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Female pheromones modulate flight muscle activation patterns during preflight warm-up

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Crespo JG, Vickers NJ, Goller F. Female pheromones modulate flight muscle activation patterns during preflight warm-up. *J Neurophysiol* 110: 862–871, 2013. First published May 22, 2013; doi:10.1152/jn.00871.2012.—At low ambient temperature *Helicoverpa zea* male moths engage in warm-up behavior prior to taking flight in response to an attractive female pheromone blend. Male *H. zea* warm up at a faster rate when sensing the attractive pheromone blend compared with unattractive blends or blank controls (Crespo et al. 2012), but the mechanisms involved in this olfactory modulation of the heating rate during preflight warm-up are unknown. Here, we test three possible mechanisms for increasing heat production: *1*) increased rate of muscle contraction; *2*) reduction in mechanical movement by increased overlap in activation of the antagonistic flight muscles; and *3*) increased activation of motor units. To test which mechanisms play a role, we simultaneously recorded electrical activation patterns of the main flight muscles (dorsolongitudinal and dorsoventral muscles), wing movement, and thoracic temperature in moths exposed to both the attractive pheromone blend and a blank control. Results indicate that the main mechanism responsible for the observed increase in thoracic heating rate with pheromone stimulation is the differential activation of motor units during each muscle contraction cycle in both antagonistic flight muscles. This additional activation lengthens the contracted state within each cycle and thus accounts for the greater heat production. Interestingly, the rate of activation (frequency of contraction cycles) of motor units, which is temperature dependent, did not vary between treatments. This result suggests that the activation rate is determined by a temperature-dependent oscillator, which is not affected by the olfactory stimulus, but activation of motor units is modulated during each cycle.

insect; behavior; muscle physiology; olfaction; thermobiology

MANY INSECTS OF MEDIUM TO large size have evolved to take advantage of the metabolic heat produced during flight muscle contraction for regulating their thoracic temperature (Heinrich 1974). The main antagonistic flight muscles, the dorsolongitudinal wing depressors (DLMs) and the dorsoventral wing elevators (DVMs) are also the main heat source prior to take-off (Kammer 1970). Their kinetics are, like that of other invertebrate and vertebrate muscles, strongly affected by temperature. For example, increased temperature accelerates muscle contraction rates (Bennet 1985; Josephson 1984) and, consequently, increases mechanical power output (Josephson 1999; Stevenson and Josephson 1990). The linear increase of thoracic temperature during the warm-up behavior has been attributed mainly to the acceleratory effect of higher temperature on various aspects of the contraction cycle, muscle metabolism, and the central nervous system activity (Kammer 1981). Kammer (1981) proposed that the rate of heat production is not regulated during warm-up, but should occur at its maximal rate. However, heat production during natural behavior is not always maximal for a given thoracic temperature. For example, in male moths heat production during preflight warm-up is modulated by olfactory information. When the female pheromone is present, male moths heat at higher rates than without pheromone, suggesting that modulation of thoracic heat production occurs during natural behavior (Crespo et al. 2012), but the mechanisms for modulating heat generation are not known.

Muscles involved in flight behavior include indirect, direct, and accessory muscles. The indirect muscles (DLMs and DVMs) generate wing movement by deforming the thoracic box, providing, in many insect orders (including Lepidoptera), much of the power for wing strokes. In general, direct (e.g., basalar, subalar, and axillary) and accessory muscles (e.g., pleurosternal and tergopleural) influence wing pronation-supination and the mechanical properties of the thorax, respectively. However, little is known about the function of these muscles during the preflight warm-up (but see Kammer 1968). During the preflight warm-up behavior of Lepidoptera, DLMs and DVMs as well as several (and probably most) of the direct flight muscles (e.g., subalar, tergosternal, anterior and posterior tergocoxals, and dorso oblique; Kammer 1968) are activated almost simultaneously (Fig. 1), generating only small-amplitude wing movements (as opposed to the alternate contraction seen in flight; Kammer 1981). It is thought that more simultaneous activation reduces work and maximizes heat generation (Kammer 1981).

The innervation of flight muscles has been studied in insect orders with synchronous (e.g., Orthoptera: Bentley 1970; Lepidoptera: Kondoh and Obara 1982) and asynchronous (e.g., Diptera: Coggshall 1978; Hymenoptera: Ikeda and Boettiger 1965a; Coleoptera: Ikeda and Boettiger 1965b; Hemiptera: Davis 1977) flight muscles. Most studies have focused on the DLMs which in moths consist of three distinct groups of muscles, the $DLM_{1a\text{-}e}$, DLM_2 (or dorsal oblique muscles), and DLM_3. In *Helicoverpa zea*, the moth utilized in the present study, there are two nerves (IIN1B and IIIN1B) with seven (6 ipsilateral and 1 contralateral) and six (5 ipsilateral and 1 contralateral) motoneurons, respectively, that innervate all the DLMs on each side of the thorax (Orona and Agee 1987, 1988). Even though the precise connection between motoneurons and specific DLMs is not known, nerve IIN1B has been shown to innervate the DLMs of the forewing, while nerve IIIN1B innervates these same muscles but of the hindwing. It is not known how the DLMs of each side, i.e., those that move the ipsilateral forewing and hindwing, are coupled to work together during both flight and warm-up activities.

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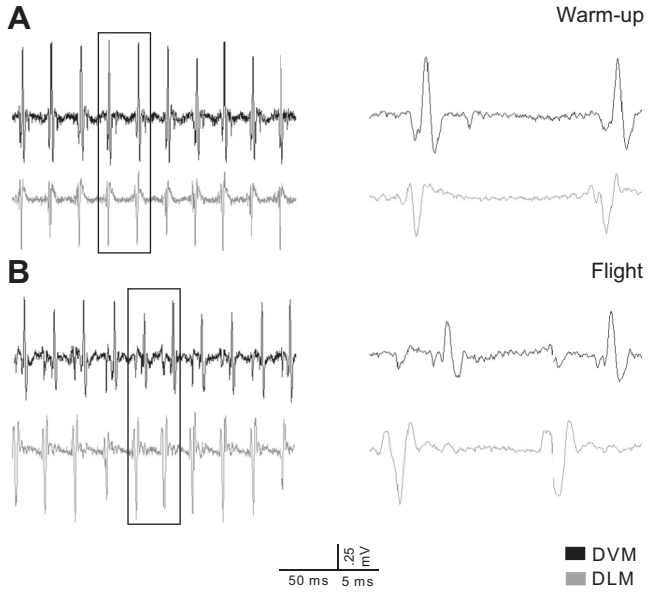

Fig. 1. Motor activation patterns of indirect flight muscles differ between warm-up (*A*) and flight (*B*). This example shows activation during both conditions in the same individual. *A*: more synchronized activation of antagonistic flight muscles (DLM: dorsolongitudinal muscles, and DVM: dorsoventral muscles) during warm-up behavior. *B*: out-of-phase activation of antagonistic flight muscles during flight. Box indicates the region of electromyogram traces that is expanded on the *right*.

