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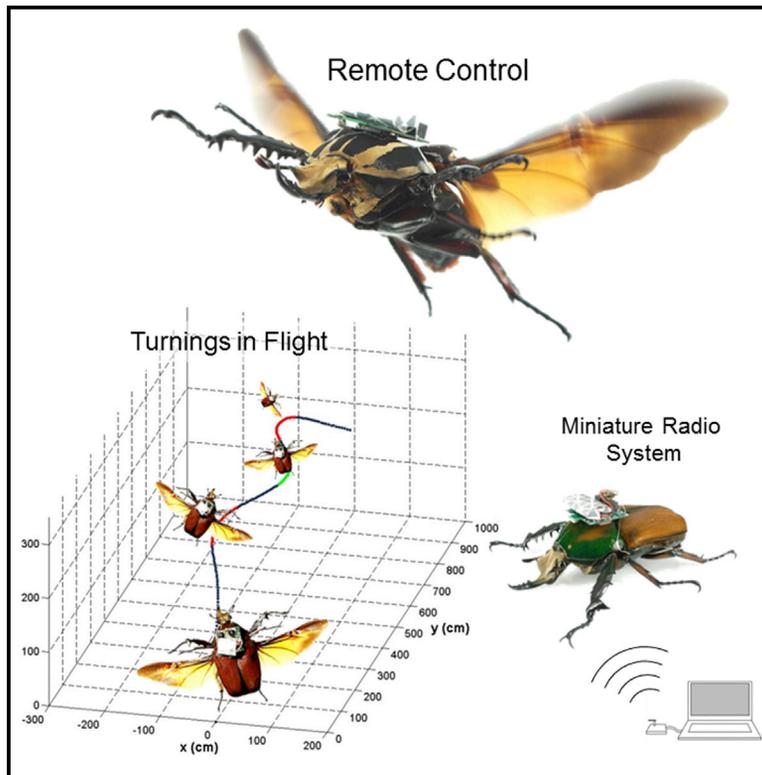
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Deciphering the Role of a Coleopteran Steering Muscle via Free Flight Stimulation

Graphical Abstract



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In Brief

The coleopteran third axillary muscle, also known as the wing-folding muscle, which has been known to function in wing folding since the 19th century, plays a key function in left-right steering. Sato et al. demonstrate graded and controlled turns by stimulating the muscle in free-flying insects using a miniature radio system.

Highlights

- Developed a miniature radio system to remotely stimulate free-flying insects
- Graded and controlled turns in free-flying insects by electrical stimulation
- Demonstrated the coleopteran third axillary muscle is tonically activated ipsilaterally during turns



Deciphering the Role of a Coleopteran Steering Muscle via Free Flight Stimulation

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Summary

Testing hypotheses of neuromuscular function during locomotion ideally requires the ability to record cellular responses and to stimulate the cells being investigated to observe downstream behaviors [1]. The inability to stimulate in free flight has been a long-standing hurdle for insect flight studies. The miniaturization of computation and communication technologies has delivered ultra-small, radio-enabled neuromuscular recorders and stimulators for untethered insects [2–8]. Published stimulation targets include the areas in brain potentially responsible for pattern generation in locomotion [5], the nerve chord for abdominal flexion [9], antennal muscles [2, 10], and the flight muscles (or their excitatory junctions) [7, 11–13]. However, neither fine nor graded control of turning has been demonstrated in free flight, and responses to the stimulation vary widely [2, 5, 7, 9]. Technological limitations have precluded hypotheses of function validation requiring exogenous stimulation during flight. We investigated the role of a muscle involved in wing articulation during flight in a coleopteran. We set out to identify muscles whose stimulation produced a graded turning in free flight, a feat that would enable fine steering control not previously demonstrated. We anticipated that gradation might arise either as a function of the phase of muscle firing relative to the wing stroke (as in the classic fly b1 muscle [14, 15] or the dorsal longitudinal and ventral muscles of moth [16]), or due to regulated tonic control, in which phase-independent summation of twitch responses produces varying amounts of force delivered to the wing linkages [15, 17, 18].

Results and Discussion

Similar to other insects, *Mecynorrhina torquata* beetles have a small muscle inserted into the third axillary (3Ax) sclerite, an

articulation near the wing base. The 3Ax muscle of the beetle is located between the basalar and subalar muscles (Figures 1 and S1). In beetles, this muscle has often been referred to as the wing-folding muscle, a name dating to 19th century observations that 3Ax muscle contraction pulls on the 3Ax dorsally and inward, which in these insects tends to pull the wing into a folded posture [19–21]. The 3Ax muscles of other insects are known to play a role in stabilizing flight or steering [14, 17, 18, 22, 23]. Electromyogram (EMG) recordings of the 3Ax muscle of the beetle show that, although it fires during wing folding (Figure 2A), it does not always do so (~30% or 66 of 216 tests, Figure 2B); this implies that the 3Ax muscle is not required for wing folding.

