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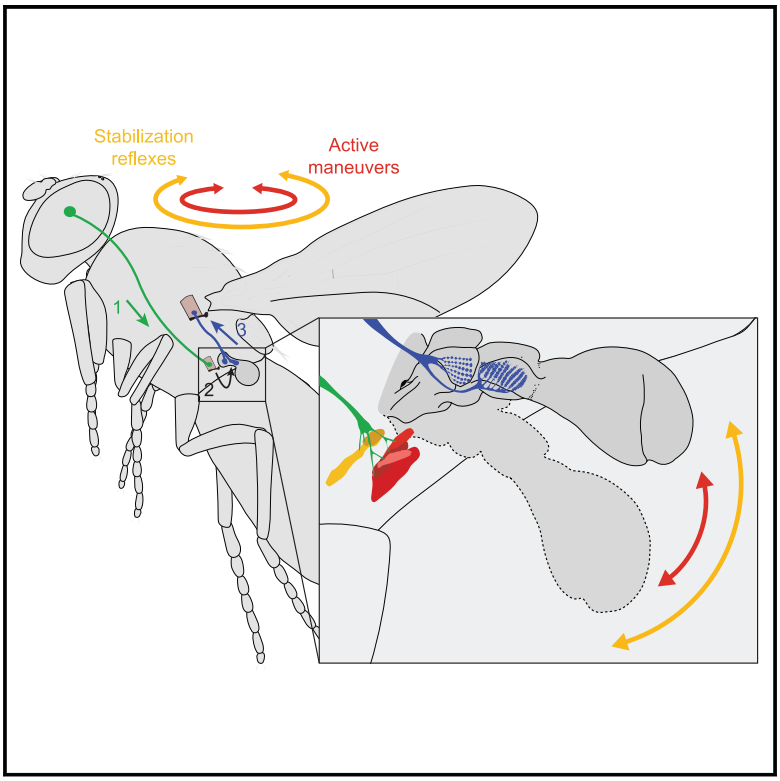
Flies tune the activity of their multifunctional gyroscope

Graphical abstract



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In brief

Halteres are multifunctional sensory organs unique to flies that help them achieve both stability and maneuverability via stereotyped arrays of embedded mechanosensors. Using an array of genetic tools, Verbe et al. demonstrate that the visual system indirectly modulates the activity of these sensors through subtle changes in haltere motion.

Highlights

- Halteres dynamically regulate wing motion via fields of biological strain gauges
- Visual input modulates the activity of the haltere's sensory fields
- The haltere steering muscles are functionally segregated into two groups
- The haltere steering muscles regulate haltere stroke amplitude

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Flies tune the activity of their multifunctional gyroscope

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SUMMARY

Members of the order Diptera, the true flies, are among the most maneuverable flying animals. These aerial capabilities are partially attributed to flies' possession of halteres, tiny club-shaped structures that evolved from the hindwings and play a crucial role in flight control. Halteres are renowned for acting as biological gyroscopes that rapidly detect rotational perturbations and help flies maintain a stable flight posture. Additionally, halteres provide rhythmic input to the wing steering system that can be indirectly modulated by the visual system. The multifunctional capacity of the haltere is thought to depend on arrays of embedded mechanosensors called campaniform sensilla that are arranged in distinct groups on the haltere's dorsal and ventral surfaces. Although longstanding hypotheses suggest that each array provides different information relevant to the flight control circuitry, we know little about how the haltere campaniforms are functionally organized. Here, we use *in vivo* calcium imaging during tethered flight to obtain population-level recordings of the haltere sensory afferents in specific fields of sensilla. We find that haltere feedback from both dorsal fields is continuously active, modulated under closed-loop flight conditions, and recruited during saccades to help flies actively maneuver. We also find that the haltere's multifaceted role may arise from the steering muscles of the haltere itself, regulating haltere stroke amplitude to modulate campaniform activity. Taken together, our results underscore the crucial role of efferent control in regulating sensor activity and provide insight into how the sensory and motor systems of flies coevolved.

INTRODUCTION

Among flying insects, true flies (order Diptera) stand out for their adept aerial maneuvers. Multiple physiological specializations in both their sensory and motor systems make flies remarkable fliers, and these adaptations helped them become one of the most diverse insect orders.[1] Among these specializations is the functional segregation of the flight muscles into two major groups: power muscles that provide the force necessary to flap the wings and generate lift and steering muscles that control the subtle changes in wing motion needed to accomplish maneuvers.[2,3] Whereas a single action potential triggers multiple contraction cycles in the power muscles,[4] the firing times of the wing steering muscles rely on mechanosensory feedback that arrives with each wingstroke. Aside from the wing base proprioceptor pterale C,[5] there are two major sources of mechanosensory feedback that structure the activity of the steering muscles: the wings themselves and vestigial hindwings unique to flies called halteres.[6–8]

Halteres are tiny, club-shaped structures found on the metathorax that do not serve an aerodynamic function but instead provide sensory information crucial to flight.[9–11] Halteres oscillate during flight, providing rhythmic input to the wing steering system via arrays of embedded mechanosensors (campaniform sensilla) that are sensitive to cuticular strain.[12] Both the wings and halteres possess campaniform sensilla, but they have hypertrophied on the haltere such that in the case of *Drosophila melanogaster*, the haltere has approximately 140 campaniforms compared with less than 50 on the wing.[13]

The haltere is also the only biological gyroscope: during rotations, it experiences Coriolis forces due to its tendency to resist changes in its plane of oscillation, triggering equilibrium reflexes of the head and wings.[9–11,14–18] Underscoring their evolutionary history as a hindwing, each haltere is equipped with a single power muscle and a set of seven control muscles that are serially homologous to those of the wings.[19–23] The motor neurons of the haltere steering muscles receive descending visual input.[20,23,24] Recent evidence demonstrates that rather than serving as passive gyroscopic sensors, halteres are multifunctional sensory organs. Through what is known as the control-loop hypothesis[3,20,23] (Figure 1A), flies modulate the activity of the wing steering system via the halteres even in the absence of body rotations to dynamically regulate the wing steering system. Furthermore, through active control of the haltere muscles, flies may even execute voluntary movements without triggering counteracting reflexes, although this has not yet been directly tested. However, how the haltere achieves its multifunctional role is poorly understood.