In contrast, the neural control of DVMs has received less attention. DVMs include an anterior and two posterior tergocoxals, one or a few tergosternals, a tergotrochanteral, and an extracoxal depressor (whose function in flight is unknown) muscle (Kammer 1985), and almost all of these are involved in the preflight warm-up behavior seen in moths (Kammer 1968). In *H. zea*, nerves IIIN1A and -B with six (five ipsilateral and one contralateral) and three (two ipsilateral and one contralateral) motoneurons, respectively, have been shown to innervate the DVMs (Orona and Agee 1988). Again, no information regarding the synchronization of these muscles during flight and warm-up behaviors has been collected so far.

Multiterminal and polyneuronal innervation in insects allows for direct, complex control of the main flight muscles. Such innervations patterns have been documented in the DLMs of a katydid (Stokes et al. 1975), as well as in the DVMs of a bumblebee (Ikeda and Boettiger 1965a). Thus, although the small number of motor units may limit fine control of muscle activation as seen in vertebrate skeletal muscle (Belanger 2005), the diversity of motoneurons might facilitate the sophisticated modulation of their activation. Furthermore, in the moth *Manduca sexta*, different subunits of DLM_1 are activated nearly simultaneously and vary in power production according to their dorsoventral position in the thorax (George and Daniel 2011; George et al. 2012). As opposed to ventrally located subunits, which produce positive power, cooler dorsal subunits act as springs (George et al. 2012). Hence, besides the variability in motoneuron innervation present in the muscles of insects, the distinct functional roles of the different DLM subunits might also contribute differentially to heat production during warm-up.

Multimodal sensory information is used to modulate motor function for locomotion and navigation in the environment, and this modulation is facilitated by the somewhat complex activity of descending neurons and motoneurons innervating the flight muscles. Motor patterns of indirect wing depressor and elevator muscles have been shown to change when insects are exposed to different sensory stimuli. For example, in the noctuid moth *Barathra brassicae*, a rhythmically active DLM motoneuron sometimes responds to ultrasonic stimulation by firing two spikes per cycle, as opposed to only one spike when no stimuli were present (Madsen and Miller 1987). Also, when looming stimuli were presented to tethered flying *Locusta migratoria*, the mesothoracic tergosternal muscles (indirect wing elevators) showed a sudden increase in firing activity during each wingbeat cycle (Santer et al. 2005, 2006), indicating that behavior is changed through sustained muscle contraction when specific sensory information is present. Sensory information may also play a role in the preflight warm-up behavior. Pheromones signaling the presence of a female during the warm-up of the moth *H. zea* induce males to start warming up earlier and faster (Crespo et al. 2012), but it is not known how changes in motor control affect modulation of the heating rate. With this current study, we aim to explore how motor control of flight muscles is altered to modulate heat production. We investigate to what degree three putative motor control mechanisms contribute to the observed increase in heating rate in the pheromone-stimulated males: *1*) increase in the rate of flight muscle contractions; *2*) increased heat production as the result of minimizing mechanical energy use through more simultaneous contraction of the antagonistic flight muscles; and *3*) differential activation of motor units.

MATERIALS AND METHODS

Insects. H. zea (Boddie 1850) were reared on a modified pinto-bean diet (Shorey and Hale 1965) at the University of Utah. Pupae were sexed and placed in separate environmental chambers (Percival Scientific, Boone, IA) at 25°C and 60% relative humidity on a 14:10-h light-dark photoperiod until adult emergence. Male moths were aged daily and separated in plastic containers with access to a 9% sucrose solution ad libitum. Virgin males 2–6 days of age were used in the experiments, which were conducted between the third and seventh hour of the scotophase (Vetter and Baker 1984). A single red incandescent light bulb positioned above the experimental setup provided

Table 1. *Individual values for linear regression parameters estimated from the temperature-dependent change in muscle potential frequency for both treatments (pheromone present or absent)*

	No Odor			Odor		
Individual	Slope	Intercept	R^2	Slope	Intercept	R^2
1	6.38	−111.66	0.98	4.92	−78.05	0.95
2	9.68	−70.81	0.90	6.40	−116.65	0.95
3	10.83	−215.67	0.92	9.03	−175.41	0.91
4	3.95	−53.96	0.95	5.13	−82.64	0.96
5	7.06	−125.70	0.86	9.69	−182.12	0.88
6	4.18	−61.78	0.54	7.12	−128.37	0.96
7	5.60	−100.09	0.87	8.03	−56.69	0.94
8	6.68	−121.06	0.98	6.85	−125.06	0.98
9	5.69	−96.18	0.94	4.99	−78.91	0.93
10	4.41	−63.73	0.98	5.24	−85.37	0.97
Mean	6.45	−102.06		6.74	−110.93	

There was no significant difference in the means of slopes [$t(9) = -0.445$; $P = 0.67$] and intercepts [$t(9) = 0.683$; $P = 0.51$] of both conditions as compared by a paired Student's *t*-test.

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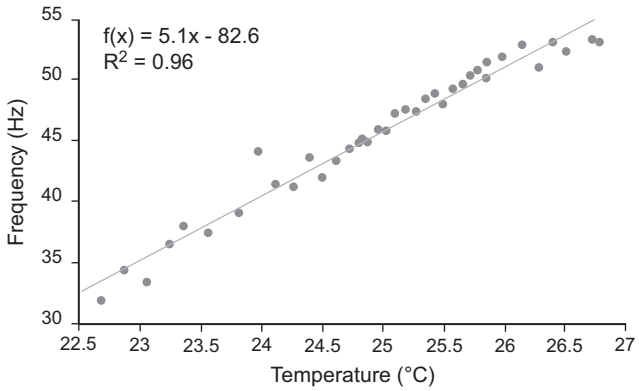

Fig. 2. The rate of flight muscle activation cycles increases linearly with increasing thoracic temperature during warm-up and is independent of the presence of female pheromone.

illumination for the experiments. Up to two males per day were prepared, left to acclimatize for 60 min, and tested under two conditions (see *Pheromone blend* section). Moths were given a small styrofoam ball (2.5 cm diameter) to hold onto, and recordings were automatically started when the electromyogram (EMG) signal surpassed a preset threshold indicating the initiation of warm-up behavior. Short flight bouts were recorded by gently pulling the styrofoam ball away from the moth after warm-up activity had been recorded.

Pheromone blend. Concentrated solutions of *cis*-11-hexadecenal (Z11–16:Ald) and *cis*-9-hexadecenal (Z9–16:Ald; Sigma-Aldrich, St Louis, MO) were maintained at −20°C and used to make dilutions in hexane of 100 ng/μl for Z11–16:Ald and 10 ng/μl for Z9–16:Ald (Vickers et al. 1991). Capillary gas chromatography (Shimadzu GC 17A, Shimadzu Scientific Instruments, Columbia, MD) was used to check all dilutions. Odor was prepared with respect to Z11–16:Ald (main pheromone component of *H. zea*), which was always 1000 ng (denoted 1) and loaded onto a 1-cm-diameter filter paper disk (Whatman no. 4). Ratio of components for the pheromone treatment was as follows: 1:0.05 Z11–16:Ald to Z9–16:Ald.

