The Journal of Experimental Biology 202, 787–796 (1999)
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JEB1929



# RESPECIFIED LARVAL PROLEG AND BODY WALL MUSCLES CIRCULATE HEMOLYMPH IN DEVELOPING WINGS OF *MANDUCA SEXTA* PUPAE

JANE L. LUBISCHER[1,*], LAURA D. VERHEGGE[2,‡] AND JANIS C. WEEKS[1,§]

[1]*Institute of Neuroscience* and [2]*Oregon Institute of Marine Biology, University of Oregon, Eugene, OR 97403-1254, USA*

*Present address: Department of Zoology, University of Texas, Austin, TX 78712, USA
‡Present address: Bamfield Marine Station, Bamfield, British Columbia, Canada VOR 1B0
§Author for correspondence (e-mail: weeks@uoneuro.uoregon.edu)

*Accepted 18 January; published on WWW 3 March 1999*

## Summary

**Most larval external muscles in *Manduca sexta* degenerate at pupation, with the exception of the accessory planta retractor muscles (APRMs) in proleg-bearing abdominal segment 3 and their homologs in non-proleg-bearing abdominal segment 2. In pupae, these APRMs exhibit a rhythmic 'pupal motor pattern' in which all four muscles contract synchronously at approximately 4 s intervals for long bouts, without externally visible movements. On the basis of indirect evidence, it was proposed previously that APRM contractions during the pupal motor pattern circulate hemolymph in the developing wings and legs. This hypothesis was tested in the present study by making simultaneous electromyographic recordings of APRM activity and contact thermographic recordings of hemolymph flow in pupal wings. APRM contractions and hemolymph flow were strictly correlated during the pupal motor pattern. The proposed circulatory mechanism was further supported by the findings that unilateral ablation of APRMs or mechanical uncoupling of the wings from the abdomen essentially abolished wing hemolymph flow on the manipulated side of the body. Rhythmic contractions of intersegmental muscles, which sometimes accompany the pupal motor pattern, had a negligible effect on hemolymph flow. The conversion of larval proleg and body wall muscles to a circulatory function in pupae represents a particularly dramatic example of functional respecification during metamorphosis.**

Key words: circulation, hemolymph, *Manduca sexta*, pupa, wing, leg, muscle, metamorphosis, insect.

## Introduction

At metamorphic stage transitions, neuromuscular systems utilized during the previous life stage may be retained unchanged, eliminated by programmed cell death or respecified for new functions. For example, motoneurons and muscles used by tadpoles for feeding undergo substantive changes to accommodate the different jaw anatomy and feeding behavior of frogs (for a review, see Alley, 1990). In insects, the fates of larval motoneurons and muscles during metamorphosis have been examined in several species (e.g. Breidbach, 1990; Fernandes and Keshishian, 1995), with the most detailed information being available in the hawkmoth *Manduca sexta*. In *Manduca sexta*, many larval motoneurons that experience the degeneration of their original target muscles are subsequently respecified to innervate new muscles that develop around the remnants of the larval muscle. During this type of respecification, motoneurons undergo a period of dendritic and axonal regression as their targets degenerate, followed by the elaboration of new dendritic and axonal arborizations as new muscles develop (e.g. Levine and Truman, 1985; Kent and Levine, 1988; Weeks and Ernst-Utzschneider, 1989; Truman and Reiss, 1995; Consoulas and Levine, 1998). Dendritic remodeling is related to the reorganization of synaptic inputs appropriate to the new behavioral roles of the motoneurons (Levine and Truman, 1982; Streichert and Weeks, 1995).

The present study examines a previously unreported form of neuromuscular respecification during metamorphosis in which larval muscles and motoneurons are retained into the pupal stage to carry out an entirely different physiological function. In *Manduca sexta* larvae, prolegs located on abdominal segments 3–6 (A3–A6) and the terminal abdominal segment (AT; Fig. 1A) participate in locomotory and other behaviors (for a review, see Weeks et al., 1997). The musculature of each larval proleg includes an accessory planta retractor muscle (APRM), which is innervated by a pair of APR motoneurons (APRs) located in the ganglion of the same segment (Weeks and Truman, 1984; Sandstrom and Weeks, 1996). Homologs of the APRs and APRMs are also present in the non-proleg-bearing segments A1

and A2 (Weeks and Ernst-Utzschneider, 1989). APRMs in all larval abdominal segments have similar dorsal insertions near the spiracle (respiratory opening) but, at their ventral end, APRMs in segments A3–A6 insert within the proximal rim of the proleg while APRMs in A1 and A2 insert on the ventral abdominal body wall (Fig. 1A). Larval APRMs in segments A3–A6 function as proleg retractors, whereas they contribute to abdominal tonus and bending movements in segments A1 and A2.

During the larval–pupal transformation, the prolegs degenerate and are not replaced by pupal or adult appendages. Regression of the dendritic arborizations of the APRs during this time is associated with the loss of synaptic inputs involved in larval behaviors (Streichert and Weeks, 1995; Sandstrom and Weeks, 1998). Within 48 h after pupation, subsets of APRs and APRMs have undergone programmed cell death in dissimilar segmental patterns (Weeks and Ernst-Utzschneider, 1989; Sandstrom and Weeks, 1998). In segments A1, A5 and A6, both the APRMs and the APRs die. In segment A4, the APRMs degenerate, while the APRs survive and are respecified to innervate new muscles that develop during the pupal stage; this type of motoneuron respecification has been well characterized by others (see above) and is not considered further. Finally, in segments A2 and A3, both the APRs and APRMs survive through the pupal stage and die after adult emergence (Sandstrom and Weeks, 1998). Similar muscles survive in the pupae of other lepidopteran species (see Discussion). In *Manduca sexta* larvae, the mass of individual APRMs in segment A3 [APRM(3)s] is approximately three times that of the APRM(2)s. During the larval–pupal transformation, however, APRMs in both segments lose mass such that all four muscles stabilize at a similar mass for the remainder of the pupal stage (Sandstrom and Weeks, 1998). The dorsal insertions of APRM(2)s and APRM(3)s are in the same location in larvae and pupae but, after the prolegs degenerate, the ventral ends of APRM(3)s attach to the abdominal body wall, as do the APRM(2)s (Fig. 1A).