The 3Ax muscle is easily isolated and removed in *Mecynorrhina* (Figures 1 and S1). In all cases (N = 5 beetles, n = 267 trials), removal of the cuticle above the 3Ax muscle did not affect the beetles' flight, and 3Ax muscle surgery did not prevent beetles from folding or unfolding their wings, nor did it appear to interfere with wing oscillations (Figure 2C). However, beetles (N = 5 beetles, n = 280 trials) lost the ability to steer and maneuver in free flight after the 3Ax muscle was isolated and removed (Movie S2; Figure 2C). These experiments further imply that the 3Ax muscle is not always required for wing folding but could be involved in flight course control.

Tonic 3Ax muscle firing occurs during visually induced ipsilateral turns and is correlated with a reduction in ipsilateral wing beat amplitude. In order to examine the behavior of the 3Ax muscle and 3Ax during turns, we unilaterally recorded 3Ax muscle EMGs during visually induced fictive turns in tethered beetles. High-speed videos (3,000 frames per second) were used to map EMG recordings to the stroke cycle and map the wing tip trajectory (Figures 3 and S2). Wide-field optic flow patterns moving either left or right (black and white stripes) produced strong optomotor responses, causing them to turn left or right, respectively, to track optic flow. The latency of optomotor response was found to be 0.81 ± 0.42 s, which is consistent with the slow and variable optomotor responses found in other insects [24, 25] (N = 17 beetles, n = 495 tests). All beetles in all cases (N = 17 beetles, n = 226 bursts) activated the 3Ax muscle on the side ipsilateral to the turn, with few spikes occurring in the contralateral 3Ax muscle (Figures 3A and 3B). This result is consistent with the hypothesis that the 3Ax muscle plays a role in course corrective turns and demonstrates that the 3Ax muscle is activated during a visually induced ipsilateral turn. In contrast, previously studied coleopteran flight muscles, such as basalar flight muscle, are activated during both ipsilateral and contralateral turns, with the firing rate varying depending on the direction [26–28]. Although the 3Ax muscle spikes showed a slight preference for phase, we found they occurred throughout the wing cycle (Figure S2). This is similar to the weak preference found in the tonic steering muscles III1 and I1 in the fly [14] and in contrast to the phasic steering muscles found in flies [14], moths [22, 23], and locusts [18], where the muscle shows clear preferred firing phases. The muscles in the latter case produce turning by altering the relative phase between the muscle's activation and the wing stroke cycle [18, 22, 23]. Thus, our results are in accord with the suggestion that the beetle performs

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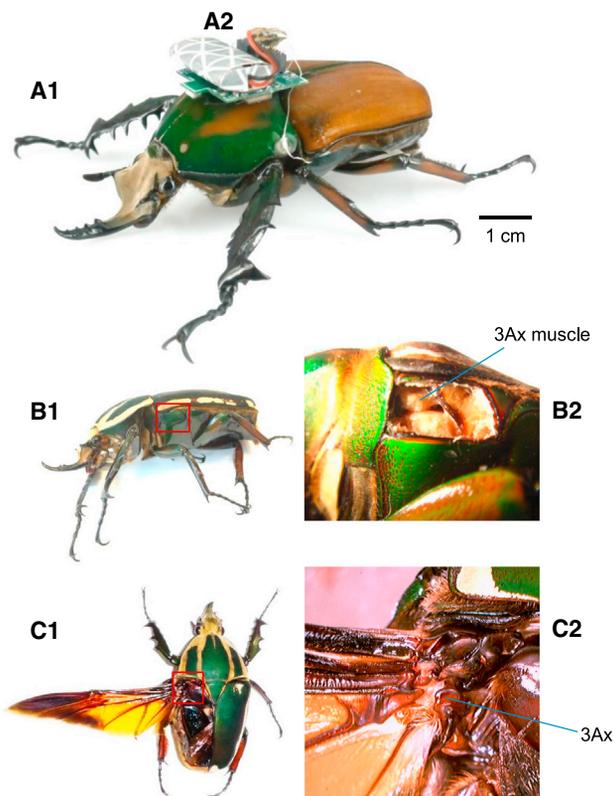