We tested moths with two treatments: *1*) conspecific female pheromone, Z11–16:Ald + Z9–16:Ald and *2*) no pheromone (i.e., hexane). Both treatments were sequentially, but randomly, applied to each tested moth. After the blend or, in the no-pheromone case, hexane was applied to the filter paper, hexane was allowed to evaporate in the fume hood. After evaporation was completed, the disk was mounted with an alligator clip at a distance of 9 cm upwind from the moth. Odors were delivered by an ~45 cm/s wind and vented out of the building at the downwind end via an exhaust duct.

Simultaneous force, temperature, and muscle activity measurements. Moths were inserted into a plastic pipette tip with the narrow end cut to allow for the male's head to pass through. Dental wax was used to immobilize the head. A small window cut on the side of the pipette tip made the dorsal part of the thorax accessible. As few dorsal thoracic scales as possible were removed to wax (Ted Pella, Redding, CA) the EMG leads, thermocouple, and entomological pin (size 2) to the dorsal surface of the thorax (see below). Once the moth was prepared, it was taken out of the tube and left to acclimatize in the rig before the recordings began.

EMG leads were constructed of polyimide-insulated 25-μm stainless steel wire (California Fine Wire, Grover Beach, CA), and the electrode tips were inserted 0.3 mm into the thorax. To record the activity of the DVMs, electrodes were placed ~0.2 mm left to the right edge and ~0.6 mm behind the anterior edge of the thorax, and for DLMs, ~0.2 mm right to the midline and ~0.6 mm behind the anterior edge of the thorax. After the experiments, males were dissected to confirm the placement of the EMG electrodes in the respective muscles.

To monitor thoracic temperature, we constructed a copper-constantan thermocouple from 75-μm wire (Omega Engineering, Stamford, CT) and waxed it to the surface of the thorax to measure thoracic surface temperature (afterwards calibrated to core thoracic temperature as in Crespo et al. 2012). The thermocouple was connected to a thermometer (Bat-12; Physitemp Instruments, Clifton, NJ), and its analog output (±0.2°C error) recorded onto the computer. The entomological pin was used to fix the moth to a force transducer (force displacement transducer FT03, Grass Technologies, West Warwick, RI) and measure vertical force production. EMG signals were amplified (×3,000) and band-pass filtered (100–3,000 Hz) with a Brownlee Precision 440 amplifier (Brownlee Precision, San Jose, CA). Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany) was used to record all four channels simultaneously to a computer at 10 kHz via a multichannel A/D board (BNC 2110, National Instruments).

Data analyses. The number of muscle potentials was counted by taking at least 20 muscle contraction cycles for each temperature value. Under the experimental conditions, moths did not heat beyond a thoracic temperature of 27.5°C. Individual slope and intercept values for temperature-related changes in muscle potential frequency were estimated from linear fits to the data points, and means for the two treatments (see *Pheromone blend* section) were compared by a paired Student's *t*-test. Since greater vertical force is produced at larger flapping amplitudes (e.g., Wu and Sun 2008), this measurement was used as a proxy for wing-flapping amplitude. We recorded data from 15 individuals, but only 10 individuals were included in these analyses (see Table 1), because lack of data over a sufficient range of thoracic temperatures did not allow a reliable estimate of the slopes

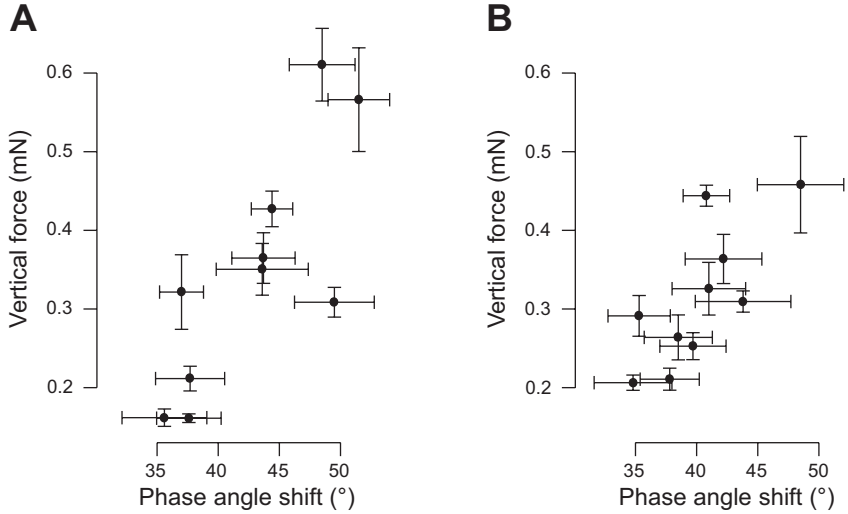

Fig. 3. Phase shifts in flight muscle activation patterns during warm-up are correlated with the generation of vertical force with no pheromone (*A*) and pheromone present (n = 10; *B*). Vertical force is used as a proxy for wingbeat amplitude. Within individuals no significant temperature-dependent variation was observed for either of the two variables (repeated-measures ANOVA, $P > 0.05$). Individual data points are means ± SE for individual moths.

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and intercepts. Thus we only utilized the data of these 10 individuals throughout the rest of the analyses.

Phase-angle shifts were calculated from the simultaneous EMG recordings of the two antagonistic flight muscles in the same individual during the two treatments in the following way. First, we determined the period of the wing cycle by using the force transducer output, which clearly displayed each wing movement cycle. This period was converted to 360°. Then we identified the first positive peak to the next positive peak of the DVM trace, and the time difference to the appearance of the first positive peak on the other trace (i.e., the DLM trace) was calculated as a phase shift (in °) in muscle activation. During the warm-up phase, angle values were low (due to the almost simultaneous contraction of both antagonistic flight muscles) as expected, and, during flight, the phase angles became higher (due to the non-overlapping activation of both flight muscles). Since no temperature-related changes were observed in vertical force and phase angle (see RESULTS), individual means across different temperatures were calculated and compared for the two treatments (and also flight) by a paired Student's *t*-test with a Bonferroni correction for multiple comparisons.

To analyze potential changes in muscle activation patterns during individual wingbeat cycles, we first calculated the number of spikes that occur in an average muscle contraction cycle during the two conditions (i.e., no pheromone and pheromone) and compared them by a Wilcoxon rank-sum test. Subsequently, all muscle potentials were classified by peak amplitude (0.25-mV intervals) and duration (1-ms intervals). The threshold voltage used was 0.15 mV, and peak duration was measured above this baseline. In general, DLM potentials of all individuals fell into two amplitude and five duration classes, and four amplitude and three duration classes were found for DVMs potentials. The following index was constructed for quantifying the occurrence of muscle potential categories in all individuals

$$I(i) = n(i)/[n(i) + m(i)] \quad \text{for } n(i) + m(i) > 0$$

where i is a particular (amplitude, duration) class, n is the percentage of peaks in the presence of pheromone, and m is the percentage of peaks in the absence of odor. This index characterizes in a normalized fashion whether particular muscle potentials exclusively occur when the pheromone is absent ($I = 0$), or present ($I = 1$) and when these occur equally in both treatments ($I = 0.5$). Thus index values below 0.5 represent the number of muscle potentials that are more common when the pheromone was absent, while index values above 0.5 indicate that a particular muscle potential category occurs more frequently when the pheromone was present.