Across dipterans, the campaniform sensilla embedded in the haltere are arranged in highly stereotyped groups, called

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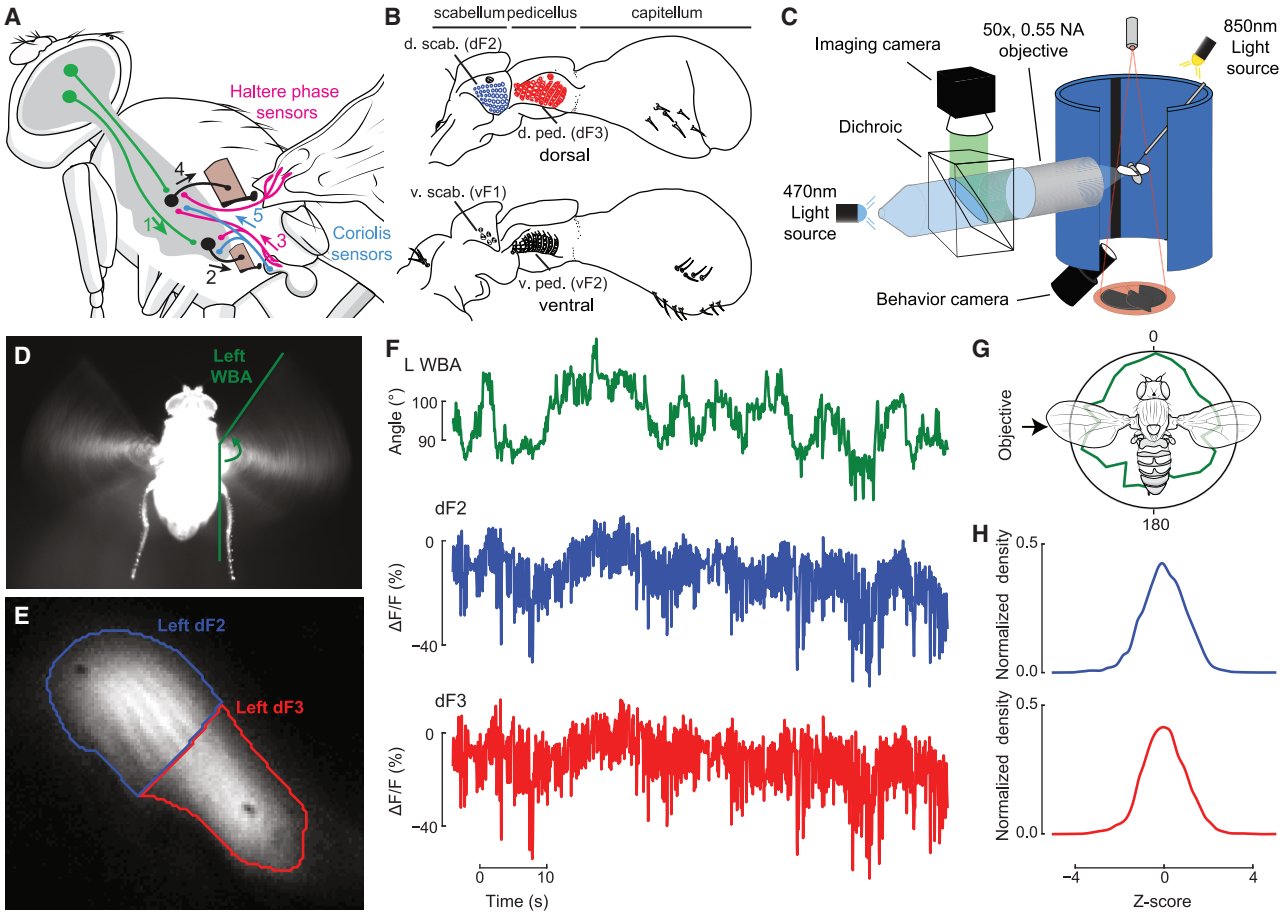

Figure 1. *In vivo* imaging of the dorsal haltere campaniform fields during tethered flight

(A) The control-loop hypothesis explains the multi-sensory activity of the halteres (redrawn from Dickerson et al.[23]). Visual signals (1) are sent to the haltere muscles (2), recruiting additional campaniform sensilla with different preferred firing times (3). This feedback alters the timing or activation of the wing steering muscles (4). The haltere's gyroscopic function may operate through a similar pathway (5).

(B) Anatomy of the *Drosophila* haltere, showing the locations of the four major campaniform fields (redrawn from Cole and Palka[13]).

(C) Schematic of setup used to image activity of the dorsal campaniform fields on the left haltere while simultaneously tracking wing motion during the presentation of visual stimuli.

(D) Image from the behavioral tracking camera showing the fly from below and the wingstroke envelope of both wings; left wing (ipsilateral) amplitude is shown in green line.

(E) Maximum intensity projection of the dorsal campaniform fields of a single experiment, clustered by fluorescence activity into regions that correspond with dF3 (red) and dF2 (blue).

(F) Extracted signals of the left wingbeat amplitude, fluorescence of the mean area of dF2 and dF1.

(G) Polar probability density histogram showing the distribution of stripe azimuth during closed-loop fixation behavior.

(H) Mean histograms of fluorescence changes during stripe fixation, normalized as *Z* scores (dF2 in blue and dF3 in red).

(I) Correlation between dF3 and dF2 (Pearson $R^2 = 0.912$, black), dF1 and L WBA (Pearson $R^2 = 0.347$, red), and of dF2 and L WBA (Pearson $R^2 = 0.376$, blue) for all trials. $N = 23$ flies.

See also Figure S1 and Video S1.

fields[9,13,25–28] (Figure 1B). Like other insect campaniform sensilla, the orientation and location of the haltere fields are hypothesized to have functional significance.[9,26,29–32] For example, the fields of sensilla that lie parallel to the haltere's long axis—i.e., dF3 and vF2—are predicted to detect the in-plane strains produced by the large vertical oscillations during flapping.[9,26] Similarly, the diagonally orientated sensilla of dF2 should be maximally sensitive to the strains produced either by lateral bending from gyroscopic torques or from active haltere movements from the activity of the haltere steering muscles.[9,20,23,26] Although these longstanding hypotheses suggest that the different arrays may provide different information relevant to flight control,[9,26] we know little about how haltere campaniform location corresponds to sensory activity.

We leveraged the genetic tools available in *Drosophila* to investigate how visual input modulates the encoding of two campaniform fields, dF3 and dF2, by imaging neural activity from a moving haltere during tethered flight. Specifically, we test the hypothesis that field dF2 is modulated—via the control loop—by visual input alone. We then probed how campaniform encoding is

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determined by the organization of the haltere steering system and its control of haltere kinematics. Our results provide an example of how efferent control of a sensory system regulates the dynamic range of sensors to mediate both stabilizing and active maneuvers.

RESULTS

In vivo imaging of the haltere's sensory fields

To image from the haltere campaniform fields during tethered flight, we used a driver line that targets most of the population of haltere campaniform sensilla, *DB331-GAL4*, driving the expression of the genetically encoded calcium indicator GCaMP7f (Figure 1). The neurons associated with each campaniform field are directly beneath the dome of each sensillum, and the cuticle in this region is quite thin. This allowed us to image GCaMP activity with an epifluorescent microscope using a previously described method[23,33] in an intact animal (Figure 1C). We simultaneously measured wing steering effort, reported here as left-minus-right wingbeat amplitude (L-R WBA), using custom machine vision software[34] (Figure 1D; Video S1). Due to the high density of campaniforms within each field, we could not resolve GCaMP activity at the level of individual sensilla. We instead report the activity of each field from the left haltere using k-means clustering of the image stream to extract signals for the dorsal campaniforms fields, dF1 and dF2 (Figure 1E). Prior electrophysiological work in larger flies shows that campaniforms fire single, phase-locked action potentials in response to periodic stimuli.[7,8,35–38] Additionally, in *Calliphora*, haltere oscillation generates phase-locked compound action potentials, which are generated by specific campaniform fields.[8] In keeping with prior work,[23] we therefore interpret increases in GCaMP signal as recruitment of additional sensilla. Preliminary experiments indicated that the ventral fields are also active in flight. However, fluorescent imaging of ventral fields is subject to substantial motion artifact, so we focused on the calcium activity of the dorsal fields.