Figure 1. Overview of Device-Mounted Beetle and Anatomy of 3Ax Muscle (A1 and A2) Overview of the miniature wireless muscular stimulator device (A1); Stimulator device mounted on a live beetle (A2). The device consisted of a custom printed circuit board (PCB) on which a microcontroller, battery with a pair of thin wires, and connector were mounted (see also [Figures S4A](#) and [S4B](#)). Four silver wires (127 μm diameter bare, 178 μm diameter Teflon coated) were tightly inserted into the headers, which were mounted on the PCB and electrically connected to the outputs of the micro-processing unit (MPU). The other terminals of the wires were implanted into the left and right wing-folding muscles (3Ax muscle, working electrodes) and the mesothorax center hemolymph (counter electrodes). (B1 and B2) Lateral view of a beetle (B1); close-up view of the red square domain of (B1) after dissection of a cuticle (B2), showing the flight muscle of 3Ax muscle (see also [Figure S1](#)). Top view of a beetle after the left elytra was removed and the hind wing was unfolded (B1), exposing the left wing base indicated by the red square. (C1 and C2) Close-up view of the red square domain of (C1) to show the 3rd axillary sclerite (3Ax) (C2) that was internally and directly connected to the 3Ax muscle and externally connected to the wing base via a tendon.

fine steering not with a single twitch contraction at a specific timing within the stroke cycle but by the summation of multiple twitch contractions (tonic control) that gradates the pull of the wing base. The 3Ax is connected to the wing base via a tendon that allows the 3Ax to transmit force on the wing base ([Figures 1C](#) and [S1B–S1E](#)). Direct extracellular electrical stimulation of the 3Ax muscle in tethered insects via a micro wire had no influence on the behavior of nearby muscles ([Figure S3](#)) but pulled the 3Ax dorsally and inward, as expected. The velocity and displacement of the 3Ax was graded as a function of stimulus frequency ([Figures 4A1](#) and [4A2](#); [Movie S3](#)), suggesting that the 3Ax muscle acts on the 3Ax via graded tonic control arising from the summation of twitch contraction forces.

Turns induced with visual stimuli were associated with either a reduction of the ipsilateral wing stroke amplitude and no

change in contralateral wing stroke amplitude, or with no change in the ipsilateral wing stroke amplitude and an increase in the contralateral wing stroke amplitude ([Figures S2S](#) and [S2T](#)) [[28](#), [29](#)]. An analysis of wing beat trajectory during electrical stimulation of 3Ax muscle shows that 3Ax muscle activation produces a stimulation frequency-dependent reduction of ipsilateral wing stroke amplitude ([Figure 4B](#)); higher stimulus frequency produced a greater reduction in wing stroke amplitude. This reaction is associated with the correlation of EMG firing rate and wing amplitude change during the fictive turn induced by visual stimulation ([Figure S2U](#)). In addition, the wing amplitude did not significantly vary as a function of the firing phase ([Figure S2V](#)).

Remote electrical stimulation of 3Ax muscle enables graded left-right turn control in free flight. Building on these findings, we set out to demonstrate that exogenous stimulation of the 3Ax muscle could produce graded turns in free flight. Our stimulation paradigm was based on three observations: 3Ax muscle firing occurs on the ipsilateral side; firing does not correlate strongly with stroke phase; and reduction of wing stroke amplitude by stimulation of 3Ax muscle is graded with frequency. A radio-enabled backpack ([Figures S4A](#) and [S4B](#)) was mounted on the pronotum, and the left or right 3Ax muscle was stimulated during free flight. Stimulation resulted in clear ipsilateral turns ([Figure 4C](#); [Movie S1](#)). Moreover, the estimated induced lateral force was graded as a function of stimulus frequency ([Figures 4D](#) and [S3E–S3G](#); [Movie S1](#)). The range of the lateral forces induced by the stimulation ([Figure 4D](#)) was of the same order as those arising from natural (unstimulated) turns ([Figure S3H](#)).