Duty cycles were calculated by using the average of at least five wing strokes for both flight muscles (at the same portion of the warm-up period) divided by the muscle activation period at three distinct temperatures. For each temperature, across-treatment and temperature comparisons were performed by means of repeated-measures ANOVA with Tukey post hoc analyses for multiple comparisons. Finally, heating rates were calculated for constant increases in temperature of at least 4°C in each individual and compared by a paired Student's *t*-test. Constant recording of at least two distinct muscle potentials per cycle in either DLM or DVM was used for determining the subset of individuals ($n = 6$) in which additional muscle potentials produce an increased heating rate.

All statistical analyses were done using R statistical package (R Development Core Team, 2011).

RESULTS

The simultaneously recorded data on thoracic temperature, lift production, and muscle activation allowed an assessment of three possible mechanisms for heating rate modulation, and the presentation of the results is structured by these mechanisms.

Higher rate of muscle contractions. One possible mechanism by which the amount of heat produced in the thorax could be controlled is a variable rate of flight muscle contractions, thus causing variable wingbeat frequencies. Figure 2 shows an example for the increase in muscle potential activation frequency with thoracic temperature rises during warm-up. Both indirect flight muscles (i.e., DLMs and DVMs) showed similar linear increases of muscle potential activation with thoracic temperature in the absence of olfactory stimuli. Thus, to assess if the increase in heating rates observed in males exposed to the pheromone is due to a higher rate of flight muscle contractions, we compared individual slopes and intercepts for both conditions (i.e., pheromone present or absent; Table 1). No signifi-

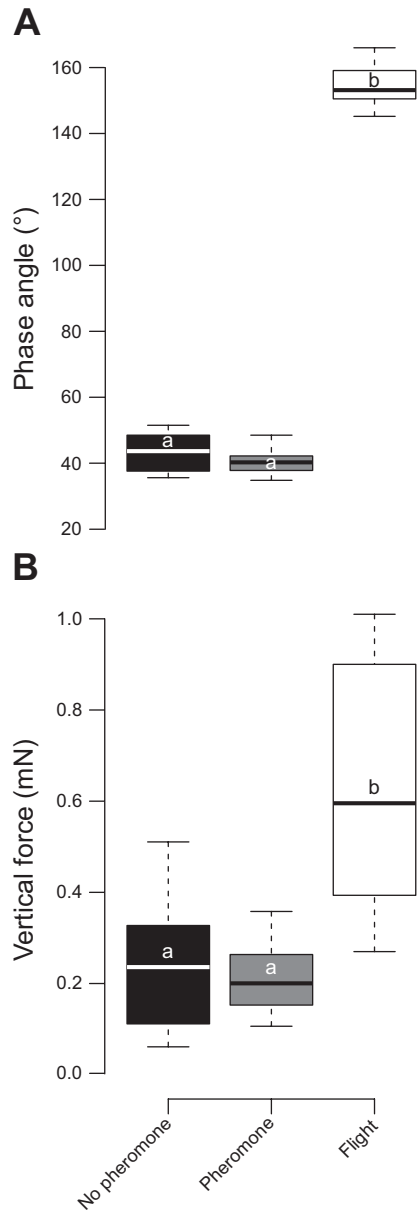

Fig. 4. Phase angles (*A*) and vertical forces (*B*) during warm-up and flight ($n = 10$). *A*: during warm-up, no differences were observed for the two treatments (i.e., no pheromone and pheromone), but a significantly higher phase angle was measured during tethered flight (paired Student's *t*-test with Bonferroni correction, $P < 10^{-10}$). *B*: vertical forces measured during warm-up did not differ for the two treatments, but significantly higher vertical forces occurred in flight (paired Student's *t*-test with Bonferroni correction, $P < 0.005$). [a,b] Different letters indicate statistical differences. Box plots show the first quartile (lower limit of the box), median, and third quartile (upper limit of the box).

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cant differences were found for either of the two variables, i.e., the slope and intercept, when males were exposed to the pheromone and when no olfactory stimulus was present ($n = 10$; paired Student's t-test, $P > 0.5$). Therefore, the higher heating rate observed in males exposed to the attractive odor is not produced by an increase in the frequency of muscle contractions.

Phase-angle shifts in contraction of antagonistic muscles. During the warm-up behavior of *H. zea*, activation of the antagonistic flight muscles (i.e., DLMs and DVMs) is not entirely simultaneous (Fig. 1). Another possible mechanism by which thoracic heat production could be modified is, therefore, a shift in the timing of the contraction of the antagonistic flight muscles. A more simultaneous activation of the antagonistic muscles should produce more heat, because simultaneous antagonistic co-contraction typically produces more energy dissipation and therefore heat production. Vertical force (proxy variable for wing amplitude) increased with greater phase differences between DLMs and DVMs during the warm-up period (Fig. 3). No significant differences in both variables (i.e., vertical force and phase shift) for changes in thoracic temperature were found within individuals when comparing means (average of at least five EMG peaks) at different temperatures (repeated-measures ANOVA, $P > 0.5$). Thus we compared means of phase angles (Fig. 4*A*) and vertical forces (Fig. 4*B*) of each individual for both treatments (i.e., pheromone present or absent) and found no differences ($n = 10$; paired Student's t-test with Bonferroni correction, $P > 0.1$). Figure 4 also shows that, as expected, more vertical force is produced (paired Student's t-test with Bonferroni correction, $P < 0.005$), and phase angles are greater (paired Student's t-test with Bonferroni correction, $P < 10^{-10}$) during short bouts of tethered flight. So, the higher heating rate observed in males exposed to the female pheromone (Crespo et al. 2012 and see below) is not produced by a more simultaneous contraction of the antagonistic flight muscles.

Motor activation. The higher thoracic heating rate of male moths exposed to female pheromone appears to be caused by a change in the activation pattern of the flight muscles within each contraction. EMG traces of DLMs (Fig. 5*A*) and DVMs (Fig. 5*B*) show that the number and amplitude of muscle potentials increase when males are exposed to the pheromone (*left* panels). Furthermore, in an average muscle contraction cycle, significantly more spikes occur in both flight muscles when the pheromone is present (Fig. 6*A*). Since these additional muscle potentials appear to form distinct new clusters (Fig. 7), we analyzed muscle potential peaks according to different amplitudes and durations (see Fig. 6, *B* and *C*). The occurrence of muscle potential categories in DLMs (Fig. 6*B*) and DVMs (Fig. 6*C*) shifted between the two conditions: pheromone absent and present. When the pheromone was present, there was not only an increase in the percentage of some of the muscle potential categories in both flight muscles, but also new potential categories appeared in high percentages (gray bars). To quantify these observations, we calculated an index (Fig. 8). For both indirect thoracic muscles (DLMs, Fig. 8*A* and DVMs, Fig. 8*B*), the higher frequency index values above 0.5 confirm the increment in muscle activation that is