Electromyographic (EMG) recordings from APRM(2)s and APRM(3)s in intact pupae reveal that all four muscles contract synchronously at approximately 4 s intervals for bouts lasting hundreds of cycles while producing no visible movements (Sandstrom and Weeks, 1998). This ‘pupal motor pattern’ is driven by synaptic activation of APRs, and the fictive motor pattern is expressed spontaneously in isolated pupal nerve cords. The pupal motor pattern is robust through the first half of pupal life but then becomes faster and weaker (see Discussion). Rhythmic contractions of APRMs during the pattern are sometimes accompanied by rhythmic contractions of intersegmental muscles and spiracular closer muscles; anemometric recordings show that activity in these latter two muscle groups produces ventilation through the tracheal system (Sandstrom and Weeks, 1998). On the basis of anatomical considerations, Sandstrom and Weeks (1998) hypothesized that APRM contractions during the pupal motor pattern function to circulate hemolymph in the developing wings and legs of pupae. The proposed model is shown in Fig. 1B. Pupal APRMs insert dorsally on rigid, sclerotized cuticle, which is immovable, while their ventral ends insert on the soft, flexible cuticle of the ventral abdomen. In segments A2 and A3, the ventral abdominal surface is covered by the developing adult wings and legs, which project caudally from their origins in the thorax. The external surfaces of the appendages are sclerotized, but their flexible inner surfaces adhere tightly to the flexible abdominal cuticle. The model shown in Fig. 1B postulates that, when APRMs contract, they work against the stationary dorsal insertion points to pull the flexible ventral structures upwards, thereby increasing the volume of the lumina of the appendages

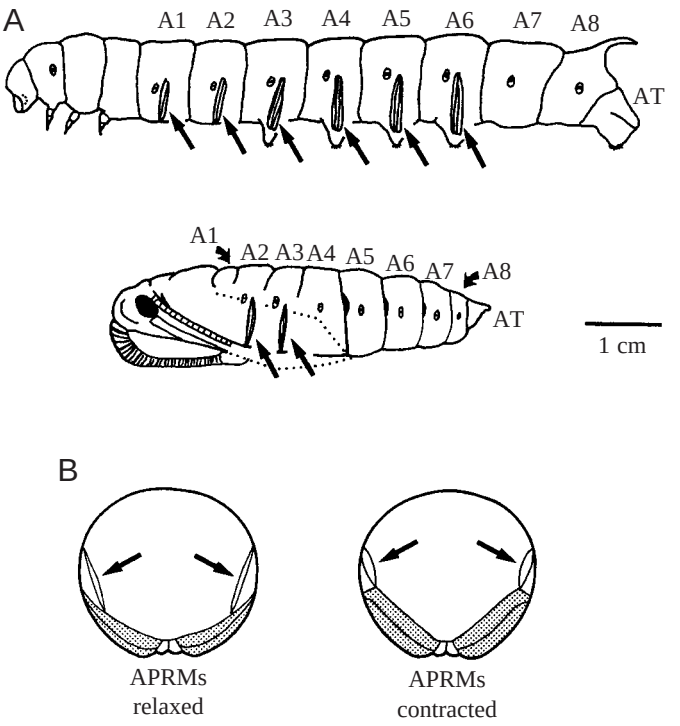


Fig. 1. Accessory planta retractor muscles in *Manduca sexta* larvae and pupae. (A) Drawings of a larva (top) and pupa (bottom), with anterior to the left and abdominal segments 1–8 (A1–A8) and the terminal abdominal segment (AT) labeled. In larvae, accessory planta retractor muscle (APRM) homologs (arrows) are present in proleg-bearing segments A3–A6 and non-proleg-bearing segments A1 and A2. In pupae, the retained APRMs in segments A2 and A3 (arrows) insert ventrally on the flexible abdominal floor, beneath the developing wings (dotted outline) and legs that extend caudally from the thorax. (B) Model of the hypothesized role of pupal APRMs in hemolymph circulation. A transverse view of segment A3 is illustrated (dorsal surface up), with the bilateral pair of APRMs (arrows) shown relaxed (left) or contracted (right). The ventral surface of segment A3 is covered by the developing metathoracic wings, which are in turn covered by the developing mesothoracic wings. The lumina of the wings are shown stippled. The segment is bounded entirely by rigid cuticle, whereas the ventral abdominal surface and adjacent wing surfaces are flexible. When the APRMs are relaxed, the wings lie in their rest positions. APRM contraction pulls the abdominal surface dorsally, which is proposed to increase the volume of the wing lumina and to draw hemolymph in from the thorax in a bellows-like action. In segment A2 (not shown), APRMs insert over the metathoracic legs, which are covered by the meta- and mesothoracic wings. Modified from Sandstrom and Weeks (1998).

and reducing the internal pressure. The lumina connect with the body cavity (hemocoel) only at the bases of the appendages in the thorax, a distance of up to 3 cm from the appendage tips (see Fig. 1A). It is proposed that the increase in the volume of wing and leg lumina caused by each APRM contraction during the pupal motor pattern draws hemolymph into the appendages from the thoracic circulation in a bellows-like manner and circulates hemolymph within the appendages.

This hypothesis was tested in the present study by using contact thermography to monitor hemolymph flow in the wings during the pupal motor pattern in young pupae. Some of these results have appeared previously in abstract form (Lubischer et al., 1995).

## Materials and methods

*Manduca sexta* L. were reared individually on an artificial diet (modified from Bell and Joachim, 1976) on a 17 h:7 h light:dark, 27 °C:25 °C cycle. Insects were staged with respect to ecdysis to the final (fifth) larval instar (day L0) and ecdysis to the pupal stage (day P0). Days following these stages were numbered; e.g. day P2 is the second day after day P0. Both male and female insects were used, and no sex differences were noted.

