Figures and figure supplements

Airflow and optic flow mediate antennal positioning in flying honeybees

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Figure 1. Antennal responses to changing airflow. (A) Inter-Antennal Angle (IAA) is measured by digitizing 4 points (red circles) on the antennae in all frames. (B) Groundspeed is obtained by tracking Point#2 from A which is static relative to head. It is the vector sum of bee-controlled airspeed and random environmental windspeed. (C) Linearly changing airflow stimulus to tethered bees. (D) Randomized airflow stimulus to freely-flying bees (Reshuffled). (E) Randomized airflow stimulus to tethered bees (Reshuffled).
Figure 1 continued

experimenter-controlled windspeed. (C) Top panel: Response to ambient airflow in tethered bees. Tethered bees were positioned at the centre of the wind-tunnel test section, and facing upwind and the windspeed was linearly varied from 0 to 5.5 m/s. Two high-speed cameras positioned dorsally and laterally filmed the bees at 500 fps. Bottom panel: Normalised IAA response as a function of airspeed (or windspeed). We normalized IAA values between 0 (defined as the mean of values between 0 and 1.5 m/s) and 1 (defined as the mean of values between 3 and 5.5 m/s). Between 0–1.5 m/s and 3–5.5 m/s, normalized IAA did not significantly change (p>0.05, Moore’s test). Between 1.5 and 3 m/s, normalized IAA sigmoidally decreased with airspeed, changing with each step (*p<0.0001, Moore’s test, N=10; each colour represents one individual) relative to the preceding and succeeding values. Non-normalized data in Figure 1—figure supplement 1B. Here and everywhere we have plotted the means, and the error bars indicate the standard deviation of the mean. (D) Top panel: IAA response of freely-flying bees to ambient airflow. Bees were trained to enter the wind tunnel through a side-door and fly upwind past the test section to a feeder. High-speed cameras placed and operated as in (C) filmed their IAA response. Bottom panel: Normalised IAA response as a function of airspeed in free flight. Between airspeeds of 1.5 to 3 m/s, IAA changed significantly (*p<0.0001, Moore’s test, N=10) relative to the preceding and succeeding values, but saturated at airspeeds less than 1.5 m/s and greater than 3 m/s. Non-normalized data in Figure 1—figure supplement 1D. (E) IAA responses to random sequence of ambient airflow in tethered bees. We presented the bees with airflow values between 0 and 5.5 m/s in a random sequence and plotted the normalised IAA response as a function of airspeed values reshuffled to lie in increasing order. As in 1C, IAA sigmoidally decreased with airspeed, significantly changing between 1.5 and 3 m/s (*p<0.0001, Moore’s test, N=5; each colour represents one individual). From 0–1 m/s and 3.5–5.5 m/s, the normalized IAA did not significantly change. For non-normalized data, see Figure 1—figure supplement 1C. (F and G insets) Experiments with sham-treated and JO-restricted bees. Red crosses indicate the location of applied glue, and blue arrow the presence of airflow. (F) Normalised IAA vs. airspeed in sham-treated bees. Sham-treated bees (N=5) show responses similar to untreated bees (compare with Figure 1C). Each coloured line represents an individual bee (Non-normalized data in Figure 1—figure supplement 1H). Change in IAA (Figure 1—figure supplement 1H) is in the same range as untreated bees (compare with Figure 1—figure supplement 1B). (G) Bees with restricted JO do not respond to airspeed change. When the pedicel-flagellum joint is glued, IAA does not vary significantly with changing airspeed (*p>0.1, Moore’s test, N=7). Each colour represents an individual (Non-normalized data in Figure 1—figure supplement 1I).