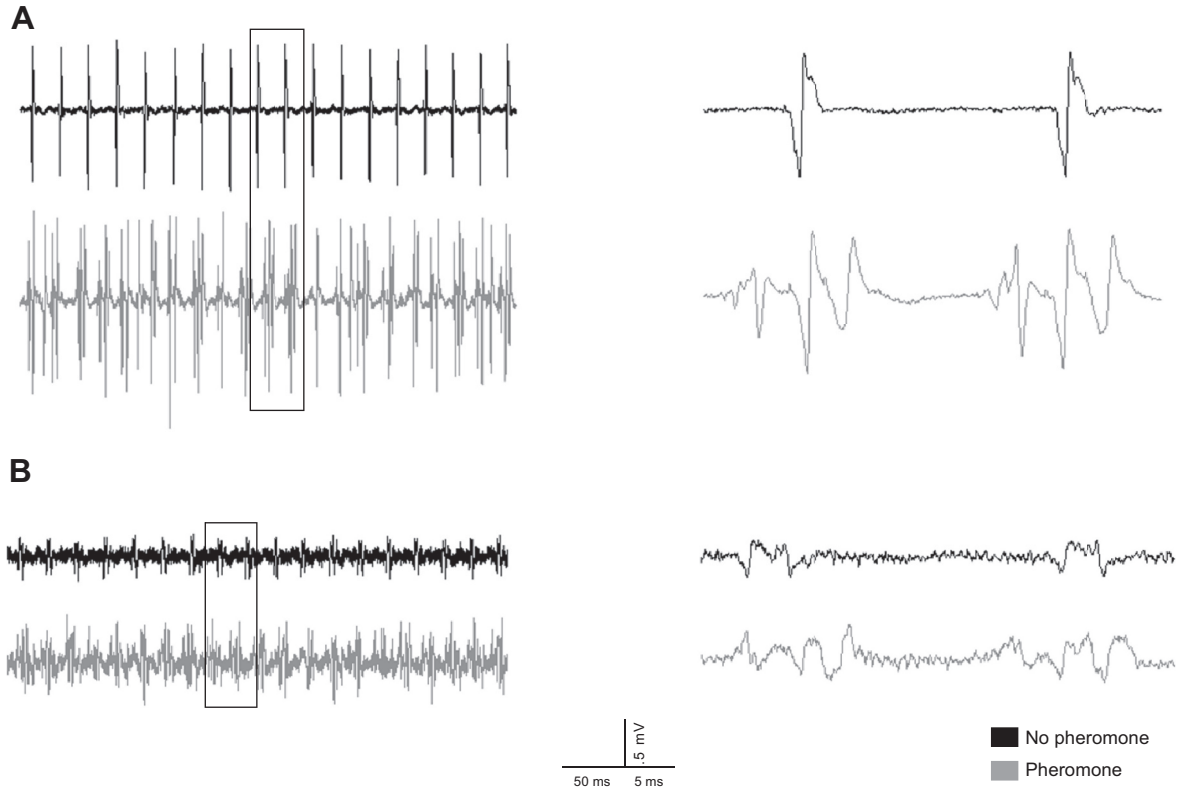

Fig. 5. Warm-up motor activation patterns of DLM (*A*) and DVM (*B*) changes when the olfactory stimulus is absent compared with when the female pheromone is present. *A*: electromyogram trace shows additional muscle potentials in DLM when the pheromone is present. *B*: as seen in *A*, additional muscle potentials are seen in DVM when the same moth is exposed to the female pheromone. Note also change in amplitude of the signal in *lower* traces (i.e., when the pheromone is present) in both flight muscles. Box indicates region of the electromyogram traces expanded on the *right*.

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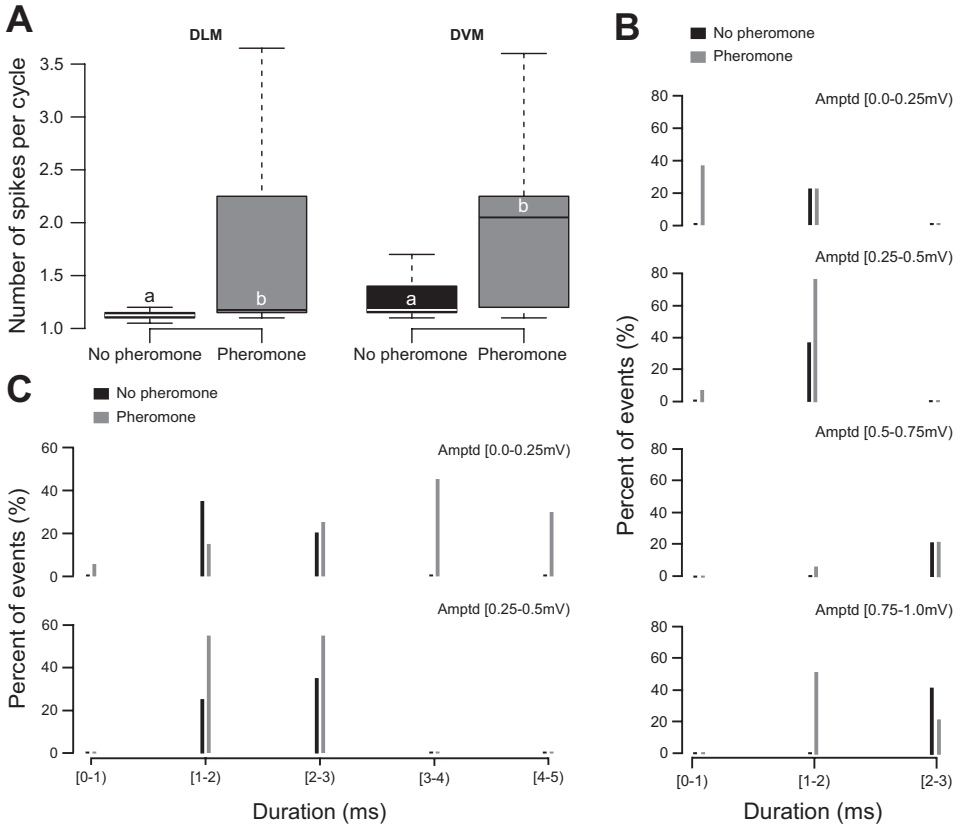

Fig. 6. *A*: higher number of muscle activation peaks occur in both flight muscles when the pheromone is present compared with the blank condition. The example of frequency distributions of muscle potential categories (classified by amplitude and duration) in both the DLMs (*B*) and DVMs (*C*) during the warm-up of one individual illustrates the occurrence of additional potentials when the female pheromone is present. [a,b] Different letters indicate statistical differences. Box plots show the first quartile (lower limit of the box), median, and third quartile (upper limit of the box). Percentages indicate the presence of muscle potentials in the 20 contraction cycles analyzed.

observed with the presence of the pheromone. Index values of zero account for either the activity of motor units that are only activated when the pheromone is absent (which might mean that different motor units are active in the two conditions) or are masked by occurring simultaneously with other muscle potentials when the pheromone is present. In any case, these data indicate strongly that a higher heating rate is achieved by a change in the activation pattern within each contraction cycle.