### *Electromyography*

EMG recordings of muscle activity were made on days P2–P4. Pupae were immobilized on ice, the cuticle was cleaned with ethanol, and holes for the insertion of EMG electrodes were made in the cuticle using an insect pin. Bipolar EMG electrodes were placed in one, two or all four APRMs in segments A2 and A3 (in the dorsal ends of the muscles near the spiracles) and in a ventral intersegmental muscle (Levine and Truman, 1985) in segment A5. In experiments examining the relationship between thermistor placement and signal amplitude (see below), intersegmental muscle electrodes were omitted. EMG electrodes were made from 76 μm diameter silver wire coated with Teflon except at the cut tip (A-M Systems, Inc., Everett, WA, USA) and anchored to the cuticle using Tackiwax (Central Scientific Co., Chicago, IL, USA). EMG signals were a.c.-coupled and amplified *via* high-gain differential amplifiers (model P-15, Grass Instruments, Quincy, MA, USA, or units fabricated by the Neuroscience Technical Support Group, University of Oregon) and recorded on magnetic tape (Vetter Instruments, Rebersburg, PA, USA) for later playback on a chart recorder (DASH 8, Astro-Med, Inc., West Warwick, RI, USA). Placement of EMG electrodes was verified by dissection at the conclusion of each experiment.

### *Contact thermography*

Contact thermography has been used previously to monitor hemolymph flow non-invasively in adult moths and butterflies (e.g. Wasserthal, 1980, 1981). We used a modification of the so-called 'C-method' in which a heated thermistor placed on the cuticular surface slightly warms the hemolymph beneath it. Hemolymph flow causes the warmed hemolymph to be replaced by cooler hemolymph, which is registered by the thermistor as a temperature decrease (and transduced into a decrease in resistance). Briefly, a pair of small bead thermistors (0.1 mm in diameter; Thermometrics B10PA202N, Edison, NJ, USA) were connected across a Wheatstone bridge circuit. A constant voltage of 1.5 V was applied to the thermistor that contacted the wing. The second thermistor, which served as a reference and was not heated, was positioned in the air near the first thermistor. The output of the bridge circuit was filtered, amplified, stored and displayed as described above for EMG recordings. A downward deflection of the trace represented a temperature decrease. Before recording from pupae, the thermographic technique was validated by placing a thermistor on the surface of a length of polyethylene tubing (1.14 mm i.d., 1.57 mm o.d.) anchored securely to a platform and gently pulsing water through the tubing using a syringe (modified from Wasserthal, 1980). There was no detectable movement of the tubing. Thermistors reliably detected fluid movement within the tubing (data not shown).

To monitor hemolymph flow and its relationship to muscle activity, pupae were implanted with EMG electrodes and positioned on a platform on their dorsal surfaces. The head, thorax and anterior abdominal segments were immobilized on the platform using Plasticine, while the posterior abdominal segments could produce shortening or limited rotary movements. A thermistor was pressed gently against the external surface of the mesothoracic wing (which covers the metathoracic wing; see Fig. 1B and Sandstrom and Weeks, 1998) on one or both sides of the body. Thermistors were typically placed near the medial (ventral) wing edge in segments A2, A3 or A4, and recordings were made at several sites on each pupa (see Results). For simultaneous bilateral recordings, left and right thermistors were placed symmetrically. The pupa and platform were enclosed within a removable box to minimize air currents and fluctuations in ambient temperature. A glass window in the box allowed the experimenter to monitor movement of the pupa during the experiment.

### *APRM ablation*

On day L0 or L1, larvae were anesthetized by immersion in water (30 min), the cuticle was cleaned with ethanol and an incision was made near the dorsal or ventral attachment sites of APRM(2) and APRM(3) on the left or right side of the insect. In experimental insects ($N$=5), all APRM fibers were severed in both segments on one side of the body. This procedure caused degeneration of the damaged APRMs, which was verified at the end of the experiment (see also Lubischer and Weeks, 1996). Control insects underwent sham surgery, during which APRM(2) and APRM(3) on one side of the body were exposed but not cut ($N$=5). Surgical incisions were closed using 6-0 gauge silk suture (Ethicon, Somerville, NJ, USA) and Tackiwax. After surgery and before being returned to the colony incubator, insects were kept at 4 °C for 1–3 h to minimize movement during wound closure. Further development was monitored daily. On days P2–P4, APRM activity on the unoperated side was recorded using electromyography, and hemolymph flow in the

wings was recorded bilaterally by contact thermography. EMG and thermographic recordings were made by an experimenter unaware of whether the insect had undergone APRM ablation or sham surgery.

*Mechanical uncoupling of wings from APRMs*

Immediately following pupal ecdysis, the cuticle is soft and flexible, and the wings, legs, antennae and proboscis gradually (over a period of several hours) extend to their full length along the ventral surface of the pupa (Truman et al., 1980). The external cuticle then tans and hardens through the process of sclerotization. On day P0, after the wings had extended to their full length but before the cuticle had hardened, pupae were immobilized on ice or with $CO_2$ gas, and a piece of Parafilm (Fisher Scientific, Santa Clara, CA, USA) was used to lift the meso- and metathoracic wings on one side of the body gently away from the abdominal surface, thereby mechanically uncoupling them from the ventral APRM attachment sites (Fig. 1B; *N*=6). To ensure that circulation through the wing bases was not blocked by this procedure, the wings were lifted only slightly away from the abdominal surface and kept parallel to the body. The two wings remained attached to each other along their lengths. The wings continued to contact the thorax, and incomplete separation from the abdomen occurred in a few cases (see Discussion). To prevent water loss through untanned cuticle, the exposed abdominal surface and underside of the wings were lightly coated with petroleum jelly, which was removed before recording sessions. In control insects, the wings on one side of the body were gently lifted away from the body wall and then placed back into position, where they reattached to the abdominal surface before cuticle hardening (*N*=6). Pupae that suffered epidermal damage and hemolymph loss during these procedures were excluded from the study. On days P2–P4, APRM activity and hemolymph flow were recorded bilaterally.

## Results

The aim of these experiments was to test the hypothesis of Sandstrom and Weeks (1998) that rhythmic APRM contractions during the pupal motor pattern circulate hemolymph in the developing thoracic appendages of *Manduca sexta* pupae. Rhythmic APRM activity is sometimes accompanied by rhythmic activity in intersegmental muscles, which produces abdominal shortening that might also cause hemolymph movement in the appendages. We focused on pupal motor pattern episodes during which the APRMs were active, the intersegmental muscles were silent and the abdomen was motionless. In each preparation, proper functioning of the intersegmental muscle electrode was confirmed by disturbing the pupa to evoke muscle activity and abdominal movements.