While we know of no previous reports detailing a flight function for this muscle in coleopterans, a comparison of these findings with those in moths, locusts, and flies seems to imply a previously unappreciated similarity in function between the coleopteran 3Ax muscle and the muscles that insert into the 3Ax in other insects. In moths, this muscle is active during straight flight and is phase shifted with regards to the dorsal longitudinal muscle during maneuvers [[17](#), [23](#)]. Furthermore, the tonic activation of this muscle maintained a certain degree of remotion and elucidated wing retraction in moth [[17](#), [23](#)]. In locusts, the similar muscle of the forewing has been shown to play a central role in steering and is part of the visual control system. It is activated on both sides during straight flight, and turns are correlated with both phase shifts from the baseline and changes in spike frequency [[18](#)]. In contrast, in dipterans (notably *Calliphora* and *Eristalis*), the 3Ax has been implicated in the control of the alula, a hinged flap present near the base of dipteran wings [[14](#), [30](#), [31](#)]. The alula accounts for a small percentage of fly wing area and is held flat or raised during flight bouts. Engagement of the alula is correlated to switching flight modes (or “gear shifting”) during flight. Moreover, dipterans are capable of operating the gear change mechanism associated with flipping their alula asymmetrically to reduce aerodynamic force, resulting in ipsilateral turning [[30](#), [32–34](#)]. In addition, the I1 muscle of fly was activated with change of firing rate in a weak phasic manner also associated with the reduction of stroke amplitude that would induce ipsilateral turn [[14](#)]. Coleopterans lack an alula, although the wings contain a similar fold near the base [[35](#)]. However, our results show that the 3Ax muscle tonically contracts (on the side ipsilateral to a turn) or does not contract (on the side contralateral to a turn), which is reminiscent of the muscles on the dipteran 3Ax. It is tempting to speculate that coleopterans possess a similar “asymmetrically

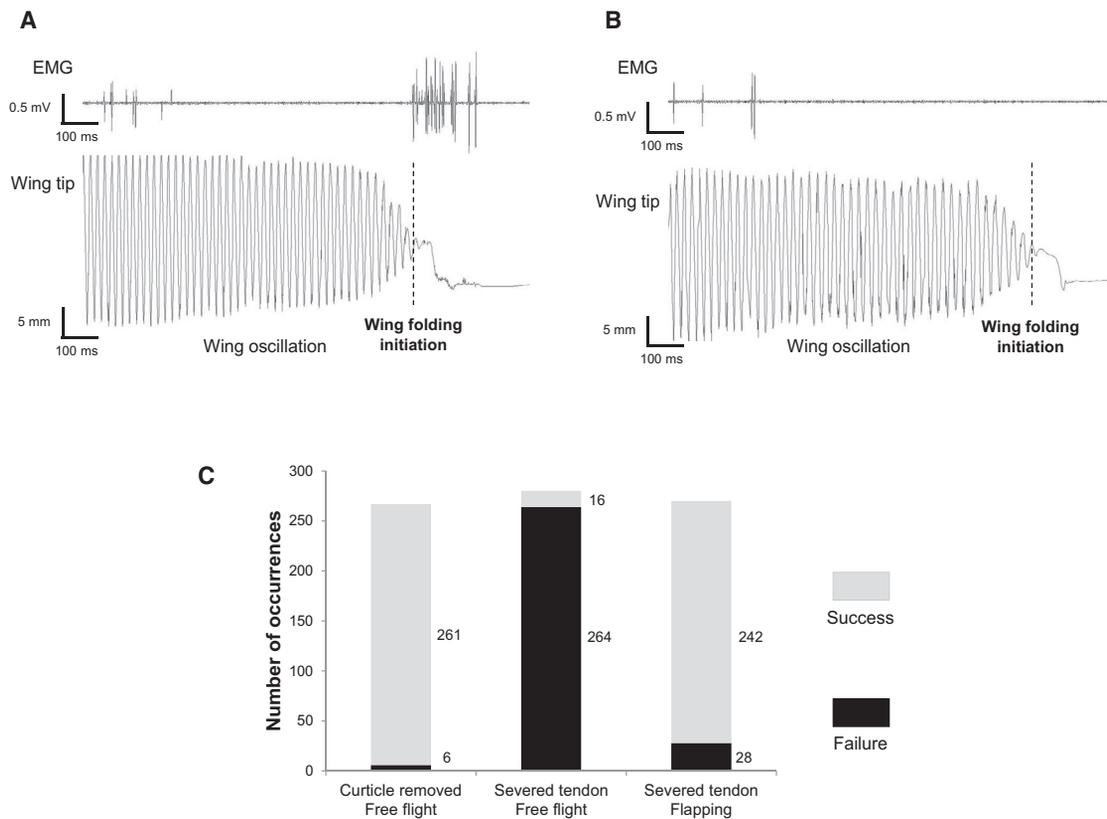


Figure 2. EMG of 3Ax Muscle Measured during Wing Flapping Followed by Wing Retraction and Free Flight Tests after Various Surgeries

(A) The 3Ax muscle was activated during the retraction and folding of the wing. Spikes are present not only during the retraction of the wings into a resting position but also while folding wings under elytra.

(B) Some cases (66 out of 216, $N = 5$ animals, $n = 216$ tests) showed that the 3Ax muscle was not activated during wing retraction and folding. Oscillations in wing tip coordinates indicate flapping. The beetle had one pair of silver wire electrodes implanted into the left 3Ax muscle and a retro-reflective marker attached immediately before the bending zone on each wing at the wing tips; a second marker on the scutellum was used as a reference point.