Additional activation of flight muscles increases muscle duty cycle and heating rates. The fraction of the contraction cycle during which muscles were activated was longer with pheromone than without it for all temperatures recorded (Fig. 9). When the pheromone was absent, duty cycles for both DLMs (Fig. 9*A*) and DVMs (Fig. 9*B*) were low, but increased with increasing thoracic temperature (black bars, e.g., when comparing 22°C and 26°C temperatures in both flight muscles). However, only the duty cycles of DLMs showed a significant difference when comparing the two extreme temperatures (repeated-measures ANOVA with Tukey post hoc analysis, $P < 0.005$). When the pheromone was present, no significant increase in duty cycle was recorded for the three temperatures (repeated-measures ANOVA with Tukey post hoc analyses, $P > 0.1$). Duty cycles (20–35% increments) were significantly different between the two treatments (no pheromone and pheromone) for the first two temperatures (repeated-measures ANOVA with Tukey post hoc analyses, $P < 0.05$ for both flight muscles). At the third temperature, duty cycles of moths not exposed to the pheromone were high enough to not significantly differ from the elevated duty cycles recorded when the stimulus was present (repeated-measures ANOVA with Tukey post hoc analyses, $P = 0.09$ and $P = 0.07$ for DLMs and DVMs, respectively). No interaction effect between temperature and treatment was responsible for the observed differences (repeated-measures ANOVA, $P > 0.5$).

To test whether a change in muscle activation pattern did indeed change heat production, we calculated heating rates for the two treatments ($n = 10$; Fig. 10). Higher heating rates occurred when the pheromone was present compared with when it was absent (paired Student's *t*-test, $P < 0.05$, Fig. 10*A*), and when more than one muscle potential per cycle was observed in the EMGs of either DLMs or DVMs ($n = 6$; Fig. 10*B*). Thus the presence of the attractive odor caused changes in the muscle activation pattern of both flight muscles, which in turn increased the duty cycle of both flight muscles producing higher heating rates.

DISCUSSION

These results show that the main mechanism for increased preflight heating rates in male *H. zea* exposed to conspecific female pheromone is the differential activation of both indirect flight muscles. The two other putative mechanisms that we investigated do not seem to play a significant role in modulating heat generation. Although the rate of muscle contractions and associated wingbeat frequency increased linearly with thoracic temperature, it did not change when the attractive odor was present. Also, the contraction cycles of the antagonistic flight muscles did not overlap more during the preflight warm-up behavior of males exposed to the female pheromone, thus presumably having similar phases of coactivation in both conditions. The main change in pheromone-stimulated males occurred in the activation pattern of the power muscles during each contraction cycle. Increased

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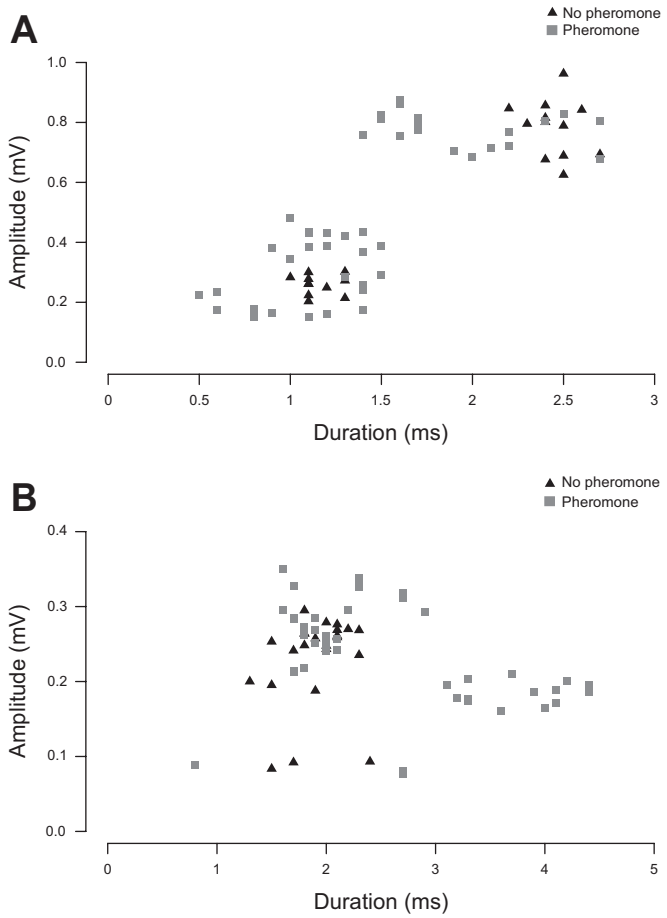

Fig. 7. Two examples of amplitude-duration plots of muscle potentials show that different clusters of muscle activation peaks emerge in both flight muscles when moths are stimulated with pheromone compared with the blank condition. In both, DLM (*A*) and DVM (*B*) distinct additional potentials occur when the pheromone is present.

duty cycles of both muscles are associated with higher heating rates. These results give insight into the neural mechanisms for modulating heat generation during preflight warm-up behavior.

In the present experiment we aimed to differentiate among three distinct motor control mechanisms that could potentially affect the modulation of heating rate previously reported for male moths detecting the female pheromone (Crespo et al. 2012). The first of these mechanisms is an increase in the rate of flight muscle activation, such that the small-amplitude wingbeat frequency during shivering is increased. Both DLMs and DVMs could be activated at higher frequency, which would account for the increase in preflight heat production when the pheromone is present. This potential mechanism, however, is not used to modulate heating rate. Although there is an increase in the activation rate of both indirect flight muscles with increasing thoracic temperature, no change in activation frequency occurred in response to the presentation of the olfactory stimulus. This result suggests that the oscillator for activation of the flight muscles operates at a temperature-dependent frequency that is not subject to behavioral modulation.

A second mechanism for modulating heat production during preflight warm-up is to minimize mechanical energy use by more simultaneous contraction of the antagonistic flight muscles. In *H. zea*, unlike insects that shiver with asynchronous flight muscles, the timing of activations of DLMs and DVMs does not overlap completely. A shift to more simultaneous contractions could therefore generate isometric contractions in both flight muscles (Esch and Goller 1991; Esch et al. 1991), thus minimizing mechanical wing movement and increasing heat production. However, our results show that, when the pheromone is present, the timing of activation of both power muscles is the same as that of males not exposed to an olfactory stimulus. Thus the timing of activation of the two antagonistic flight muscles does not change in response to pheromone stimulation. Although this proposed mechanism could produce an increase in heat generation, the data presented here show that it is not used. Over the temperature range that we were able to record shivering behavior, no change in phase activation of both antagonistic muscles was observed. Kammer (1968) showed that some insects transition from a warm-up to a flight muscle activation pattern abruptly, while others do it gradually. In *H. zea*, the switch between preflight warm-up and flight behavior appears to be more abrupt than gradual. Interestingly, differences in the firing time of indirect flight muscle