*EMG activity and hemolymph flow in normal pupae*

EMG and thermographic recordings were made from five normal pupae on days P2–P4 during pupal motor pattern episodes. The pupal motor pattern is intermittent, with long bouts of rhythmic activity interspersed with periods of quiescence (Sandstrom and Weeks, 1998). Fig. 2 illustrates

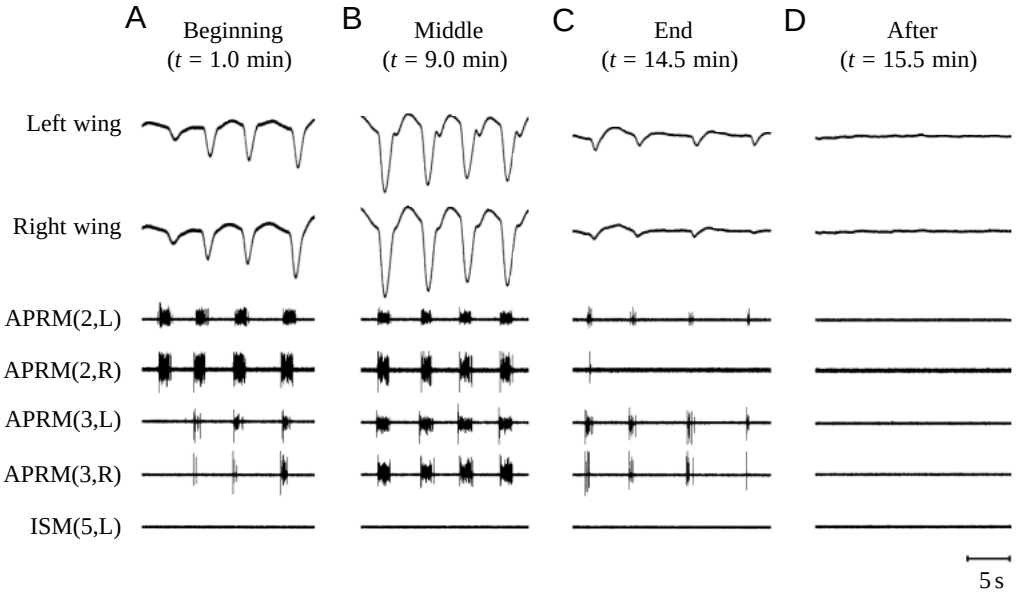


Fig. 2. Contractions of accessory planta retractor muscles during the pupal motor pattern are correlated with hemolymph flow in the wings of a normal pupa. In this and subsequent figures, the two upper traces are thermographic recordings from the wings, while the lower traces are electromyographic (EMG) recordings from accessory planta retractor muscles (APRMs) or an intersegmental muscle (ISM); the segment and body side (R, right; L, left) of each EMG recording is indicated in parentheses. In this example, thermographic recordings were made on day P2 from the ventral margins of the mesothoracic wings in segment A3. The traces shown are taken at the times indicated from a continuous recording of a 15 min episode of the pupal motor pattern to show the beginning (A), middle (B) and end (C) of the episode and a period after the motor pattern terminated (D). (A) The pupal motor pattern initially involved only APRM(2)s; hemolymph flow (indicated by downward deflections of the thermistor traces) became stronger as the APRM(3)s began to contract. (B) During the middle of the episode, all four APRMs contracted strongly and synchronously, with each contraction cycle correlated with strong hemolymph flow. (C) Near the end of the episode, APRM contractions and hemolymph flow became weaker, but activity in APRMs remained synchronous. (D) After the end of the episode, no activity was seen in the APRMs or the thermistors. The intersegmental muscle was silent throughout the recording period.

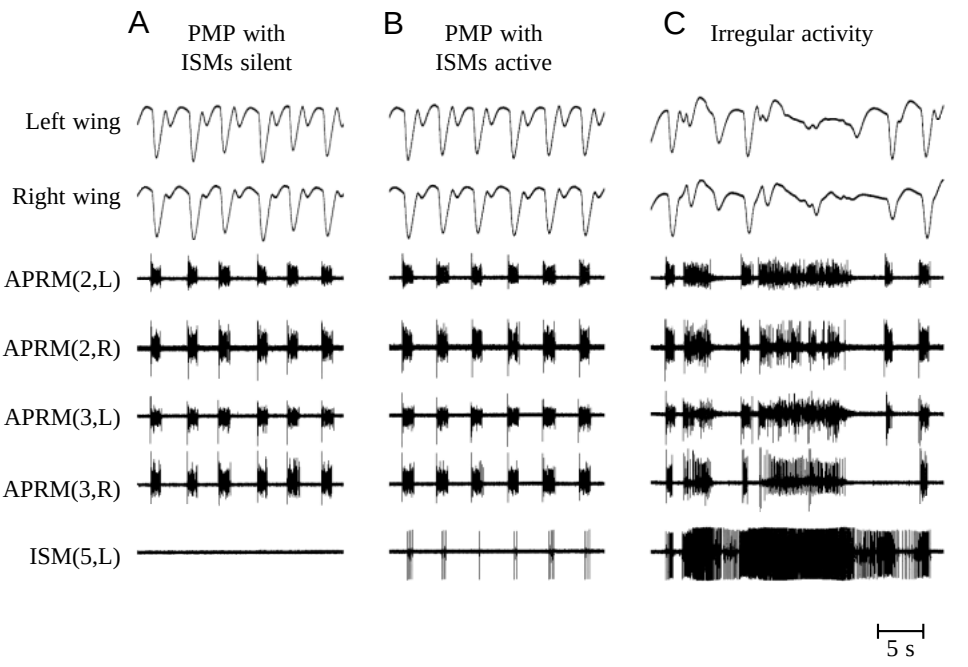


Fig. 3. Relationship between intersegmental muscle contractions and wing hemolymph flow in a normal pupa. Traces are as in Fig. 2; thermographic recordings were made on day P2 from the ventral margins of the mesothoracic wings in segment A2. (A) Hemolymph flow during the pupal motor pattern (PMP) in the absence of intersegmental muscle (ISM) activity. In this pupa, a multiphasic deflection of the thermistor trace was recorded during each accessory planta retractor muscle (APRM) contraction cycle (compare with other figures, which show predominantly monophasic deflections); the first downward deflection corresponded to the onset of an APRM burst, whereas the second deflection corresponded to APRM relaxation. (B) Later during the same motor pattern episode, the intersegmental muscle became active but hemolymph flow in the wings was not noticeably altered. (C) During irregular muscle contractions and abdominal movements, hemolymph flow was irregular; nevertheless, it was more closely correlated with APRM contractions than with intersegmental muscle contractions.