Under closed-loop conditions in which the fly controls the position of a dark stripe in its visual field via changes in L-R WBA, haltere feedback from both dF3 and dF2 is modulated throughout flight (Figures 1F and 1G). Control experiments expressing GFP in the haltere campaniforms demonstrated that these signal fluctuations were not motion artifacts. Pooling data across flies allowed us to test whether these modulations were consistent. We normalized each fluorescence trace, creating *Z* scored versions of the data from all 23 flies, and subsequently created population histograms of dF3 and dF2 activity during closed-loop stripe fixation (Figure 1H). The fluorescence activity of both dF3 and dF2 was normally distributed, indicating that the activity of each field fluctuated about some baseline level and was correlated with changes in WBA (Figures 1I and S1). Our observation that dF2 campaniforms are tonically active is particularly informative because this field is thought detect Coriolis forces due to body rotation.[9,26] However, we imaged from rigidly tethered, non-rotating flies, and as a result, Coriolis forces are absent our experiments. Thus, this result confirms that dF2 activity is modulated by the control loop[20,23] and forces a reevaluation of the classic model of haltere function.

Haltere feedback is directionally tuned

We next tested the sensitivity of the dorsal haltere campaniform sensilla to an array of open-loop visual stimuli. Previous calcium imaging of the haltere axon terminals demonstrated that their activity is modulated by rotations of wide-field visual motion.[23] We subjected flies to simulated rotations in the sagittal (yaw-roll) and azimuthal (pitch-roll) planes while simultaneously tracking changes in wingstroke amplitude. Using random starfield patterns, we shifted the center of rotation in 30° increments about the midsagittal plane and compared the kinematic changes to the patterns of dF3 and dF2 activity elicited by the corresponding patterns of visual motion (Figures 2A and S2).

From these data, we calculated tuning curves for L-R WBA as well as dF3 and dF2 activity (Figures 2B and 2C). For presentations of wide-field motion about the yaw-roll axis, L-R WBA, and the calcium activity of both dF3 and dF2 varied sinusoidally with the stimulus rotation angle, with a maximum response near yaw motion to the right and a minimum response around yaw to the left (Figures 2B and 2C). Notably, the tuning of dF3 and dF2 matches the sensitivity of flies' steering effort, yet the campaniform fields demonstrate tuning in the opposite direction of the haltere steering muscles.[23] Although visual rotations about the pitch-roll axis elicited changes in wing kinematics, neither dF3 nor dF2 show significant changes in calcium activity or directional selectivity (Figure S2).

Haltere fluorescence signals rise linearly for visual stabilization reflexes and during active maneuvers

We next compared haltere campaniform recruitment under two conditions in which flies regulate wing motion: responses to optomotor stimuli and during spontaneous maneuvers. Haltere campaniform activity being modulated during both conditions would mean that haltere feedback—in addition to correcting mechanical and visual perturbations—helps the wing steering system execute active maneuvers, called saccades.[33,39–41] Such a result would also mean that the haltere not only shortens saccades via gyroscopic input[42] but also helps initiate them through the control loop.

We first investigated the relationship between haltere campaniform field activity and steering maneuver magnitude. The specific nature of this relationship could take many forms (e.g., linear, sigmoidal, or exponential), all of which would be consistent with the halteres' role in regulating the activity of the wing steering system in response to optomotor stimuli. To test which strategy is used, we sorted the L-R WBA data from the responses to wide-field visual motion, irrespective of the direction of stimulus motion, along with the corresponding calcium signals for dF3 and dF2. We sorted 1,260 responses from 21 flies from the most leftward to the most rightward responses during the 3 s following stimulus onset (Figure 3A). We then divided the sorted data into amplitude deciles, ranging from the largest leftward responses (90^{th}–100^{th} percentile) to the largest rightward responses (0^{th}–10^{th} percentile). We then calculated the mean signal for each decile during the stimulus (Figure 3B). The average peak haltere responses during the stimulus windows in each stroke amplitude decile are plotted in Figure 3C. For both dF3 and dF2, we observed similar patterns of roughly linear recruitment as a function of L-R WBA (Figures 3B and 3C). These results suggest that as a fly turns, the number of campaniforms

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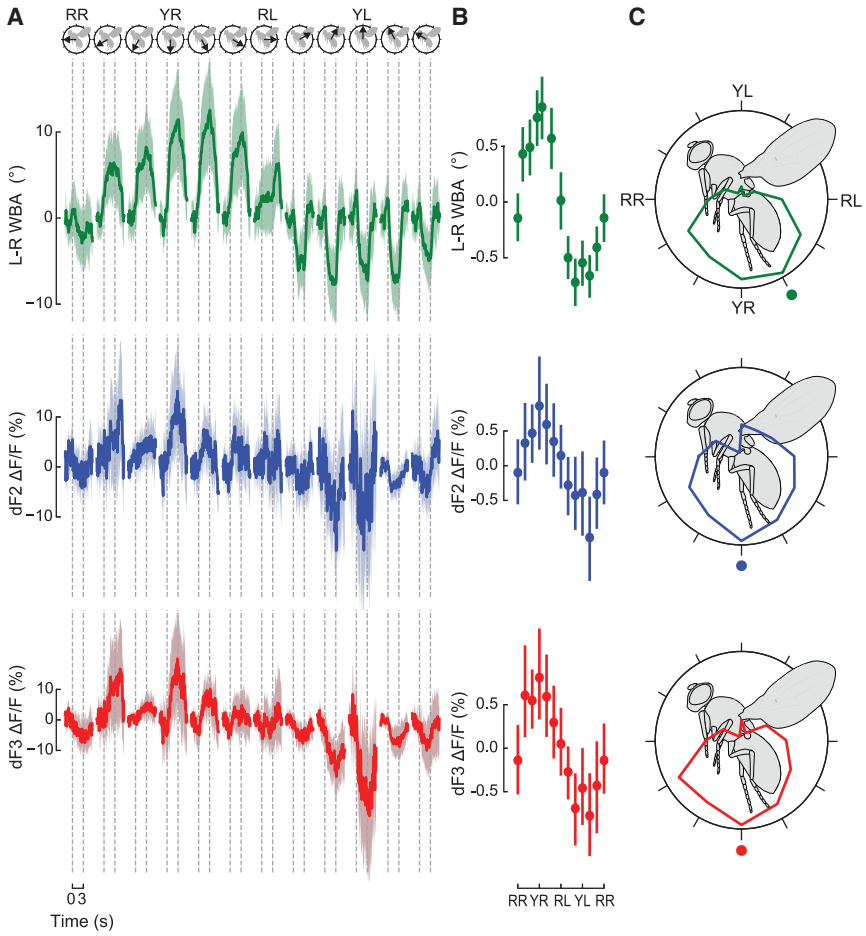

Figure 2. Haltere sensory feedback is directionally tuned by visual motion

(A) Tuning dynamics of the dorsal campaniform sensilla fields in response to 3 s presentations of wide-field visual rotational motion about the yaw-roll axis. Stimulus direction (top), wingbeat amplitude responses, and campaniform fields fluorescence where the stimulus center of rotation was shifted in 30° increments. Data represent means ± 95% confidence intervals (CIs). RL, roll left; RR, roll right; YL, yaw left; and YR, yaw right. Stimulus direction is indicated by the right-hand rule.