(C) Severed tendon control for inactivating 3Ax muscle in free flight showed no influence on wing folding/unfolding and flapping but caused the steerage loss. The removal of cuticle had little influence on the free flight since all the beetles ($N = 5$ beetles, $n = 267$ trials) flew well at a rate of 97.5% “successful flight” ($p < 0.01$, binomial test). After 3Ax muscle was cut, all the beetles lost the ability to steer (94.33%, $p < 0.01$, binomial test) (Movie S2), although they still showed high capability and motivation in flapping wing (89.45%, $p > 0.01$, binomial test) ($N = 5$ beetles, $n_{\text{flight}} = 280$ trials, $n_{\text{flap}} = 270$ trials).

switchable” muscle even lacking the clear “gear shifting” structures present in dipterans.

Conclusions

While insect flight has been examined under tethered conditions traditionally, there have been few studies that test whether tethering introduces artifacts. Here, we have shown that the results from the tethered and free flight experiments are in close agreement. By mounting miniature electronics equipped with radio communication and capable of delivering electrical stimulation on demand, we were able to induce exogenous firing events in flight muscles involved in left-right turnings during free flight. Coupling this data with classical anatomical and electrophysiological studies revealed the role of the 3Ax muscle of beetle in flight. Further experiments could elucidate the effects of the 3Ax muscle contraction on wing dynamics and 3D wing beat trajectory in more detail (for example, focusing on the importance of changes in the angle of attack to carry out rotations). Overall, remote stimulation in free flight enabled by the technology presented provides a powerful experimental paradigm that will likely see increased application as these technologies become universally accessible.

Experimental Procedures

Study Animal

Mecynorrhina torquata (approximately 6 cm, 8 g) beetles were kept in separate terrariums (20 cm × 15 cm × 15 cm) containing woodchips. The beetles were fed a cup of sugar jelly every 2–3 days. The temperature and relative humidity in the terrariums were maintained at 25°C and 60%, respectively. The use of this animal is permitted by the Agri-Food and Veterinary Authority of Singapore (AVA; HS code: 01069000, product code: ALV002). Invertebrates, including insects, are exempt from ethics approval for animal experimentation according to the National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines.

Electrode Implantation

A beetle was anesthetized by being placed in a small chamber containing CO₂ for 1 min; it was then placed on a wooden plate and immobilized with dental wax (Set Up Modeling Wax, Cavex), which had been softened in hot water (80°C) for 10 s. The dental wax was molded gently around the beetle to ensure it was immobilized. A small hole was made in the cuticle (exoskeleton) above the wing-folding muscle using an insect pin (enamel-coated #5, Indigo Instruments). One side of an approximately 10-cm segmented Teflon-insulated silver thin wire (127 μm diameter bare, 178 μm diameter coated; A-M Systems) was flamed to expose the bare silver; the exposed end was then implanted into the small hole to a depth of 3 mm. After insertion, melted beeswax was dropped onto the implanted site. The beeswax cooled quickly, solidified, and fixed the implanted wire.