representative events during and after an episode of the motor pattern. This episode lasted 15 min and consisted of 181 APRM burst cycles, with no accompanying intersegmental muscle activity. When active, all four APRMs contracted together, with exceptions occurring only when individual muscles were inactive at the beginning or end of the episode (e.g. Fig. 2A, segment A3). At the beginning of an episode, APRMs in one segment usually began to contract before those in the other segment, and bursts were shorter and less intense than during the established motor pattern (Fig. 2A,B). Similarly, at the end of an episode, bursts became shorter and weaker, and APRMs in one segment sometimes stopped contracting before those in the other segment (Fig. 2C). APRMs were never observed to contract asynchronously during the pupal motor pattern.

Deflections in the thermographic recordings, indicating hemolymph flow in the wings (see below), were strictly correlated with APRM activity in their timing and magnitude (Fig. 2A–C). When APRMs were inactive, no hemolymph flow was detected (Fig. 2D). Relatively weak flow was detected at the beginning and end of episodes, when APRM activity was less robust (Fig. 2A,C). These features were observed in each of the five pupae examined. In four of these pupae in which the thermistor was placed along the ventral margin of the mesothoracic wing over segment A2 or A3 (see below), we measured the latency between the onset of APRM bursts and the onset of the corresponding deflection in the thermistor trace (measured for 10 cycles during a robust period of the motor pattern). The mean latency was 0.21±0.02 s (mean ± S.D.; $N$=4 pupae). The reliable correlation between APRM contractions and hemolymph movement in the wings is consistent with the hypothesized role of the pupal APRMs in hemolymph circulation. The delay between the onset of APRM contractions and thermistor deflections presumably reflected the time required to lift the abdominal floor (Fig. 1B) and increase hemolymph flow to a detectable level at the thermistor site.

We did not examine in detail the properties of hemolymph flow during intersegmental muscle contractions. However, casual observations suggested that intersegmental muscle activity during the pupal motor pattern had little effect on hemolymph flow. Fig. 3A,B shows the motor pattern in a control pupa in which intersegmental muscle bursts sometimes accompanied APRM bursts. In this example, the thermographic traces showed two deflections (one large, one small) for each APRM contraction cycle, reflecting the normal variability in these recordings (see below). As reported previously (Sandstrom and Weeks, 1998), when intersegmental muscles were active during the pupal motor pattern, they contracted in synchrony with APRMs (Fig. 3B). Thermographic recordings were similar with or without intersegmental muscle activity (Fig. 3A,B), consistent with APRM contractions being the key determinant of hemolymph flow. When the pupa exhibited irregular muscle activity and abdominal movements, hemolymph flow in the wings was likewise irregular, although thermistor deflections continued to correspond to APRM bursts even during strong intersegmental muscle activity (Fig. 3C). Further experiments would be required to evaluate fully the relationship between intersegmental muscle contractions and hemolymph flow in the wings. The important result in the present context is that APRM contractions are sufficent to produce robust

hemolymph flow without the contraction of intersegmental muscles or abdominal movements.

The recordings described above were made with thermistors placed along the ventral edge of the mesothoracic wings over segment A2, A3 or A4. The features of the thermographic signal varied with thermistor position on the wings ($N$=8 pupae; data not shown). Deflections tended to be larger along the ventral wing edge and smaller or undetectable along the dorsal wing edge or at the distal wing tip. The shape of the thermographic deflections also varied with position, with multiphasic waveforms (Fig. 3) most commonly seen along the ventral wing edge over the thoracic segments and segment A1; these variations in waveform presumably represent regional differences in the pattern of wing hemolymph flow caused by each APRM contraction cycle (Wasserthal, 1980). When the thermistor was positioned on the abdominal surface rather than on the wing during APRM activity, no hemolymph flow was detected. Hemolymph flow was also detected at the distal tip of a mesothoracic leg in one pupa. For all experiments described below, thermistors were placed along the ventral edge of the wing over segment A2, A3 or A4. During simultaneous bilateral recordings, left and right thermistors were placed symmetrically.

### *EMG activity and hemolymph flow in pupae with ablated APRMs*

To test whether the relationship between APRM contractions and thermistor deflections was causal, APRMs were ablated unilaterally in segments A2 and A3 during the final larval instar (see Materials and methods). Sham-operated insects served as controls. On days P2–P4, EMG recordings were made of APRM activity contralateral to the surgery, and thermographic recordings were made bilaterally on the wings. An EMG electrode in segment A5 monitored intersegmental muscle activity. Data were accepted only when the intersegmental muscle was inactive and the abdomen motionless. After recording, the presence or absence of APRMs was verified by dissection.

Similar results were obtained in five pupae with ablated APRMs, with a representative recording shown in Fig. 4. In this pupa, the right APRMs in segments A2 and A3 were ablated. The intact APRMs on the left side of segments A2 and A3 showed normal rhythmic activity during the pupal motor pattern, and robust hemolymph flow was recorded from the left wing. In contrast, the thermographic signal was essentially flat over the right wing. Recordings from five sham-operated pupae were similar to those described above for normal pupae (data not shown). In a small number of cases (one or two wing recording sites in two of five pupae), weak hemolymph flow was recorded from a wing overlying ablated APRMs, presumably as a result of weak mechanical coupling to APRM contractions on the unoperated side (intersegmental muscles were silent during these recordings; data not shown). In three pupae with ablated APRMs, we recorded instances of the pupal motor pattern when intersegmental muscles were active with APRMs and hemolymph flow in the wing on the ablated side was undetectable (data not shown). These results support the hypothesized relationship between APRM contractions and hemolymph circulation proposed by Sandstrom and Weeks (1998; Fig. 1B) and are consistent with thermographic deflections representing hemolymph movement in the wing.