(B) Stroke amplitude and haltere campaniform field activity plotted as functions of rotational orientation in the midsagittal plane. The curves were constructed by determining the average signal over the 3 s following stimulus onset. Roll right is plotted twice to emphasize the cyclical nature of the data.

(C) Mean tuning functions from (B) plotted in polar coordinates. Solid dots indicate the orientation of the maximum response for each signal. N = 21 flies.

See also Figure S2.

that fire each wingstroke increases in proportion to the stroke amplitude of the ipsilateral wing.

If haltere input mediates active maneuvering, then campaniform field activity should increase during saccades. Although most saccades are triggered by external visual stimuli,[43,44] flies also exhibit spontaneous saccades,[45] and they are conspicuous features of tethered flight preparations.[46] We used a simple classifier[47] (based on L-R WBA) to identify saccades and the corresponding calcium signals of dF3 and dF2. We applied our classifier to a total of 4,521 saccades from 23 flies that we imaged during closed-loop stripe fixation (Figure 4A). We again sorted these data into amplitude deciles based on L-R WBA magnitude and calculated decile means (Figure 4B). In contrast to the pattern of linear recruitment we observed during wide-field visual motion (Figure 3C), we found a less clear relationship between steering effort and campaniform field activity. Whereas L-R WBA monotonically increased, we find that dF3 and dF2 are either inactive or show low activity for the bottom 50% of identified saccades and linearly recruited for the remaining 50% (Figure 4C). Notably, these modulations in dF3 and dF2 activity occur before the peak changes in L-R WBA during a saccade. We also looked at spontaneous saccades during presentation of a static visual pattern but saw no consistent trends in dF3 or dF2 activity (Figure S3). Together, these results indicate that dF3 and dF2 are recruited during visually mediated reflexes (Figure 3D) and prior to executing saccades (Figure 4C).

The haltere muscles are differentially recruited

The most parsimonious explanation for haltere feedback being modulated during both visually mediated stabilization reflexes and active maneuvers is that haltere steering muscles are functionally segregated like their forewing counterparts. The wing steering system is comprised of tonically active muscles, which are linearly recruited for stabilization reflexes, and phasically active muscles, which have a steep nonlinear activation threshold and are recruited for active maneuvers.[33,39–41] Campaniforms generally exhibit either rapid or slow adaptation to applied static loads,[29,36,48–50] yet they all fire single spikes at different preferred phases when stimulated at wingbeat frequency.[35,36,51] Thus, campaniform physiology in the context of the high flapping frequencies associated with flight suggests that the haltere motor system is functionally stratified to help regulate wing motion.

To test this hypothesis, we expressed GCaMP6f in a driver line (*R22H05-GAL4*) that targets all of the haltere steering muscles. The haltere steering muscles are divided into two major anatomical groups: the basalares (hB1 and hB2) and the axillaries (hI1, hI2, hIII1, hIII2, and hIII3; Figure 5A).[23] We imaged haltere muscle activity using the same method we applied to the haltere campaniform fields (Figure 5B) and again identified spontaneous saccades. We classified a total of 2,627 saccades from 7 flies during the presentation of a closed-loop visual pattern. From the identified saccades, we sorted the calcium signals associated with each muscle group into deciles and again calculated averages for each group (Figure 5C). The activity of the haltere basalares is linearly related to saccade amplitude, consistent with these muscles being tonically active (Figure 5D).[33] The activity of the

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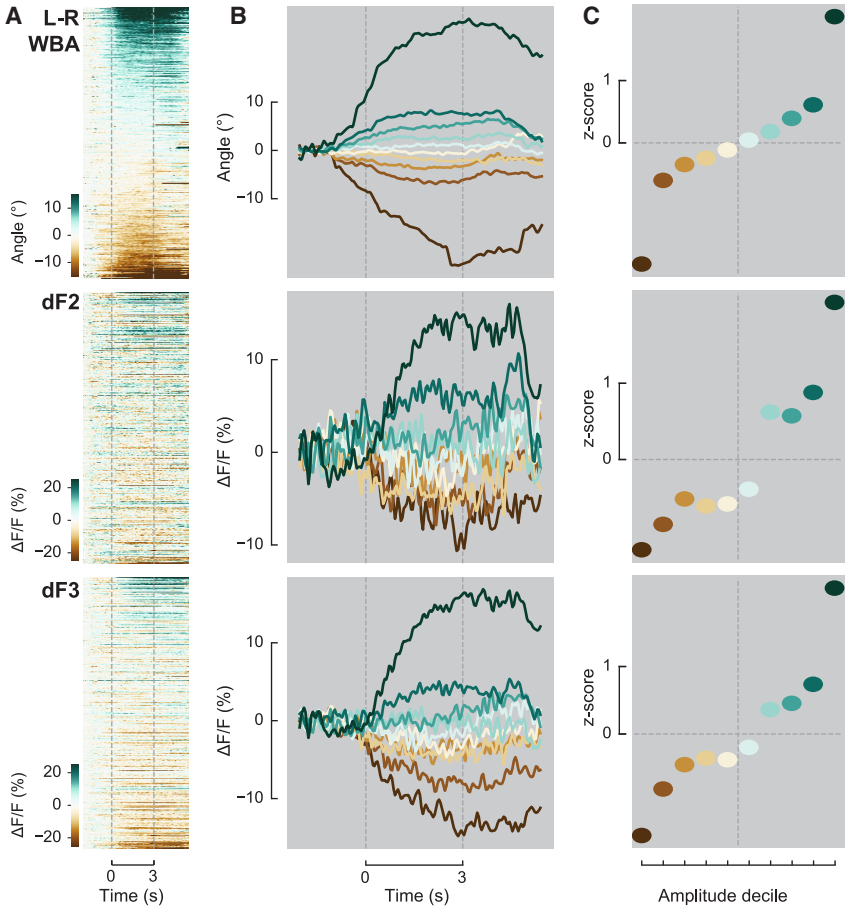

Figure 3. Haltere feedback is linearly recruited for visual stabilization reflexes

(A) Raster plots showing the left-minus-right wingbeat amplitude and the corresponding fluorescence signals of dF3 and dF2 in response to a 3 s episode of wide-field motion about the yaw-roll axis for all trials. Data are sorted in descending order according to the average magnitude of ipsilateral stroke amplitude during the 3 s following stimulus onset.

(B) Decile mean responses of wing kinematics and campaniform field fluorescence, ranging from the largest rightward turns (0^{th}–10^{th} percentile, green) to the largest leftward turns (90^{th}–100^{th} percentile, brown).

(C) Plots showing peak responses in the 3 s episode for each decile. *N* = 21 flies.

haltere axillaries, however, is nonlinearly related to saccade amplitude, showing a step-like increase in activity for the largest 30% of turns to the right (Figure 5D). This recruitment profile is consistent with the haltere axillaries being phasically active muscles. Thus, the haltere motor system is both anatomically and functionally stratified.