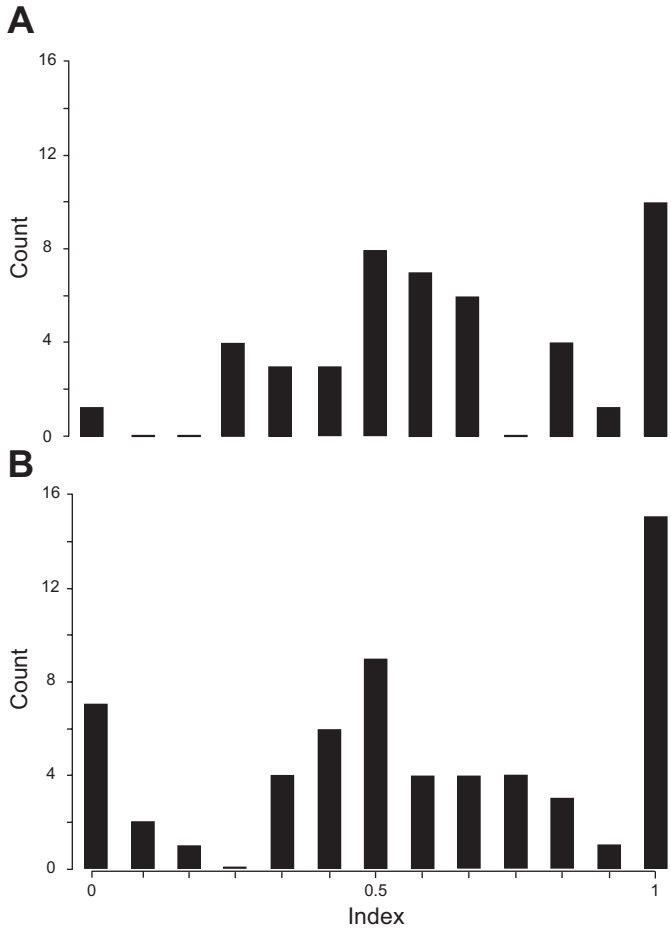

Fig. 8. Quantification of new muscle activity found in both the DLMs (*A*) and DVMs (*B*) during warm-up for all individuals ($n = 10$). The index is the ratio of all muscle potentials observed when the pheromone is present, divided by the total muscle potentials observed (i.e., those that appeared when the pheromone was present and absent). The higher frequencies of index values above 0.5 (compared to those below 0.5) depict the increase in muscle activity that the pheromone stimulus evoked in male moths.

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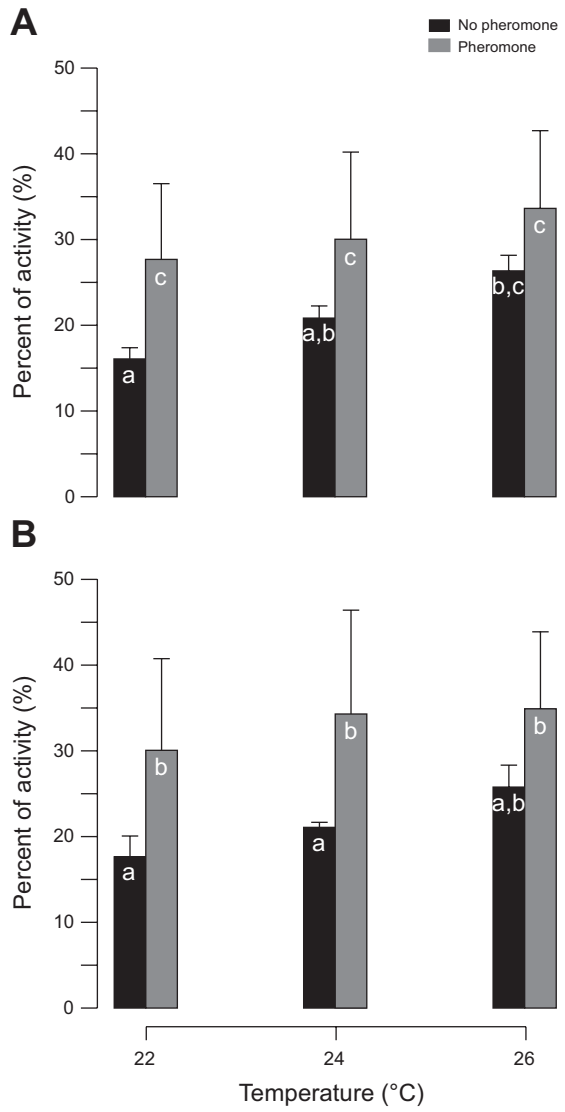

Fig. 9. Duty cycle of DLMs (*A*) and DVMs (*B*) in male moths exposed to the two treatments (i.e., no pheromone and pheromone) at three different warm-up temperatures ($n = 10$). Percentage of DLM (repeated-measures ANOVA with Tukey post hoc analyses, $P < 0.05$) and DVM (repeated-measures ANOVA with Tukey post hoc analyses, $P < 0.005$) activity per cycle is greater for the first two temperatures when males are exposed to the female pheromone. Duty cycles for the third temperature were not significantly different for both flight muscles ($P = 0.09$ and $P = 0.07$ for DLMs and DVMs, respectively). Also, significant differences across temperatures were found within the no-pheromone treatment, at least for the DLMs. When the pheromone was absent, the duty cycle of DLMs was significantly different for 26°C compared with 22°C ($P < 0.005$). Duty cycle differences are not due to the interaction of both factors (i.e., treatment and temperature; repeated-measures ANOVA, $P > 0.5$). [a,b,c] Different letters indicate statistical differences. Bars are means ± SD.

activities of hawkmoths have been shown to occur during flight and are correlated with changes in wing position, i.e., with changes in yaw and roll (e.g., Springthorpe et al. 2012; Sponberg and Daniel 2012).

The final mechanism examined for generating more heat in males exposed to female pheromone was that of changing the activation pattern of the indirect flight muscles within each activation cycle. This physiological mechanism accounts for the modulation of heat production in *H. zea*. We recorded an increase in the number and amplitude of flight muscle potentials when the pheromone was present, indicating that the longer time of activation of the antagonistic muscles during each activation cycle generates more heat. This longer activation period of both flight muscles translates into a higher muscle duty cycle. Whether this change in activation pattern is the result of recruitment of additional motor units, as demonstrated in the flexor tibiae muscle of *Schistocerca gregaria* (Page et al. 2008), or repeated activation of the same motor units cannot be answered unambiguously. However, our data suggest that additional recruitment most likely occurs. Although muscle potentials in insect muscles are known to vary in amplitude, we propose that such variation cannot completely account for the changes in potential amplitude, duration, and shape that we observed in pheromone-stimulated moths. The emergence of new pulses, as determined by peak amplitude and duration, is more easily explained by activation of new motor units compared with different temporal activation of the same motor units. Anatomical evidence on the innervation pattern of flight muscles in *H. zea* is not inconsistent with this potential mechanism. It is, therefore, possible that the change in muscle activation following pheromone stimulation is caused by repeated activation of single motor units and recruitment of additional motor units, allowing for a more complex interaction between the various elements of this neuromuscular network (Brezina et al. 2000). The resulting increase in duty cycle accounts for the increased heat generation in the thorax of male moths when the female pheromone is present.