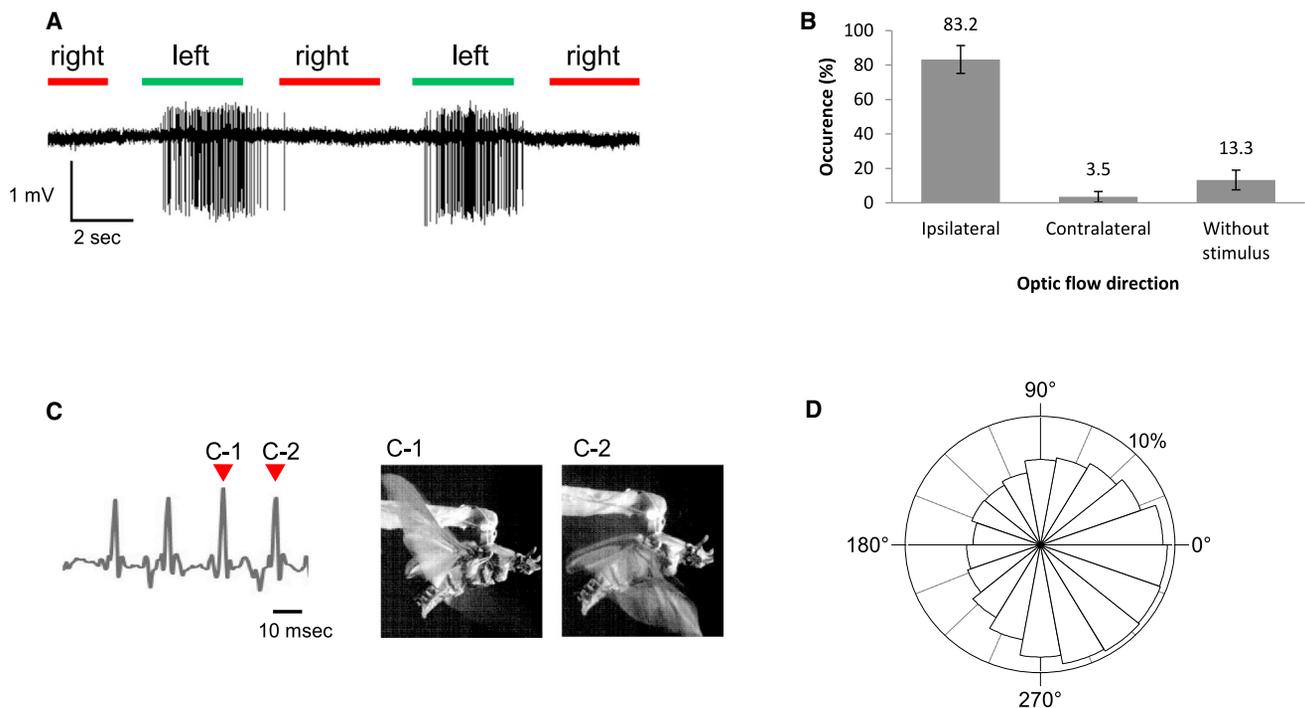


Figure 3. EMG of 3Ax Muscle in Tethered Flight Experiment

(A) Representative EMG of left 3Ax muscle measured during left and right turns induced by the visual stimulation (projected movie of black and white stripes moving to the left and right, alternately). The 3Ax muscle was activated while the beetle turned to the left. The same tendency was confirmed for the corresponding right side 3Ax muscle. EMG spikes were seen from the left 3Ax muscle during the left turn while no spikes appeared during either right turns or no turns (no moving in the stripes) (and vice versa for the right 3Ax muscle). The green and red bars indicate timings of the visual stimulation for left and right turns, respectively.

(B) The spikes appeared during the ipsilateral flow of the pattern at a rate of 83.2% while that of the contralateral one was 3.5% and without stimulation was 13.3%. The error bars represent SD ($N = 17$ beetles, $n = 14,476$ spikes).

(C–C2) Wing beat trajectories were filmed at 3,000 frame per second and synchronized with EMG of 3Ax muscle, indicating the phase of the wing beat at which each spike occurs. For instance, the spikes indicated by the arrows (C) were fired at the timings of wing beat shown in (C1) and (C2), respectively.

(D) Histogram of wing beat phase (0° : beginning of downstroke or end of upstroke; 180° : end of downstroke or beginning of upstroke) at timing when muscle spikes were fired ($N = 17$ beetles, $n = 14,476$ spikes/226 bursts).

To measure the muscle potential (unilateral EMG), we connected the exposed (non-implanted) end of the implanted wire to the input pin of a battery-driven CC2431 Microprocessor Development Kit board using alligator clips. The data acquired by the board were transferred wirelessly to a computer via a guidance and inertial navigation assistant (GINA, provided by Professor Kris Pister's laboratory at University of California, Berkeley) base station (using the 2.4-GHz IEEE 802.15.4 wireless standard). In the free flight control experiments (i.e., electrical stimulation in free flight), a wireless backpack was mounted on the pronotum of the beetle using a piece of double-sided sticky tape after the insertion of the wire (see "Wireless Backpack Assembly" below). The wire was then cut to a length sufficient to allow it to be extended from the implant site to the female connector on the wireless backpack. The non-implanted end of the wire was then flamed to expose the wire silver and inserted into the appropriate pinhole of the female connector so the wire was connected to the appropriate output pin of the backpack microcontroller. The resistance of the recording electrodes was estimated from the measured current (Figure S3A) as 69.4 kOhm ($SD = 12.4$ kOhm, $N = 3$ beetles, $n = 150$ trials).