Optogenetic activation of haltere steering muscles changes haltere amplitude

Changes in haltere campaniform field activity result from the activity of the haltere steering muscles, which regulate either haltere kinematics or the biomechanics of each campaniform field.[3,20,23] These two modes of regulating haltere sensing are both feasible, but wing campaniform activity is correlated with changes in L-R WBA in response to wide-field visual motion stimuli, much like haltere campaniforms.[23] Changes in wing kinematics and the resulting strain on the wing are the direct result of wing steering muscle activity. Given their serial homology with the wings, this suggests that the haltere muscles regulate the stroke amplitude of the haltere to modulate campaniform recruitment and, ultimately, wing steering muscle activity. To test if the haltere steering muscles control haltere kinematics, we optogenetically activated two haltere muscle motor neurons during tethered flight and recorded haltere motion at 2,000 fps using a high-speed camera (Figure 6A). We expressed Chrimson in a driver line (*SS41075-SplitGAL4*[52]) that targets two haltere muscle motor neurons: hDVM, the asynchronous power muscle,[53] and hI1, an axillary steering muscle (Figure 6B). Optogenetic activation of these motor neurons decreased haltere stroke amplitude (Figures 6C–6F; Video S2). The reduction in amplitude is a result of the haltere's failure to reach its full ventral extent during the stimulus; the dorsal extreme is not as strongly affected.

DISCUSSION

The decades-long hypothesis that the haltere campaniform fields should exhibit different directional sensitivities to haltere motion is based on the observation that these fields show specific spatial orientations.[9,26] We directly tested these hypotheses for the first time using *in vivo* calcium imaging during tethered flight to obtain population-level recordings of the haltere afferents in specific fields of sensilla, including dF2, which was thought to be highly specialized for detecting Coriolis forces. In contrast to the canonical model of haltere function, we found that dF2 is continuously active during flight—even when the fly is not subject to Coriolis forces—and that its activity is modulated by visual input. We also found that haltere input from both dorsal fields is recruited during saccades. These different roles for flight control are determined by the haltere motor system, which subtly alters haltere kinematics to modulate campaniform activity. Overall, our results demand a reevaluation of the functional organization of the haltere campaniform fields and demonstrate how descending efferent control can filter sensory stimuli to help produce flexible and robust behavior.

Role of haltere in visually mediated flight

We found that the activity of both dF3 and dF2 is continuously regulated during flight (Figure 1). These results are significant in two respects. First, the observation that activity from both fields is modulated by specific directions of wide-field visual motion (Figure 2) provides further confirmation of the control-loop hypothesis.[20,23] Furthermore, the tuning characteristics of dF2 and its alignment with steering responses suggest that the haltere

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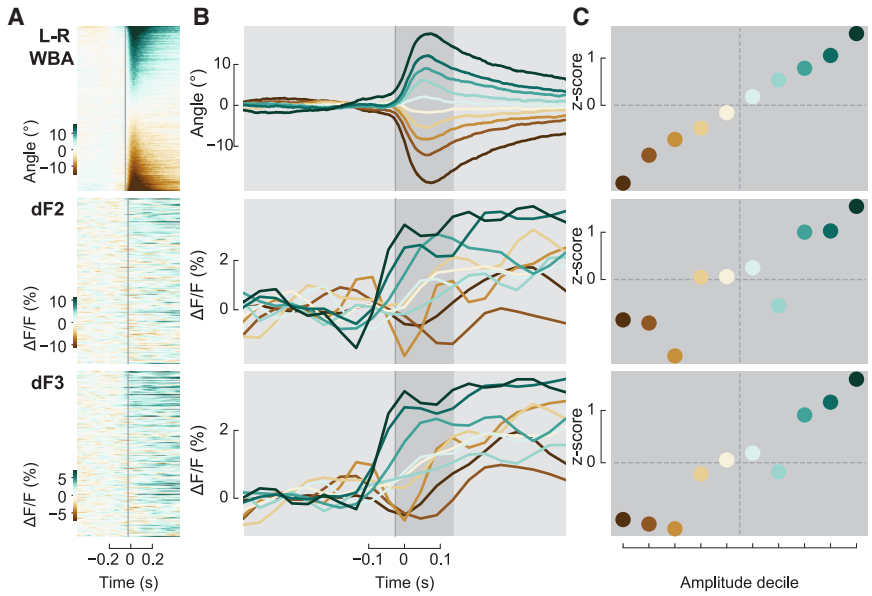

Figure 4. Haltere feedback is recruited for active maneuvers

(A) Raster plots of all saccades for all trials sorted by the change in left-minus-right wingbeat amplitude.

(B) Saccade-triggered averages of wing kinematics and dorsal halteres signals sorted into amplitude deciles, ranging from the largest rightward turns to the largest leftward turns. Dark gray bar denotes saccade initiation.

(C) Plots showing peak responses for each decile during the saccade. n = 4,521 saccades from 23 flies.

See also Figure S3.

steering muscles manipulate haltere motion to modulate haltere feedback. Indeed, our experiments provide the first direct evidence that the haltere steering muscles regulate stroke amplitude during flight (Figure 6). This finding is consistent with previous work showing that the haltere steering system is tuned to visual motion.[23] The peak sensitivity of the haltere motor system is in the opposite direction of wing steering responses. As a result, during a visually mediated turn to the right, as the left WBA increases, the stroke amplitude of the right haltere would decrease.

Second, our results suggest that dF3 may mediate flight control. Previous anatomical and physiological work in the blowfly *Calliphora* showed that haltere afferents provide excitatory input to the wing motor neuron b1 through a mixed electrotonic and chemical synapse.[7,8,54] However, this feedback is provided by a single field of campaniforms, dF2: ablating dF2 and stimulating the remaining campaniform fields, including dF3, fails to generate postsynaptic potentials in the b1 motor neuron.[8] However, these experiments were conducted in quiescent flies, ignoring context-dependent phenomena that can influence circuit function, such as octopaminergic modulation of the sensitivity of sensory systems, including wing mechanosensors.[55,56] Nevertheless, in our experiments, dF3 is recruited in much the same manner as dF2, for both visually mediated reflexes and active maneuvers (Figures 2, 3, and 4), indicating that dF3 is not merely a passive sensor for the haltere's large oscillation as previously predicted.[9,26] Moreover, anatomical work shows extensive projection patterns of each field within the fly central nervous system,[54] suggesting that all fields play an important part in controlling flight, though these functional roles remain unclear. New connectomics data will reveal the organizational logic of the haltere campaniform fields with respect to the wing steering motor neurons and other targets.

More broadly, the observation that both dorsal fields are continuously active is not surprising as the haltere undergoes large oscillations at a high frequency, creating complex patterns of strain across the surface. These strain patterns are transduced by the embedded campaniform sensilla on both the dorsal and ventral aspects of the haltere, which are exquisitely sensitive to the slightest deformations of the cuticle.[12,36,38,57] Our use of a calcium indicator to monitor haltere activity allowed us to obtain population recordings from the two dorsal fields. However, the kinetics of our calcium indicator are too slow to capture crucial information about spike timing from individual sensilla. Intracellular recordings from sensilla axons demonstrate that changes in haltere motion are reported via either shifts in the preferred firing phase of active campaniforms or recruitment of additional campaniforms that have unique preferred firing phases.[38] By adjusting haltere stroke amplitude via modulation of the haltere steering muscles, flies may modulate the strength of this feedback by subtle changes in stroke amplitude to rapidly adjust wing kinematics with precisely timed mechanosensory feedback.

