle changes, such as synaptic rearrangement, have been documented throughout development[51]. In an earlier study of locust species *Schistocerca gregaria*, Anton and colleagues[25] found that, as the animals matured, the fraction of PNs responding to aggregation pheromone components increased, and the overall response profiles of PNs became less selective. In juvenile locusts these authors also observed that PNs responded to odors with patterns including inhibition and excitation. Our analysis of the temporal dynamics of these responses revealed that olfactory neurons in hatchlings responded to odors very much like those of the adults: PNs fired spikes in similarly complex sequences of excitation and inhibition and coordinated in oscillatory synchrony, and, across development, LNs, which do not generate sodium action potentials in locusts, showed very similar patterns of calcium spikelets atop excitatory and inhibitory membrane fluctuations.

Notably, though, PNs in hatchlings showed less spontaneous and odor-elicited spiking than those in adults, and odor-elicited oscillations were slower in frequency in the hatchling. A previous study[32] demonstrated that the olfactory sensory neurons (OSNs) in the moth *Manduca sexta* underwent sensory adaptation when exposed to lengthy olfactory stimuli. Adaptation in OSNs led to a reduction in the strength of their response to odors and a corresponding decrease in the frequency of LFPs. (This downward frequency shift can also be observed here in spectrogram traces in Fig. 8B). A computational model incorporating OSN adaptation established this intensity reduction slowed the LFP frequency by decreasing excitatory drive to the oscillator circuitry in the AL[32]. Here, we hypothesized that LFP oscillations are slower in hatchlings than adults because hatchlings have fewer OSNs, collectively contributing less excitatory drive to the AL oscillatory circuit. To test this idea, we sought to return the adult antenna to the hatchling state by removing successive segments, and thus OSNs, from the antenna. Consistent with our hypothesis, we found LFP oscillation frequency decreased as segments of the adult antenna were removed. We speculate the relatively weak excitatory drive from the hatchling antenna may also underlie the lower levels of spontaneous and odor-elicited activity observed downstream in hatchling PNs (Fig. 4C), as spontaneous activity in PNs has previously been shown to arise almost entirely from OSN spiking[52].

In postembryonic rats, odor elicited oscillations in the olfactory cortex (analogous to the insect mushroom bodies[53], undergo a frequency shift during development similar to what we characterize here in locusts. In rats, oscillations shift from 12 to 15 Hz peak in P0-P12 animals, to ~ 25 Hz by P20[54]. However, the frequency transitions in locusts and rats are caused by very different processes: in locusts, the transition is due to an increase in the number of OSNs, while in rats it reflects changes in centrifugal input from the cortex to the olfactory bulb[54].

In principle, the lower oscillatory frequency and amplitude we observed in hatchlings could be due to relatively strong inhibition in the hatchling circuitry that decreases with age. Such a mechanism appears to occur in rats, where inhibition within the olfactory bulb is stronger in neonates than in adults[55]. Our results show that LNs, the inhibitory neurons of the AL, appear to be fully formed at hatching: they are anatomically similar to those in adults, and show similar physiological responses to odorants. The inhibitory transmitter GABA released by LNs onto PNs causes both fast inhibition (mediated by GABAa-type receptors) underlying oscillations, and slower inhibition (mediated by GABAb-type receptors), shaping the overall excitatory and inhibitory patterns of odor-elicited responses of PNs[10]. Our finding that the overall response patterns of PNs are similar in hatchlings and adults suggests they receive similar input from LNs. Together, these results suggest that, unlike the rat olfactory bulb, the level of inhibition in the locust AL remains stable throughout development.

In many hemimetabolous insect species, although the central brain is fully formed at hatching, the number of peripheral sensory neurons increases through development; visual[56,57], auditory[49,58,59], wind-sensitive[50,60], and olfactory sensory neurons[42] have all been observed to increase in quantity throughout postembryonic life. As additional sensory neurons are added in these systems, the combined strength of their outputs onto follower neurons increases[49,61]. This matches our observations in locusts, where increased odor elicited EAG amplitude occurs together with increased spontaneous and evoked firing in PNs. The increased sensory drive onto oscillator circuits in the AL also led to increased oscillatory frequency measured in the LFP. This phenomenon may be analogous to burst frequency in motor circuits organizing wing beats in the cricket, which increases through development as sensory input increases[62].

Notably, as we observed in the elaborate firing sequences of PNs and the oscillatory synchronization of AL neurons, the response properties of olfactory neurons remain consistent throughout development, despite dramatic changes in both the intensity of sensory drive[60], and the size of the follower neurons[56,57]. In cockroaches, growing neurons adjust the lengths and diameters of their processes to maintain consistent electrical properties[63], whereas crustacean neurons appear to achieve functional stability throughout development by adjusting membrane conductances[64]. It would be interesting to determine how the locust antennal lobe generates consistent output despite dramatic developmental changes in size and input.

In other species, the olfactory system has shown only a limited ability to change in response to early life exposure to odorants, including maintaining memories formed during development[65–67]. However, it is not clear that such plasticity, shown to occur under laboratory conditions, is functionally relevant to animals under more natural conditions[68].

In summary, our results show that locusts hatch with a well-developed olfactory neural circuit—one with neurons resembling and functioning like those of the adult. Despite their small size and their lack of prior experience with odorants, OSNs in hatchlings respond with similar tunings to odorants, and second order neurons display complex temporal patterns of activity organized into oscillating ensembles in response to olfactory stimulation. These results are consistent with our earlier finding that newly hatched locusts are innately attracted to the odors of food sources[24]. Fully assembled olfactory systems help enable hatchling locusts and other hemimetabolous insects to interact effectively with their environments.

Data availability

All data generated by this study are available here: Zenodo (https://doi.org/10.5281/zenodo.10213707).

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Author contributions

K.S. and conducted experiments and analyzed data, N.G. designed the project, conducted experiments, analyzed data, and and revised the manuscript, S.R. analyzed data and wrote the manuscript, Z.A. conducted experiments and revised the manuscript, and M.S. designed and supervised the project and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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