### *EMG activity and hemolymph flow in pupae with wings mechanically uncoupled from APRMs*

Another set of experiments tested the role of mechanical coupling between the abdominal surface and inner wing surfaces in producing hemolymph flow. In the model of Sandstrom and Weeks (1998), APRM contractions lift the flexible abdominal floor and tightly adherent wing surfaces, thereby increasing the volume of the wing lumina and drawing hemolymph in *via* the wing bases (Fig. 1B). The model predicts that if APRMs were active but mechanically uncoupled from the wings, no hemolymph flow would occur. Following pupal ecdysis and before cuticle hardening, the wings on one side of the body were lifted away from the abdomen and either kept in this position (experimental pupae) or replaced in their original positions (sham-operated pupae; see Materials and methods). On days P2–P4, EMG recordings of APRM activity and thermographic recordings from the wings were made bilaterally. An EMG electrode in segment A5 monitored intersegmental muscle activity. Data were

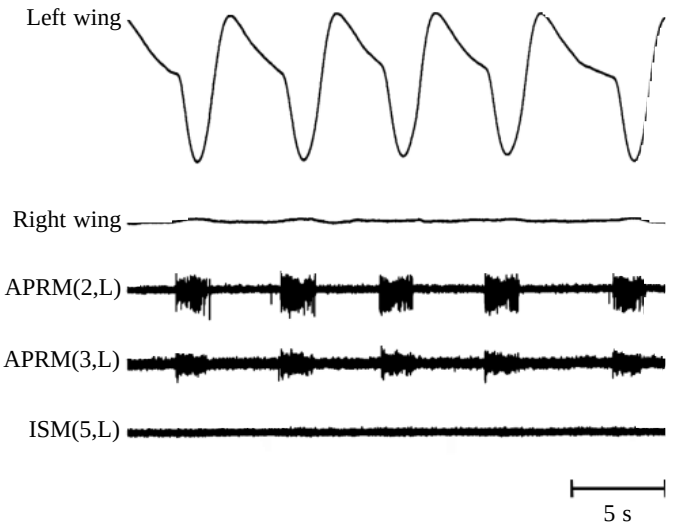


Fig. 4. Ablation of accessory planta retractor muscles eliminates detectable wing hemolymph flow during the pupal motor pattern. In this example, both accessory planta retractor muscles (APRMs) on the right side of the body were removed during the larval stage (see Materials and methods). Traces are as in Fig. 2; thermographic recordings were made on day P3 from the ventral margins of the mesothoracic wings in segment A2. APRM activity on the left (unoperated) side of the body was normal during the pupal motor pattern, as was hemolymph flow in the left wing. In contrast, negligible hemolymph flow was seen in the right wing. Similar results were obtained from at least five other recording sites tested on the right wing (not shown). The intersegmental muscle (ISM) was silent throughout the recording period.

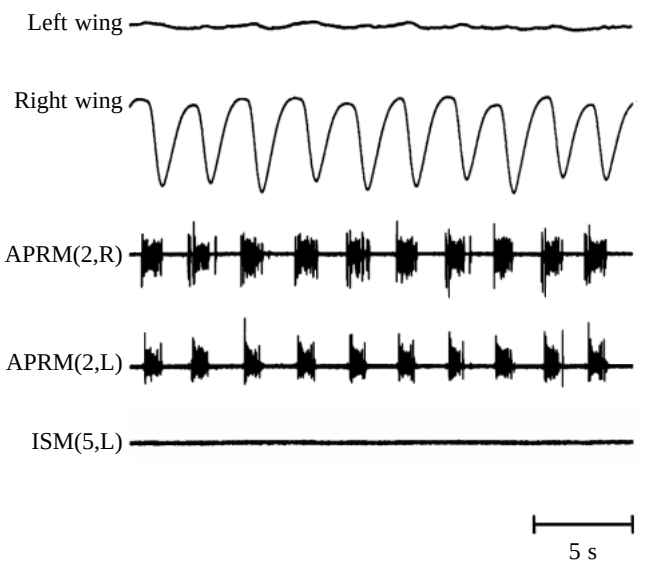


Fig. 5. Mechanical uncoupling of the wings from the abdomen eliminates detectable wing hemolymph flow during the pupal motor pattern. In this example, the left wings were lifted away from the ventral abdominal surface shortly after pupal ecdysis, where they hardened in place (see Materials and methods). Traces are as in Fig. 2; thermographic recordings were made on day P4 from the ventral margin of the mesothoracic wing on the right side of segment A2 and at a similar location on the left (lifted) mesothoracic wing. Accessory planta retractor muscle (APRM) activity was normal during the pupal motor pattern, as was hemolymph flow in the right (unmanipulated) wing. In contrast, negligible hemolymph flow was seen in the left wing. Similar results were obtained from at least five other recording sites tested on the left wing (not shown). The intersegmental muscle (ISM) was silent throughout the recording period.

accepted only when the intersegmental muscle was inactive and the abdomen motionless.

Similar results were obtained in six pupae with lifted wings, with a representative recording shown in Fig. 5. There was normal, bilateral APRM activity during the pupal motor pattern, and the right wing, which was attached to the abdominal surface, showed robust hemolymph flow. In contrast, hemolymph flow in the left wing, which was lifted away and hence mechanically uncoupled from the abdominal surface, was negligible. In all six pupae, weak hemolymph flow was detected at some recording sites on lifted wings (data not shown), probably as a result of incomplete separation of the wings from the abdominal surface. During abdominal movements, strong hemolymph movement was recorded from lifted wings (data not shown), indicating that the opening between the wing lumina and the body cavity remained open. Recordings from six sham-operated pupae were similar to those described above for normal pupae (data not shown). In three pupae with lifted wings, we recorded instances of the pupal motor pattern when intersegmental muscles were active with APRMs, and in two cases hemolymph flow was detected in the lifted wing (data not shown); possibly, the lack of normal contact between the flexible undersurface of the wings and abdomen permitted hemolymph to be driven into the wing lumina by intersegmental muscle contractions and abdominal shortenings during the motor pattern (see Discussion). This issue aside, the important finding is that APRM contractions during the pupal motor pattern produce robust hemolymph flow in the wings only if the wings are mechanically coupled to the abdominal surface, as predicted by the model in Fig. 1B.