Haltere feedback during active maneuvers

In addition to suggesting that visual information modifies haltere mechanosensory feedback and consequently wing motor output, the control-loop hypothesis predicts that the haltere control muscles and sensilla are active during voluntary maneuvers, e.g., body saccades. Consistent with their role in sensing the Coriolis forces that result from body rotations,[42] feedback from the halteres shortens the motor program regulating saccades. However, whether haltere feedback is modulated during the initiation of saccades remains an open question.[58]

We found that both the haltere motor system as well as the campaniform fields are recruited during spontaneous saccades (Figures 4 and 5). Moreover, these changes in muscle and campaniform field activity occur prior to the changes in wing kinematics. This suggests that haltere motion can be controlled independently of the wings to initiate a voluntary maneuver. Recent evidence shows that flies tune the magnitude of a saccade according to the angular velocity of a visual stimulus.[59] Similarly, we found that the strength of haltere mechanosensory feedback varies with the magnitude of the saccade (Figure 4).

Recent work in insects suggests a role for efference copy in suppressing visually mediated reflexes during voluntary flight maneuvers.[47,60,61] For example, electrophysiological recordings of lobula plate tangential cells (LPTCs) in *Drosophila* show predictive, scalable inhibition or excitation that is correlated with spontaneous saccades.[47,61] Yet in all cases, the source of these

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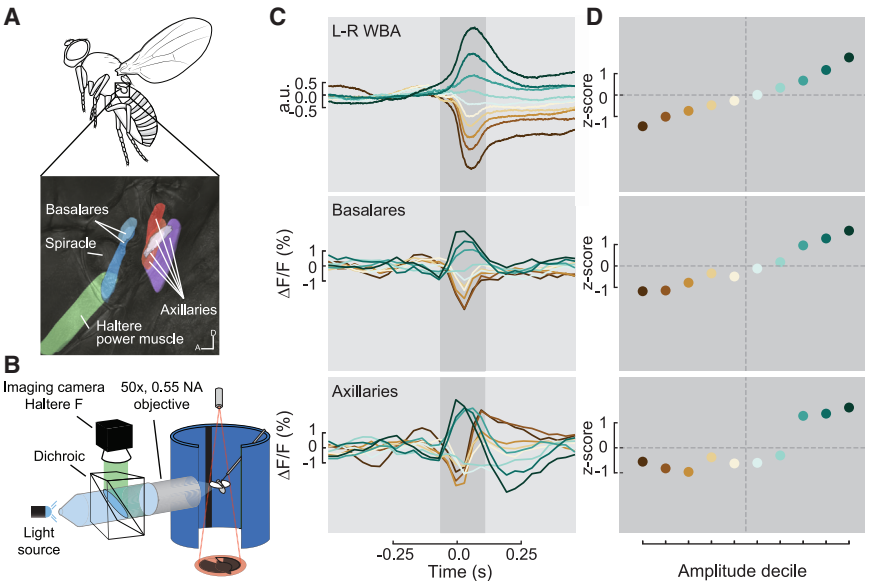

Figure 5. The haltere muscles are differentially recruited

(A) The haltere motor system of *Drosophila* consists of a power muscle and seven steering muscles that can be divided into two anatomical groups, the basalares and the axillaries.

(B) Schematic of setup used to simultaneously image muscle activity and track wing motion in response to visual stimuli. (A) and (B) are reproduced from Dickerson et al.[23]

(C) Saccade-triggered averages of left-minus-right wingbeat amplitude and haltere muscle fluorescence for each anatomical group. Saccade-triggered averages are plotted as means of each amplitude decile, sorted from the largest rightward turns to the largest leftward turns.

(D) Plots showing the peak decile responses during a saccade (dark gray bar in C). n = 2,627 saccades from 7 flies.

efference copies remains unidentified. By contrast, our results suggest that rather than cancel out expected haltere feedback during a turn, flies co-opt existing haltere reflex loops to actively maneuver. An efference copy signal would render a fly susceptible to mechanical perturbations. Instead, by actively manipulating the haltere during a turn, flies can remain sensitive to gyroscopic forces during voluntary maneuvers.

How does a fly reconcile internal mechanics with Coriolis force sensing in flight?

The multifunctional capability of the haltere system suggests that flies can be maneuverable at low cost to their stability.[3] However, how flies can distinguish self-motion mechanosensory feedback from external whole-body mechanical perturbations remains unclear. Campaniform sensilla function through a generic encoding mechanism, termed derivative pair feature detection (DPFD).[62] DPFD may be an inherent property of neurons with non-specialized Hodgkin-Huxley dynamics, like many mechanosensors. As a result, the anatomical placement and mechanics of a DPFD neuron, rather than the specialized neural computation and membrane dynamics, act as a biomechanical filter and may confer specialized encoding. Although the activity of dF2 is dynamically regulated even in the absence of body rotations, we hypothesize that the arrangement of campaniforms within this field ensures that it is the primary source of gyroscopic feedback in flight. In this scheme, descending visual commands to the haltere steering system can initiate a turn by causing small changes in haltere kinematics and by recruiting campaniforms from all fields. Then, as the fly begins to rotate and the haltere undergoes its hypothesized changes in trajectory due to Coriolis forces, the activity of dF2—which should be maximally sensitive to the resulting shear strains—would increase, triggering a corrective maneuver. This hypothesis is consistent with both our observation that haltere feedback is constant and the haltere's well-established role as a gyroscopic sensor.

In free flight, both stabilization and active maneuvers consist of banked turns that involve coordinated changes about all three cardinal axes.[63,64] Modeling and behavioral evidence show that the haltere can detect any combination of body rotations.[11,15,17,65,66] In this regard, it is surprising that visual modulation of campaniform sensilla activity seems to be only present in the yaw-roll plane and nonexistent for rotations about the pitch-roll plane. This is of particular interest as the direction of motion that elicited the strongest response, for both dF1 and dF2, is pure yaw, which is the weakest axis for gyroscopic responses.[17] It is possible that, in addition to helping initiate active turns, one major role for the control loop is to help mediate straight flight about the azimuth by instituting rapid corrections. Indeed, flies dedicate a great deal of neural circuitry to descending interneurons that are hypothesized to maintain a straight flight trajectory.[67] Presumably some of those commands are directed to the haltere motor system.

Co-evolution of the haltere and flight

Although the haltere is a unique sensory structure, its core function—regulating the timing of the wing steering system—and control are much like the aerodynamically functional forewings. Indeed, the haltere evolved from the hindwing,[1] and past genetic work confirms that it is a serially homologous structure.[13] Moreover, in other flying insects, this homology includes detecting perturbations.[68] Fields dF2 and vF1 in flies are serial homologs of campaniform fields on the radial vein of the wing (dorsal radius A and ventral radius A, respectively).[13] We found that this homology extends to the organization of the motor system for each structure, as the haltere motor system is functionally segregated like the wing steering muscles[33] (Figure 5). Interestingly, along with their distinct morphologies, fields dF3 and vF2 have no clear homolog on the wing. It is possible that the development of these two fields is an evolutionary novelty that—combined with the existing control loop—allowed the haltere to become a multifunctional sensor that provides reafference for controlling flight maneuvers. The breadth of tools available in *Drosophila* combined with classic approaches drawn from biomechanics and biophysics will enable for a fuller appreciation of this enigmatic sensory structure.