Wireless Backpack Assembly

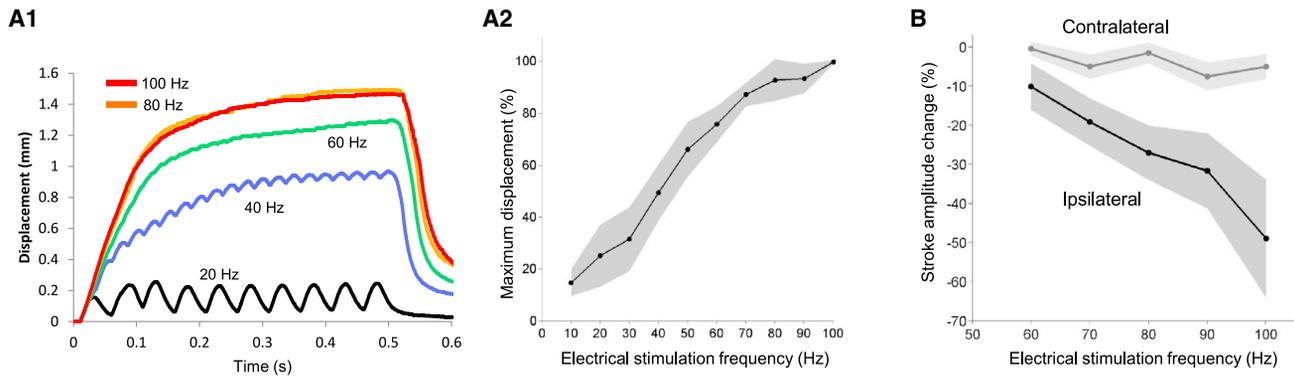
The circuit diagram and photographs of the radio frequency (RF) receiver used for *M. torquata* are shown in Figures S4A and S4B. The RF system comprised two Chipcon Texas Instruments CC2431 microcontrollers (6×6 mm, 130 mg, 32 MHz clock, 2.4-GHz IEEE802.15.4-compliant RF transceiver), one of which acted as the beetle-mounted RF receiver while the other was used as a computer-driven RF transmitter base station. Based on the circuit diagram, we designed and manufactured a custom printed circuit board (PCB; FR4 [rigid], 500 mg) for the receiver. The microcontroller and other components, including the surface-mounted antenna, resistors, capacitors, inductors, oscillator, and two female wire connectors, were

assembled on the PCB, as shown in Figure S4B. The microcontroller was then loaded with a custom-made signal generating software (BeetleBrain v.0.99b). The backpack was driven by a rechargeable micro lithium ion battery (Micro Avionics, 3.9 V, 350 mg, 8.5 mAh). The battery was wrapped with retro-reflective tape (Silver-White, Reflexite), which was required by the 3D motion capture system (VICON, see Remote Radio Control of a Freely Flying Insect under the Experimental Procedures). The anodic and cathodic terminals of the battery were soldered and electrically connected to a two-pin male connector via two thin wires. When the wireless backpack was in use, the male connector was plugged into the female connector of the PCB such that the anodic and cathodic terminals of the battery were connected to the ground (GND) and digital voltage supply (DVDD) pins of the microcontroller, respectively. The backpack has a mass of 1.2 ± 0.26 g, including the battery.

Remote Radio Control of a Freely Flying Insect—Electrical Stimulation during Free Flight

The flight commands were generated by custom control software (BeetleCommander v.1.8e), which ran on a personal computer, via a serial port interface with the GINA base station. BeetleCommander v.1.8e facilitated the in-flight control of the frequency (10–100 Hz) and the number of pulses of the electrical stimulus (monophasic square pulse, 3-ms pulse width, 3.5 V amplitude) at the stimulated sites. The measured current showed a mean of $43.2 \mu\text{A}$ ($SD = 9.4 \mu\text{A}$, $N = 3$ beetles, $n = 150$ trials) (Figure S3A). Such electrical stimulation of 3Ax muscle was confirmed to have no influence on the nearby muscles (Figures S3B–S3D) ($N = 5$ beetles, $n = 400$ tests). The command signals were transmitted using CC2431's built-in 2.4-GHz IEEE 802.15.4-compliant RF transceiver, which was broadcast on a single channel (1A, 2.480 GHz) using direct sequence spread spectrum RF modulation. The transmitter sent a command to the receiver every 1 ms for