## Discussion

The present study provides evidence for a dramatic example of neuromuscular respecification during metamorphosis of *Manduca sexta* in which motoneurons and muscles retained from the larval stage are used during pupal life to circulate hemolymph in the developing adult appendages. The present experiments focused on wing circulation, but the same mechanism is also likely to circulate hemolymph in the developing adult legs (see below). Such a role for APRs and APRMs was first proposed by Sandstrom and Weeks (1998), who reported that these larval motoneurons and muscles survive only in pupal segments A2 and A3 and become active in a new, rhythmic motor pattern after pupal ecdysis. The present study used contact thermography to measure hemolymph flow during the pupal motor pattern to test directly the hypothesis of Sandstrom and Weeks (1998) that APRM activity causes hemolymph circulation in developing adult appendages.

### *Contact thermographic measurement of hemolymph movement*

The contact thermographic technique for measuring hemolymph flow in insects was pioneered by L. T. Wasserthal, who measured hemolymph circulation in the thorax, abdomen and wing veins of adult moths and butterflies (Wasserthal, 1976, 1980, 1981, 1982). In the present study, we used his ‘C-method’, in which a heated thermistor is placed on the cuticular surface to detect the cooling effects of hemolymph flow below the thermistor site. The magnitude of the temperature change detected by the thermistor depends on the volume of hemolymph transported and, when hemolymph flow stops, the site under the thermistor is rewarmed at an exponential rate (Wasserthal, 1980). The shapes of thermistor deflections recorded in the present study varied and included monophasic downward deflections (Fig. 5), downward deflections followed by a small ‘overshoot’ [Fig. 4; proposed by Wasserthal (1980) to represent backflow of heated hemolymph to the recording site] and double downward deflections corresponding to the onset of contraction and relaxation of the APRMs (Fig. 3). These waveforms varied with the location of the recording site on the wing. Hemolymph flow within the wings during the pupal motor pattern is likely to be complex; the lumina connect with the general hemocoel only at their bases in the thorax, and their cross-sectional profile varies at different locations within the wings as well as during the APRM contraction–relaxation cycle (Sandstrom and Weeks, 1998; Fig. 1B). Further studies

would be required to identify the specific characteristics of hemolymph flow within the wing lumina that produce the different types of thermographic signal.

### *Causal relationship between APRM contractions and wing hemolymph flow*

In the model proposed by Sandstrom and Weeks (1998; Fig. 1B), APRM contractions during the pupal motor pattern lift the flexible ventral abdominal wall in segments A2 and A3, to which the flexible inner surfaces of the wings and legs adhere tightly. This upward movement is hypothesized to increase the volume of the lumina of the appendages, producing a reduction in pressure that draws hemolymph in from the hemocoel and circulates hemolymph within the appendages. We provide several lines of evidence in support of this hypothesis, all based on observations made when the intersegmental muscles were silent during the pupal motor pattern. First, there was a strict correlation between each APRM contraction (as measured by EMG activity) and each thermographic deflection during the motor pattern (Figs 2–5). Importantly, this constant relationship was observed in the absence of intersegmental muscle activity or abdominal movement. Second, the amplitude of each thermographic deflection was strongly correlated with the strength of APRM activity. This relationship was particularly apparent during the beginning or end of motor pattern episodes (Fig. 2); weak APRM contractions produced weak hemolymph movement, whereas strong APRM contractions produced strong hemolymph movement. Third, after unilateral ablation of APRM(2) and APRM(3), wing hemolymph flow on the operated side was negligible or absent (Fig. 4); the small deflections that were occasionally observed may have resulted from mechanical coupling to abdominal wall movements produced by contralateral APRMs. Fourth, mechanical uncoupling of the wings from the abdominal surface on one body side caused hemolymph flow in these wings to be greatly reduced or eliminated (Fig. 5); the small deflections occasionally seen in uncoupled wings may have resulted from incomplete separation of the lifted wings from the abdominal surface. It is also possible that minute increases in hemocoel pressure in the general body cavity caused by APRM contractions pushed hemolymph into the lifted wings *via* their openings at the wing bases. Taken together, these findings provide convincing evidence that thermographic deflections measured on the wings of *Manduca sexta* pupae represent hemolymph movement and that APRM contractions during the pupal motor pattern produce robust hemolymph flow in the wings independently of intersegmental muscle contractions or abdominal shortening. The model in Fig. 1B is based on segment A3, in which the APRMs insert over the wings; in segment A2, the APRMs insert over the developing metathoracic legs (which are in turn covered by the wings) and APRM contractions are expected to circulate hemolymph in the legs by the same mechanism.

As shown by Sandstrom and Weeks (1998) and the present study (Fig. 3B and data not shown), contractions of intersegmental muscles and abdominal shortening sometimes accompany APRM activity during the pupal motor pattern. When present, intersegmental muscle contractions occur synchronously with APRM contractions. Using anemometric recordings of air flow through the spiracles during the pupal motor pattern, Sandstrom and Weeks (1998) showed that intersegmental muscle contractions, in coordination with activity in spiracular closer muscles, produce ventilation through the tracheal system. Our experiments suggest that intersegmental muscle activity during the motor pattern has little effect on wing hemolymph flow (Fig. 3B); even during intense intersegmental muscle activity and abdominal movements, hemolymph flow remained most closely correlated with APRM activity (Fig. 3C). Although the increase in hemocoel pressure caused by abdominal shortening might be expected to force hemolymph into the appendages, the same pressure increase might also press the flexible abdominal wall out against the inner surface of the appendages, thereby opposing entry of hemolymph into the lumina from the hemocoel. The ability of intersegmental muscle contractions to produce wing hemolymph flow independently of APRM contractions in normal pupae was not examined in the present study. However, indirect insight into this issue was provided by the finding that, in wings overlying ablated APRMs, contractions of intersegmental muscles did not produce detectable hemolymph flow (see Results). In contrast, in lifted wings that were mechanically uncoupled from the abdominal surface, contractions of intersegmental muscles did produce hemolymph flow, and the amplitude of thermographic deflections in lifted wings during the pupal motor pattern increased when both the intersegmental muscles and the APRMs contracted (see Results). Further experiments would be required to evaluate whether contractions of intersegmental muscles in the absence of APRM activity can produce hemolymph flow in the appendages of normal pupae, but our observations suggest that their ability to do so may be limited.