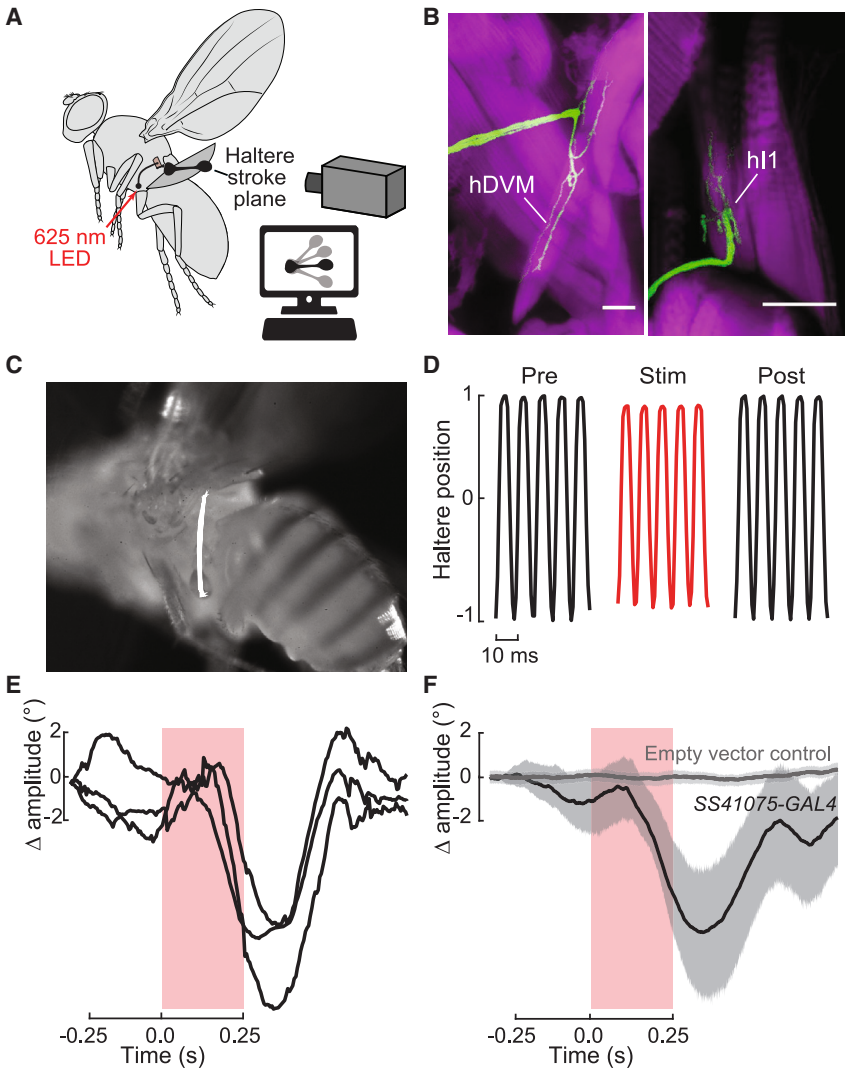

Figure 6. Optogenetic activation of haltere steering muscle motor neurons decreases haltere stroke amplitude

(A) Schematic of setup used to record haltere motion of tethered, flying flies during optogenetic activation of haltere motor neurons.

(B) Maximum intensity projections of the haltere power muscle, hDVM, and one steering muscle motor neuron, hI1, expressing GFP (green) driven by *SS41075-GAL4*. Magenta shows phalloidin staining of muscles. Images reproduced from Dickerson et al.[23]

(C) Sample frame of haltere tracking with the trajectories of 10 tracked strokes overlaid (white).

(D) Example traces of normalized haltere stroke amplitude 25 ms before or after (black) and during optogenetic activation (red).

(E and F) Three representative traces (E) and population data (F) showing the change in haltere stroke amplitude following optogenetic activation of *SS41075-GAL4*. Changes in haltere stroke amplitude for empty vector control flies are also plotted on the right (gray). Data shown represent mean ± 95% CI, $N = 54$ flies.

See also Video S2.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2024.06.066.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.V., K.M.L., J.L.F., and B.H.D.; methodology, A.V., K.M.L., J.L.F., and B.H.D.; investigation, A.V., K.M.L., and B.H.D.; resources, J.L.F. and B.H.D.; analysis, A.V., K.M.L., J.L.F., and B.H.D.; writing, A.V.,

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K.M.L., J.L.F., and B.H.D.; supervision, J.L.F. and B.H.D.; funding acquisition, J.L.F. and B.H.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Dataset and code	This paper	https://github.com/AnnaVerbe/Flies-tune-the-activity-of-their-multifunctional-gyroscope
Experimental models: Organisms/strains		
D. melanogaster: DB331-GAL4	FlyBase	FBti0115113
D. melanogaster: w[1118]; P{20XUAS-IVS-jGCaMP7f}su(Hw)attP5;+	Bloomington Drosophila Stock Center	RRID: BDSC_80906
D. melanogaster: w[1118];+; P{y[+t7.7] w[+mC] = GMR22H05-GAL4}attP2	Bloomington Drosophila Stock Center	RRID: BDSC_49002
D. melanogaster:; P{$y^{+t7.7}$ w^{+mC} = 20XUAS-IVSGCaMP6f}attP40	Bloomington Drosophila Stock Center	RRID: BDSC_42747
D. melanogaster: SS41075-SplitGAL4	Gift from M. Dickinson	N/A
D. melanogaster: w-; ScO/CyO; UAS-Chrimson.mVenus(w+)/(TM6B,Tb,Hu,e)	Gift from E. Heckscher	N/A
D. melanogaster: w[1118]; P{y[+t7.7] w[+mC]=p65.AD.Uw}attP40; P{y[+t7.7] w[+mC]=GAL4.DBD.Uw}attP2	Gift from E. Heckscher	N/A
D. melanogaster: w-; ScO/CyO; UAS-Chrimson.mVenus(w+) /(TM6B,Tb,Hu,e)	Gift from E. Heckscher	N/A
Software and algorithms		
Python	https://www.python.org/	RRID:SCR_008394
Matplotlib	https://matplotlib.org/	RRID: SCR_008624
MATLAB	https://www.mathworks.com/products/matlab.html	RRID:SCR_001622

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to lead contact, Bradley Dickerson (bdicker@princeton.edu).

Materials availability

This study did not generate new unique genetic reagents.

Data and code availability

The data from this manuscript are published on GitHub at:
https://github.com/AnnaVerbe/Flies-tune-the-activity-of-their-multifunctional-gyroscope

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals and tethering

All flies used in this study were 2-to-5-day-old females. For imaging experiments, we raised flies on standard cornmeal medium at 25°C on a 12:12 hour light/dark cycle. For the Chrimson activation experiments, we raised both the parents and offspring in the dark. We added 100μL of 100mM all-trans retinal to the bottles of the parents and 200 μL of the same concentration to the bottles of the progeny.

We expressed GCaMP7f in the haltere afferents by crossing *DB331-GAL4* to w[1118]; P{20XUAS-IVS-jGCaMP7f}su(Hw)attP5; +. We anesthetized the flies at 4°C and tethered them at the anterior notum to a tungsten pin using UV-curing glue. We allowed at least 30 min for recovery before performing experiments.