Multiple muscle potentials per wingbeat cycle have been recorded in flight muscles of a wide array of insects with synchronous flight muscles (Kammer 1985), and in some cases this type of activation has been shown to be correlated to sensory input (e.g., Madsen and Miller 1987). However, these observations were mostly made during flight, and the nature of the stimulus-altering muscle activation was not specified. In the present study, we show that the change in the activation of synchronous flight muscles during the preflight warm-up behavior of male moths is caused by the presence of an attractive stimulus, signaling the opportunity to mate. Interestingly, in another synchronous flier, *Manduca sexta*, the work produced by DLMs is higher when muscles are activated multiple times during each contraction cycle than during single activation, at least under certain cycle frequency and temperature conditions (Stevenson and Josephson 1990). If there is an increased activation of both antagonistic flight muscles during warm-up, i.e., when these muscles are contracting almost simultaneously, this could account for the higher heating rate observed in male *H. zea* during olfactory stimulation. Whether the rate of heat production should always be the highest possible during preflight warm-up was discussed previously by Kammer (1971). Despite citing data supporting variable warm-up rates, it was reasoned that, since preflight warm-up only sets the stage for subsequent behavior, as little time and energy as possible should be expended, and, therefore, heating rate should always be maximal. However, Crespo et al. (2012) showed that males increase their heating rates during shivering by more than 1°C/min when exposed to conspecific female pheromone, and that this modulation may be important in reproductive competition. As to why less than maximal warm-up rates might have evolved, we can only speculate on an "unknown" cost to high heating rates in behavioral situations. Since sensory input can be ambiguous (this is especially true for olfactory cues in

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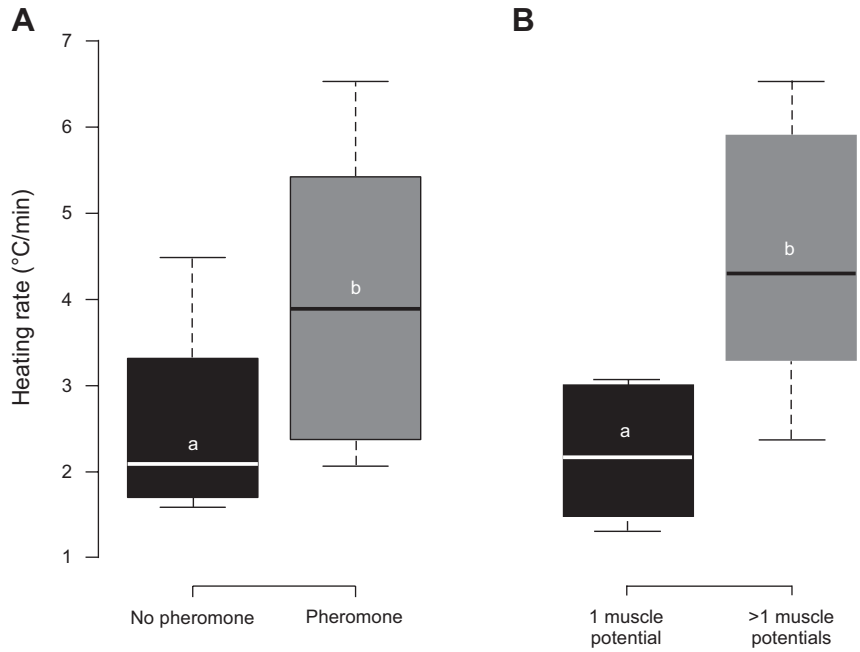

Fig. 10. Additional muscle potentials account for the increase in heating rate during warm-up. Significantly higher heating rates are observed when males were exposed to the female pheromone ($n = 10$, paired Student's t-test, $P < 0.05$; A) and more than one muscle potential per cycle is recorded ($n = 6$, paired Student's t-test, $P < 0.05$; B) in either the DLMs or DVMs. [a,b] Different letters indicate statistical differences. Bars are means ± SD.

changing wind conditions), low heating rates may allow for optimization of energy use. A longer sampling time may be required to process and confirm the acquired information, and an initial low-level heating may confer a timing advantage in exploiting resources (i.e., a food source). On the other hand, in cases where information is unambiguous, e.g., in the presence of a sex pheromone, maximal heating rates will guarantee a rapid take-off, allowing a male to outcompete other males when searching for a mate.

Generating different behaviors with the same muscle group is often achieved by different contraction and coordination patterns, which, therefore, requires the ability to modulate motor control at the level of pattern generation and motor output (e.g., Santer et al. 2006). The indirect flight muscles are a good example, because they not only produce power for active flight, but also generate the high thoracic temperatures observed in endothermic insects (Kammer 1981). It has been proposed that the central neural mechanisms controlling muscle activity during warm-up probably derived from those producing the patterned motor output of flight (Kammer 1968). The multiterminal and polyneuronal innervation observed in both indirect flight muscles of some insects (e.g., Ikeda and Boettiger 1965a, Stokes et al. 1975) appears to play a role in the repertoire of behaviors displayed by these insects. However, it is worth noting that only fast neurons have been described in the flight muscles of insects so far. Thus the mechanism by which this modulation of muscle activation occurs must involve repetitive activation and/or recruitment of fast motor units.

The fact that only the duty cycle, but not the contraction rate, is modulated in response to olfactory information gives some further insight into the neural control of flight muscles during shivering. The rhythm of the pattern-generating circuitry, although dependent on temperature, is not modulated by sensory information. Instead, this modulation must involve differential activation of the polyneuronal multiterminal motoneuron network. In addition to direct neural control of this process, a neuromodulatory control mechanism involving octopamine or other biogenic amines is also possible (Kammer 1985). Neurosecretory pathways have been confirmed in the DLMs of several insects (e.g., Wasserman 1985), indicating that a complete characterization of the flight muscle "circuitry" might be more complex than previously thought. Most octopaminergic/tyraminergic neurons of the ventral nerve cord ganglia, including those innervating flight muscles, have a median unpaired cell body (usually called dorsal or ventral unpaired median neurons). Thus it is likely that the median unpaired neurons described in the flight muscles of *H. zea* (Orona and Agee 1988) are octopamine-releasing neurons that can modulate muscle activity. Octopamine has been shown to modulate neuromuscular operation in different ways. For example, its effects increase twitch amplitude and change the relaxation rate of muscle (Pflüger and Duch 2011). However, the functional role of the rich innervation of insect flight muscles by octopamine-releasing neurons remains unknown. It would not be surprising if such a putative mechanism may play a role in modulating flight muscle activity in *H. zea* males sensing the pheromone.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.G.C., N.J.V., and F.G. conception and design of research; J.G.C. performed experiments; J.G.C. and F.G. analyzed data; J.G.C.

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and F.G. interpreted results of experiments; J.G.C. and F.G. prepared figures; J.G.C. and F.G. drafted manuscript; J.G.C., N.J.V., and F.G. edited and revised manuscript; J.G.C., N.J.V., and F.G. approved final version of manuscript.

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