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Figure 1—figure supplement 1. Antennal responses to changing airflow. (A) IAA values at 0.5 m/s airflow for a tethered (blue plot) and freely-flying honeybee (red plot). IAA values are maintained for all 250 frames for tethered bees (Mean=96°, S.D.= 0.8°) and freely-flying bees (Mean=85°, S.D. = 3°) (B) Response of tethered flying bees to linearly changing airflow cues in the wind tunnel. The response is sigmoidal between 1.5 and 3 m/s. Each colour represents the same individual as in Figure 1C. Solid black lines represent the mean IAA at which saturation occurs. (C) Randomized airflow stimulus to tethered bees (Reshuffled). (D) Randomized airflow stimulus to freely-flying bees (Reshuffled). (E) Comparison with previous data. Current study (blue plot) and Heran (1957) (black plot) data are shown. (F) Ground speed (m/s). (G) Windspeed = Airspeed (m/s). (H) Sham treated. (I) JO blocked.
Figure 1—figure supplement 1 continued

occurs. Normalized data shown in Figure 1C. (C) Response of tethered flying bees to randomly changing air flow cues in the wind tunnel. Each bee received a different sequence of random airflow value, and the x-axis was reshuffled in a linearly increasing fashion. The response is similar to that seen with linearly changing cues (Figure 1—figure supplement 1B). (D) Response of freely flying bees to changing air flow cues in the wind tunnel. The response is sigmoidal between 1.5 m/s and 3 m/s. Each colour in the plot represents the same individual as in Figure 1D. (E) Comparison of antennal responses to airflow from Heran (1957) against data from Figure 1—figure supplement 1B. (F) Groundspeed of a freely flying bee as it flies against increasing windspeeds, shown as notched plots. Honeybees maintained a constant groundspeed at approximately an average of 0.43 m/s with increasing windspeeds, consistent with previously reported values (Barron and Srinivasan, 2006). (G) Interantennal angle as a function of the groundspeed of the freely flying bees. Different colours indicate different individuals. (H and I) Raw data for response of sham-treated tethered bees to changing airflow and JO-glued bees to changing airflow.

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Figure 2. Antennal responses to changing temporal frequency of optic flow. (A) IAA response to optic flow in tethered bees. Bees were positioned central and at 10 cm from the two screens separated by 2 cm at the apex. (B) Sample IAA response to linearly increasing (red) or decreasing (green) optic flow. (C) Optic flow response to increasing frequency. (D) Optic flow response to decreasing frequency. (E) Reordered response to optic flow. (F) Normalised IAA response to optic flow. (G) Normalised IAA response to optic flow. (H) Normalised IAA response to optic flow. (I) Normalised IAA response to optic flow. (J) Normalised IAA response to optic flow.

Figure 2 continued on next page.
optic flow stimulus. (Top panel) Stimulus comprises of a visual grating moving from front to back at temporal frequencies ranging between 0–25 cycles/s (cps), in steps of 1 cps. Each step lasts for 1 s (blue). The IAA response to optic flow saturates beyond the threshold of 10 cps (grey bar; also Materials and methods for threshold calculation). (C–E) IAA Response to randomized optic flow values. The above honeybee (the individual shown in Figure 2B–E, bee #1), was presented with randomized optic flow stimulus (C). The precise temporal sequence of randomized stimulus varied between bees. Dotted line shows the 10 cps threshold. Each 1 cps step lasts for 1 s from 1–25 cps. IAA response is plotted in two ways: the IAA response to the randomized stimulus (D, olive green bars), and the response reshuffled in increasing order of temporal frequencies (E, blue bars). The peak at 20 cps is due to the sharp IAA readjustment at stimulus onset. Grey lines between C and D indicate step transitions in temporal frequency values. IAA is predicted to change when stimulus changes occur below or across threshold. Predicted changes in IAA are marked by black circle and no change by white circle. IAA is predicted to decrease (down arrowhead) when optic flow changes from high-to-low under or across threshold, and increase (up arrowhead) when values change from low-to-high under or across threshold. Changes in temporal frequency above threshold (horizontal line) yield no antennal response. In both tests, correct predictions are marked by green and wrong predictions by red circles, and fraction of correct predictions vs. total number indicated beside each test. In this instance, we correctly predicted when IAA would change with 83% accuracy (19/23=0.83), and the direction of its change with 74% accuracy. (F, G) Summary figures showing the IAA response of three individuals (shown in Figure 2B–E, Figure 2—figure supplement 1A–D and E–H) (F) IAA responses for optic flow rates increasing linearly from 0 to 25 cps. The mean response from all three bees is shown in bold red and the spread of the data is shown in the background of the plot. (G) IAA responses for optic flow rates decreasing linearly from 25 to 0 cps. The time axis is shown in the opposite manner because the first optic flow rate that is presented to the tethered bee is 25 cps. The mean of the IAA responses from all three individuals is shown in bold green and the spread of the data shown in the background. (H) IAA response to sinusoidal moving visual gratings. Sinusoidal moving grating (orange, amplitude=1.8 cps; period =10 s) stimulus elicits correlated IAA responses (blue). (I) Normalized IAA response vs. optic flow rate between 0 and 1.8 cps. Each step increase of 0.3 cps in the optic flow rate elicits significant changes in IAA (*p<0.05, Moore’s test, N=9). IAA values were normalized relative to maximum and minimum values for each individual (raw data in Figure 2A). (J, inset) IAA Responses of the bees with restricted Johnston’s organs. Red crosses indicate glue location. To the set-up in Figure 2A, we added a ducted fan to provide collimated airflow. Red dot indicates absence of airflow, and blue arrows indicate presence of optic flow. (J) Normalized IAA response to changes in optic flow. IAA changes with change in optic flow (*p<0.0001, Moore’s test, N=7). In Figure 1G and 2J, the same individuals share color. Normalisation procedure is the same as in Figure 2G and the raw data has been shown in Figure 2—figure supplement 2B. 