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We expressed GCaMP6f in the haltere steering muscles by crossing w[1118];+; P{y[+t7.7] w[+mC] = GMR22H05-GAL4}attP2 with +[HCS]; P{20XUAS-IVS-GCaMP6f}attP40;+. The haltere muscles are subject to significant motion artifact. Thus, to help stabilize our images, we removed the first two pairs of legs and tethered flies ventrally to a tungsten pin using UV-curing glue placed between the femur of the prothoracic legs and coxae of the mesothoracic legs.

To express Chrimson in the haltere steering muscle motor neurons, we crossed *SS41075-SplitGAL4* with w-; ScO/CyO; UAS-Chrimson.mVenus(w+)/(TM6B,Tb,Hu,e). For the empty vector control, we crossed the empty stable split line w[1118]; P{y[+t7.7] w[+mC]=p65.AD.Uw}attP40; P{y[+t7.7] w[+mC]=GAL4.DBD.Uw}attP2 with w-; ScO/CyO; UAS-Chrimson.mVenus(w+) /(TM6B,Tb,Hu,e). We tethered the flies as in our haltere afferent imaging experiments.

METHOD DETAILS

Flight arena and visual stimuli

For imaging of the haltere campaniform sensilla and steering muscles, we placed flies in the center of a previously described arena[69] composed of blue light-emitting diodes (LEDs; 470 nm peak wavelength). The arena spanned ± 60° in elevation from the fly's horizon (32 pixels) and 270° around its azimuth (72 pixels; 3.75/pixel). To accommodate the imaging objective, there was a 90° gap in azimuth on the left side of the arena. We placed one layer of blue filter (Rosco no. 59 indigo) to prevent light from the display from leaking into the camera used for imaging GCaMP activity. Visual stimuli consisted of either wide-field, random dot starfields or a dark stripe that subtended 22.5° on the fly's retina. Rotational patterns simulated motion at an angular velocity of π rad/s. To test rotational tuning about the yaw-roll and pitch-roll axes, we altered the center of rotation in 30° increments. To test tuning in the yaw-roll plane, we shifted the stimulus from the vertical body axis to the longitudinal axis. To test tuning in the pitch-roll plane, we shifted the stimulus from the longitudinal axis to the transverse body axis. We displayed patterns in random blocks for a duration of 3 s each, five repetitions for each stimulus. To promote flight, we presented flies with a dark stripe on a bright background under closed-loop conditions for 5s between each trial. The pattern then appeared and was still for 1 s before and after each stimulus presentation.

Flight behavior

To track steering behavior during haltere afferent and muscle imaging experiments, we placed flies within an optoelectronic wingbeat analyzer.[70] The moving wings cast shadows onto an optical sensor that converts instantaneous wingbeat amplitude into a voltage signal. We acquired wingbeat amplitude data at 2 kHz using a Digidata 1550B or 1440A amplifier (Molecular Devices) for the afferents and muscles, respectively. In our haltere afferent imaging experiments, we also illuminated flies from above with a single IR LED attached to a collimating lens and used a custom MATLAB machine vision script to calculate and record the left-minus-right wingbeat amplitude.[34] In cases where flies stopped flying, we softly blew on them to resume flight.

Functional imaging

Our method for imaging campaniform sensilla activity on the dorsal side of the left haltere was similar to that described for recording wing muscle activity.[23] We imaged the haltere campaniform sensilla with a 50x, 0.55 NA objective (Mitutoyo) mounted to 0.75x zoom tube lens on a Thorlabs WFA2001 epifluorescence module, giving us an effective magnification of 37.5x. We collected GCaMP fluorescence using a Retiga R1 camera. The amplifier we used to collect wingbeat amplitude data sent a TTL pulse to an Arduino Due, which triggered the camera at a phase of 0.5 relative to the upstroke of the wings. The Arduino also controlled the excitation LED (M470L3, Thorlabs), which provided 470 nm light to the haltere in three, 1 ms pulses during each exposure of the camera. Images were band-passed filtered by an ET535/50m emission filter (Chroma Technology). We collected TIFF stacks at an exposure time of 22 ms using μManager.

We used a similar method to image the haltere steering muscles. We placed flies beneath a 50x, 0.55 NA objective (Mitutoyo) mounted to a Nikon Eclipse FN1 epifluorescence microscope. In this setup, the fly, arena, and wingbeat analyzer were all mounted sideways to image the muscles. We excited GCaMP6f within the muscles with continuous 470 nm light (M470L3, Thorlabs). We collected images with a QIClick camera (QImaging) after they were band-passed filtered using the same emission filter as in our haltere imaging experiments. We triggered the imaging camera at a phase of 0.75 and used an exposure time of 33 ms.

Optogenetic activation of the haltere steering muscles

To track haltere movement, we tethered flies and mounted them within an optoelectronic wingbeat analyzer[70] with the wingbeat amplitude and frequency sampled at 1 kHz. We mounted a high-speed video camera (Fastec IL5) with a 4x magnification to view the haltere amplitude and frequency, sampling at 2 kHz. We activated the haltere steering motor neurons during tethered flight using a 250 ms pulse of 625 nm light (M625F2, Thorlabs) at a stimulus intensity of 2.8 W/m^2. We then tracked haltere motion using DeepLabCut[71] and edited with custom MATLAB software. We analyzed all data using custom scripts in MATLAB and Python.

QUANTIFICATION AND STATISTICAL ANALYSIS

We analyzed our imaging and flight behavior data using custom scripts written in Python. For the haltere campaniform imaging experiments, we rigidly registered each image to the mean of all images for the full experiment. We then segmented the mean image of each experiment into two areas of interest, which we interpret as dF1 and dF2, using k-means clustering. After segmenting our

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images, we computed the change in GCaMP fluorescence F_t for each time point. For each campaniform field, we computed the mean baseline fluorescence F_0 for 0.5 s prior to stimulus motion before computing $(F_t-F_0)/F_0$, which we term ''ΔF/F''. We constructed 95% confidence intervals by resampling the population average 1,000 times with replacement from the individual means. To construct tuning curves, we summed each fly's individual mean fluorescence and wingbeat amplitude signals during the 3 s stimulus period for each stimulus direction.

Saccade identification

We recorded wing motion and GCaMP activity while flies flew in near-complete darkness to elicit spontaneous turning maneuvers. To record haltere campaniform or muscle activity during spontaneous saccades, we imaged flies while they attempted to fixate a dark, 30° bar under closed-loop conditions.

We identified saccades using a method previously described.[47,72] Briefly, we first computed the left-minus-right wingstroke amplitude (L-R WBA) and low-pass filtered these data (cutoff frequency of 6 Hz). We then took the derivative of the filtered signal and identified steering events as the local maxima and minima. We defined saccades as the maxima and minima that exceeded both velocity and magnitude thresholds. We then identified the signatures of spontaneous saccades in the haltere campaniform fields and muscles by finding the fluorescence signals that corresponded to a given saccade.

For each saccade, we calculated the mean change in L-R WBA for the 100 ms windows preceding and following each event, triggered at the peak. We then sorted these data to compute decile means. We conditioned our wingbeat amplitude signals by baseline-subtracting the mean of the first 250 ms of a given decile mean. We used this same window preceding the peak in L-R WBA to calculate ΔF/F for the haltere afferent terminals and steering muscles.