Tethered experiments



Free flight experiments

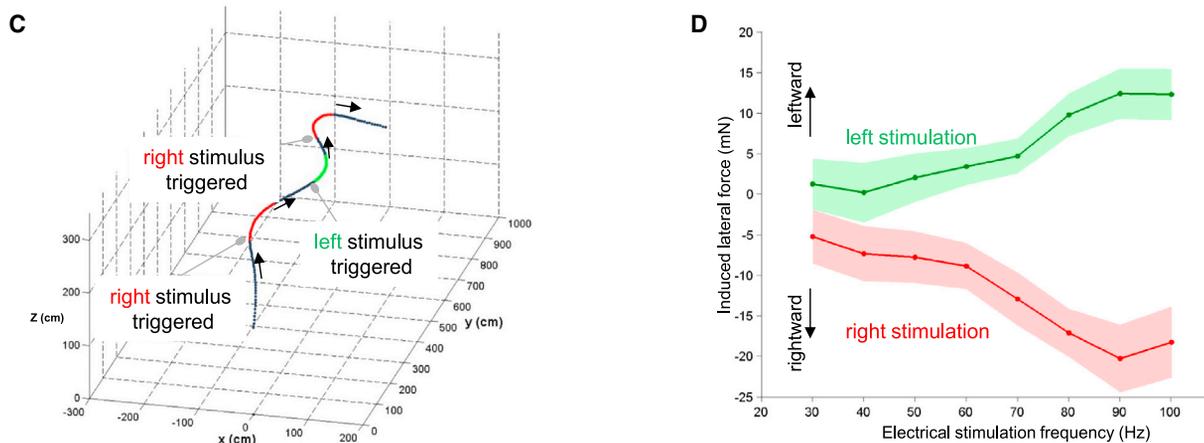


Figure 4. Responses of Beetles to Electrical Stimulation of 3Ax Muscle in Tethered and Free Flight Experiments

(A1) Displacement of the 3Ax sclerite in response to electrical stimulation of the 3Ax muscle for 500 ms at different stimulus rates (20, 40, 60, 80, and 100 Hz). (A2) Maximum displacement of the 3Ax in response to electrical stimulation of the 3Ax muscle ($N = 5$ beetles, $n = 150$ trials). The displacement of 3Ax is normalized by the largest (mechanical limitation of) displacement, which was obtained at the 100-Hz stimulation frequency. The shade area denotes 95% confidence interval.

(B) Reduction of the stroke amplitude in response to electrical stimulation of the wing-folding muscle at different rates (60–100 Hz, $N = 5$ beetles, $n = 91$ trials). The ipsilateral stroke amplitude was reduced in a graded manner when the wing-folding muscle was stimulated, whereas the contralateral stroke amplitude was retained or fluctuated by an insignificant amount. The shade areas denote 95% confidence interval.

(C) Electrical stimulation of the left 3Ax muscle for left turn and the right 3Ax muscle for right turn in sequence produced a zigzag flight path. The black trajectory segments indicate no stimulation periods; red and green trajectory segments indicate right and left stimulation periods, respectively.

(D) Lateral force (F_l) induced by the electrical stimulation of 3Ax muscle was graded as a function of stimulus frequency, with the most effective range from 60 Hz to 90 Hz ($N_{\text{right}} = 12$ beetles, $n_{\text{right}} = 758$ trials; $N_{\text{left}} = 10$ beetles, $n_{\text{left}} = 810$ trials). The shade areas denote 95% confidence interval. Values of F_l are positive when the direction of force is toward the left and negative when the direction is toward the right.

300 ms, as required. The flight commands were mapped to the appropriate amplitude pulse trains of the wireless backpack by BeetleBrain v.0.99b, which ran on the receiver. The applied amplitude of 3.5 V, which differed from the original voltage supplied (3.9 V) by the lithium ion battery, was regulated via pulse width modulation (PWM), a built-in functionality of the micro-controller. During free flight experiments, a Nintendo Wii remote was used to issue left or right and sleep or awake commands that were communicated to the base station using the Wii remote's bluetooth transceiver.

After the wireless backpack was mounted on the pronotum of the beetle and the implanted wire electrodes were set in the female connector of the backpack, the beetle was gently released into the air in a closed room (Figure S4C), and the beetle started to fly. The room was equipped with a 3D motion capture system (VICON) that comprised eight T160 cameras. The system detected the retro-reflective tape marker (see "Wireless Backpack Assembly" above) on the flying beetle and fed the 3D coordinates in real time to a nearby computer, which was synchronized using BeetleCommander v.1.8e. This software was used to visualize the flight coordinates (flight path) of the insect as well as the electrical stimulation

type and timing. Different colors on the plots indicated stimulation events or normal flight (i.e., black trajectory segments indicated periods with no stimulation, whereas red and green segments indicated electrical stimulation of the beetle's right and left muscular sites, respectively, as shown in Figure 4C).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2015.01.051>.

Author Contributions

H.S., M.M.M., and T.T.V.D. designed the research; H.S., K.I., P.A., and M.M.M. provided substantial technical and experimental design advice; S.K. developed the hardware; S.K., N.A.H., and C.Z. developed the

software; H.S., T.T.V.D., S.K., and T.L.M. conducted the experiments; H.S., T.T.V.D., S.K., J.v.K., and M.M.M. analyzed and interpreted the data; and H.S., T.T.V.D., T.L.M., J.v.K. and M.M.M. wrote the paper.

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