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Figure 2—figure supplement 1. Antennal responses to changing optic flow rates. Data for two additional individuals. Figure 2—figure supplement 1A–D show data from one individual and Figure 2—figure supplement 1E–H for a second individual. (A and E) IAA responses of linearly changing optic flow rates in tethered bees. Data from two bees showing the IAA response to linearly ascending (red plot) and linearly descending (green plot) temporal frequencies between 0 and 25 cps. The step changes in optic flow rates have been shown in blue at the top. In both cases, the IAA response to changing rates of optic flow saturates after 14 cps. (B and F) Optic flow stimulus provided to the tethered bees. Each optic flow stimulus lasted for 1 second with a 0.1 second inter-stimulus interval. (C and G) Gaussian kernel with central IAA degree values and their standard errors. (D and H) Reordered response.
Figure 2-figure supplement 1 continued

s. Black dotted line indicates the threshold after which the response to changing optic flow saturates (threshold calculated from Figure 2-figure supplement 1A and E). Grey lines indicate the transition from one value of temporal frequency to the next. (C and G) IAA responses of the bees to randomly changing optic flow rates between 0 and 25 cps. Olive green bar graphs show the actual response of the bees to the stimulus pattern shown in Figure 2C and Figure 2-figure supplement 1H respectively. The threshold in both cases is 14 cps. Predictions and scores are described in Figure 2C,D. The score for predicting motion was 17/23=0.74 (74% accuracy) and the score for prediction change in direction of antennal motion was 11/23=0.48 (48% accuracy, Figure 2-figure supplement 1C) and 18/23 = 0.78 (78% accuracy) and 17/23=0.74 (74% accuracy, Figure 2-figure supplement 1G). (D and H) IAA responses to randomized optic flows between 0 and 25 cps in two bees. IAA responses of the bee (blue) are reordered and represented against optic flow. Bar graphs show the mean and standard deviation of the IAA response to each optic flow rate value. The first optic flow experienced by the bee in Figure 2-figure supplement 1B was 8 cps and in Figure 2-figure supplement 1F was 5 cps. In both these cases, the IAA at the first optic flow rate stands out from the rest of the dataset. No significant trend emerges from the IAA response of the bee to randomised optic flow rates.