The available information thus suggests that, during early pupal life, APRM contractions produce hemolymph circulation in the appendages but not tracheal ventilation, whereas contractions of intersegmental muscles produce ventilation but little or no hemolymph flow in the appendages. The coordinated pattern of ventilatory and circulatory activity during the pupal motor pattern may be related to the ‘coelopulse’ or ‘extracardiac pulsations’ reported in the pupal stages of other insects (Slàma, 1988; for a review, see Miller, 1997). From an energetic standpoint, the metabolic costs of perfusing the appendages by rhythmic contractions of the small APRMs should be lower than by utilizing contractions of the considerably larger intersegmental muscles (assuming a similar contraction cycle period and duration). APRM(2)s and APRM(3)s both lose mass at pupation and remain at this reduced size for the entire pupal stage (Sandstrom and Weeks, 1998), suggesting that relatively small contractile forces are needed to produce hemolymph flow during the pupal motor pattern. Hemolymph circulation does appear to require smaller forces than does ventilation, as illustrated by the observation

of Slàma (1988) in another moth species (*Actias selene*) that intersegmental muscle contractions cause changes in hemolymph pressure 100 times greater than those produced by the pupal heartbeat. Although intersegmental muscle contractions are used to produce air flow through the tracheal system, the use of smaller, more specialized muscles such as the APRMs for circulation may be metabolically advantageous.

### *Physiological significance of hemolymph circulation during the pupal stage*

Although APRM contractions during the pupal motor pattern perfuse the appendages during early pupal life, this function is likely to be transient. The motor pattern is faster and weaker during the second half of the pupal stage (Sandstrom and Weeks, 1998), by which time accessory pulsatile organs have developed in the thorax (S. K. Davidson and J. C. Weeks, unpublished observations; for a review, see Krenn and Pass, 1995). Accessory pulsatile organs, which are muscular pumps, circulate hemolymph in the wings of Lepidoptera and other insects during adulthood and may also do so during the latter period of pupal life. Ensuring adequate perfusion of developing appendages, including the early pupal period prior to the development of accessory pulsatile organs, is important for many reasons. The thoracic legs and wings arise from imaginal anlage (Nardi et al., 1985; Consoulas et al., 1997), requiring the delivery of ecdysteroids to promote development and morphogenesis (Nijhout, 1994) and metabolic fuels to support this process. The most dramatic period of development occurs during the first half of pupal life, when the pupal motor pattern is most strongly expressed. Hemolymph and hemocytes play other important roles including the removal of metabolic wastes, phagocytosis of cellular debris, immune responses, wound healing and coagulation, metabolism and the delivery of cryoprotective factors (e.g. Zachariassen and Hammel, 1976; Chapman, 1982; Nardi and Miklasz, 1989; Gillespie et al., 1997). Simple diffusion is unlikely to be sufficient to perfuse the developing appendages adequately, because the distance from the tips of the appendages to their small-diameter connections with the thoracic hemocoel can be as great as 3 cm in *Manduca sexta* (see Fig. 1A).

Muscles that resemble the APRMs of *Manduca sexta* are found in pupal segments A2 and A3 of other lepidopteran species (Finlayson, 1956; Randall, 1968; see Sandstrom and Weeks, 1998). For example, a drawing by Finlayson (1956) shows muscles essentially identical to the APRMs that insert beneath the developing wings in segments A2 and A3 of a silkmoth, *Telea polyphemus*. However, the activity of these muscles during the pupal stage has not been examined in species other than *Manduca sexta*. That muscles similar to the APRMs are retained in many species provides additional, although circumstantial, support for the proposal that they play an important role during pupal life. In the beetle *Tenebrio molitor*, elimination of all patterned abdominal motor activity by ablation of the mesothoracic ganglion causes severe developmental defects of the appendages (Slàma et al., 1979). When the APRM(2)s and APRM(3)s are ablated in *Manduca sexta* larvae, no obvious defects are seen in the legs or wings after adult emergence (Sandstrom and Weeks, 1998). However, this lack of a deleterious effect of APRM removal may result from the relatively benign rearing conditions experienced in the laboratory, in which the insects are well nourished, do not experience extreme temperature fluctuations and do not enter diapause (a prolonged period of developmental arrest early in the pupal stage that permits overwintering). There may also be compensatory mechanisms to produce hemolymph flow in the absence of APRM contractions although, as discussed above, intersegmental muscle contractions do not seem well suited for this role. Wing and leg muscles do not become functional until day P6 or later (Kammer and Rheuben, 1976; Consoulas et al., 1996), so could potentially contribute to hemolymph movement only during the latter portion of the pupal stage (which lasts approximately 18 days under typical laboratory conditions). Further experiments are required to evaluate the physiological role of APRM-induced hemolymph circulation under more naturalistic conditions.

In conclusion, we have shown that a neuromuscular system that produces proleg and abdominal movements during the larval stage of *Manduca sexta* is selectively retained for a circulatory function during the pupal stage. This dramatic transformation represents yet another example of the remarkable structural and functional plasticity that accompanies metamorphosis (Gilbert et al., 1996).

This research was supported by NIH grants F32 NSO9687 to J.L.L. and R01 NS23208 to J.C.W. The authors thank Dr David Sandstrom and Dr Jack Gray for comments on a draft of the manuscript, Dr Dan Kimble for providing laboratory space to accommodate a recording rig, Don Pate and the late David Brumbley of the Neuroscience Technical Support Group for consultation and equipment construction, and Gwen Gage and Kristina Schlegel for help with graphics.

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