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Figure 2—figure supplement 2. Antennal responses to changing optic flow rates. (A) Raw data for response of tethered flying bees to changes in optic flow. Each colour represents in the plot the same individual as the corresponding colour in Figure 2I. (B) Raw data for response of JO-glued bees to changing optic flow cues. Normalised data are shown in Figure 2J.
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**Figure 3.** IAA responses to combinatorial stimuli. (A) IAA response curve of a tethered bee to changing airflow (blue) and optic flow (red). Regime A represents the combination of low optic flow rate and airspeed (temporal frequency=0.3 cps; airspeed=0.5 m/s); Regime B represents combination of decreasing airspeed and optic flow; Regime C represents increasing airspeed and optic flow.
intermediate optic flow rate and airspeed (temporal frequency = 0.9 cps; airspeed = 2.5 m/s), and Regime C represents high optic flow rate combined and airspeed (temporal frequency = 1.8 cps, airspeed = 4 m/s). The dashed line (grey) at the top of the plot indicates the IAA response at 0 optic flow. (B, C) Representative data from a single individual when values transition from Regime B→A (B) or from Regime B→C (C). IAA responses to airspeed (blue), and optic flow (red), and combination of airspeed and optic flow (green) plot. (D, E) ΔIAA response to step changes in airspeed, optic flow or combined cues. We have represented the mean ΔIAA values as notched plots. The extent of box shows the inter-quartile range and the lower and upper bounds of the box represent the 25th and the 75th quartiles. The line in the box represents the median of the data and there is a ‘notch’ around this median for easy comparison of the notched boxes with each other. If the notches of two boxes do not overlap, their medians are statistically significantly different from each other. The red crosses indicate the outliers in the data. The whiskers extend to the most extreme data point that is not considered to be an outlier. * represents statistically significant difference (*p<0.001, Moore’s test, N=8). Mean ΔIAA is significantly different from a hypothetical mean of zero (*p<0.05, ANOVA; post hoc Tukey’s HSD test, N=8) when only airspeed or optic flow are varied, but not when a stimulus combination is co-varied (*p>0.5, ANOVA; post hoc Tukey’s HSD test, N=8).

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**Figure 4.** IAA responses to changes in optic flow in presence of constant airflow (A, B), and to changes in airspeed in presence of constant optic flow cues (C, D). (A) IAA responses to optic flow rates varying from 0 to 1.8 cps in presence of still air (IAA_{Op}; solid green line) vs. steady 1 m/s airflow (IAA_{W+Op}; dotted green line). Mean difference between IAA_{Op} and IAA_{W+Op} is significant at each optic flow rate value (*p<0.0001, Moore’s test, N=8). (B) We have represented the mean ΔIAA values as notched plots. The range of ΔIAA (=IAA_{W+Op} - IAA_{Op}) is not significantly different over various optic flow values. Each mean ΔIAA is significantly different from a hypothetical mean of 0 (*p<0.05, ANOVA and post hoc Tukey’s HSD test, N=8). (C) IAA responses to varying airspeed at 1, 2.5 and 4 m/s in presence of no optic flow (IAA_{W}; solid red line; top left panel) vs. steady optic flow of 1.8 cps (IAA_{W+Op}; dotted red line; top right panel). Again, mean IAA values at each rate of optic flow are significantly different (*p<0.0001, Moore’s test, N=9) for the two cases. (D) The range of ΔIAA plotted as notched plots are again not significantly different across the various airspeeds, but each mean ΔIAA value is significantly different from a hypothetical mean of 0 (*p<0.05, N=9, ANOVA and post hoc Tukey’s HSD test). (E) A general model of the antennal positioning response to airspeed and optic flow cues, including the role of mechanosensory hair plates (Böhm’s bristles) in antennal positioning response. (F) The crossmodal calibration hypothesis proposes that insects simultaneously sample airflow and optic flow, and use response characteristics of airflow sensing to calibrate optic flow. Determining how the sampled optic flow varies in the dynamic range of airspeeds enables...
insects to linearly extrapolate the optic flow response curve over a greater range. The grey bar represents the stimulus range in which such simultaneous airflow (blue) and optic flow (black on red) measurements are made. According to this hypothesis, once a specific airflow value is correlated against the observed optic flow, which could be slow or fast, it can then be used to make measurements over much greater range of airspeeds.

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