

# Modulation of foregut synaptic activity controls resorption of molting fluid during larval molts of the moth *Manduca sexta*

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## Summary

We examined the role of the foregut in the resorption of molting fluid (MF) from the exuvial space during the last larval–larval molt of the moth *Manduca sexta*. In intermolt larvae, the activity of the foregut is characterized by robust peristaltic contractions. With the onset of the molt, MF is secreted into the exuvial space where it digests and weakens the old cuticle. The appearance of MF in the exuvial space is accompanied by a dramatic reduction in the amplitude of the foregut contractions. Foregut peristalsis returned about halfway through the molt, followed shortly by the appearance of MF in the gut. These observations suggested that larvae use their foreguts to remove MF from the exuvial space. Animals whose foreguts were surgically inactivated did not resorb their MF and most failed to successfully shed their old cuticles. The reduction in foregut motility at the

onset of the molt was correlated with a sharp decline in the amplitude of the excitatory junctional potentials. With the onset of the molt there was also a decline in the number of presynaptic terminals on the foregut that loaded with the activity-dependent dye FM1-43. In the second half of the molt, the appearance of MF in the foregut and the return of foregut motility was correlated with an increase in FM1-43 loading. These data reveal that during a larval–larval molt, vesicle release and/or recycling of the presynaptic endings on the foregut muscles is modulated to assure the proper timing of MF resorption.

Key words: molting, foregut synaptic activity, molting fluid resorption, *Manduca sexta*, insect, ecdysis.

## Introduction

The regulation and organization of a variety of stereotyped behaviors are essential for many aspects of animal development. One such collection is a series of ecdysial-related motor programs that lead to the shedding of an insect's old cuticle during a molt. Like all insects, the growth of larval *Manduca sexta* moths is limited by its inelastic cuticle. Consequently, growing larvae undergo a series of molts in which the old cuticle is shed and replaced by a new, larger cuticle. The two ecdysial-related motor patterns that have been the focus of much attention are the pre-ecdysis and ecdysis motor programs, which serve to loosen and then propel the old cuticle off the insect at the end of the molt, respectively (Copenhaver and Truman, 1982; Miles and Weeks, 1991; Žitňan and Adams, 2000). The analysis of these two simple motor programs has served as a model system for the role of hormones in coordinating stereotyped behaviors. However, pre-ecdysis and ecdysis behaviors define the end of a series of ecdysial-related motor patterns that can stretch over several days. The first external sign that a *Manduca* larva has entered the molt cycle is apolysis, or the detachment of the old and new cuticles from one another as molting fluid (MF), a cocktail of digestive enzymes, is secreted by the epidermis into the

exuvial space (the space between the old and new cuticles) where it degrades and weakens the old cuticle. Once degraded, the digested components of the exuvia and the MF are absorbed, leaving behind a thin, papery outer cuticle to be shed during ecdysis.

The secretion of MF and its resorption many hours later are among a series of molt-related events that must be precisely coordinated during the molt cycle. While the removal of the MF prior to ecdysis is a feature common to all insect molts, there is no consensus on the mechanisms used to remove the MF from the exuvial space. It has often been reported that MF is resorbed across the newly formed cuticle and epidermis (Jungreis, 1979; Reynolds and Samuels, 1996; Wigglesworth, 1972). This conclusion is based on the studies of the larval molt of the bug *Rhodnius prolixus* (Wigglesworth, 1933, 1972) and the pupal–adult molt of the silkworm *Hyalophora cecropia* (Lensky et al., 1970; Passonneau and Williams, 1953). These studies concluded that MF is absorbed into the hemocoel by passing through pores in the new cuticle.

There is also evidence suggesting that the gut serves as a route for the removal of MF. Wachter (1930) noted that during the larval molt, silkworms of *Bombyx mori* appeared to drink

their MF. Cornell and Pan (1983) were the first to demonstrate that the gut played a role in the removal of MF. By dyeing the MF during the larval–pupal molt of *Manduca sexta*, they tracked the movement of MF from the exuvial space into both the foregut and hindgut just prior to ecdysis. The gut also appears to be a major route used for the removal of MF during the pupal–adult molt of *Manduca*. Approximately 6–12 h before the emergence of the adult moth, the foregut swallowing motor pattern is activated, resulting in the ingestion of the MF (Miles and Booker, 1998). Moths whose foreguts were denervated failed to absorb their MF and often failed to emerge from their pupal cuticle.

We examined the mechanism employed by *Manduca* to remove MF during its final larval–larval molt. We have found that the modulation of the ongoing rhythmic motor activity of the foregut is crucial for the successful completion of a larval–larval molt. Our results revealed that during the molt between the 4th and 5th larval instars, foregut activity is modulated in order to ingest MF prior to the onset of ecdysis. When foregut peristalsis was experimentally blocked, larvae failed to resorb their MF and most did not successfully shed their old cuticle. The changes in foregut activity that occur during the molt cycle result from a decline in the amplitude of excitatory junctional potentials (EJPs) recorded from the foregut musculature. Using the activity-dependent dye FM1-43 to examine presynaptic activity revealed that the loss of foregut activity following the onset of the molt resulted from a dramatic decline in vesicle release and/or recycling at the synaptic terminals on the foregut musculature. The return of foregut peristalsis prior to ecdysis was accompanied by an increase in FM1-43 uptake into foregut synapses. The work presented here suggests that the presynaptic endings onto the foregut musculature serve as targets for one or more neurohormones and/or neuromodulators during a larval–larval molt.

## Materials and methods

### *Experimental animals*

*Manduca sexta* L. larvae were reared in individual plastic cups on a wheatgerm-based artificial diet modified from Bell and Joachim (1976). A 27°C, 16h:8h L:D photoperiod regimen was provided for the *Manduca* colony. Under these conditions, the durations of the 4th and 5th instar stages were 3–4 days and 4–5 days, respectively. The molt between the 4th and 5th instars lasted about 25 h. Head capsule slippage (HCS) is an obvious marker for the beginning of the molt cycle (Copenhaver and Truman, 1982). It occurs when the old head capsule slips forward and covers the developing mouthparts of the pharate fifth instar larva. We used HCS as a developmental reference point for the initiation of the molt cycle. Under our colony conditions, we found that, beginning at 15.7±0.3 h after HCS ( $N=43$ ), the pharate mouthparts started to tan and appeared yellow through the slipped head capsule. Over the remainder of the molt cycle, the mouthparts of the pharate 5th instar larva continued to tan until they were dark brown. At

21.2±0.2 h after HCS ( $N=95$ ), air bubbles collected in the old head capsule, indicating that MF had begun to be removed from the exuvial space. Over the next 4 h, air continued to fill the exuvial space, and the old cuticle dried slightly. At 25.2±0.2 h after HCS ( $N=67$ ), the molt cycle ended when the animal shed its old cuticle during ecdysis.

### *Immobilization of foregut peristalsis*

Foregut motility was surgically blocked by removal of the frontal ganglion (FG), which is solely responsible for generating the rhythmic peristaltic movements. Larvae that were selected for surgery were late-stage 4th instar larvae that were already committed to enter the molt cycle. The heads of the larvae were positioned through a hole in the cover of a deep plastic Petri dish and then immobilized with dental wax (Surgident Periphery Wax; Heraeus Kulzer, Inc., Hanau, Germany). CO<sub>2</sub> gas was fed into the Petri dish to anesthetize the larvae. Using a breakable razor blade (Fine Science Tools, North Vancouver, BC, Canada), a V-shaped incision was made inside the epistimal ridge of the head capsule. The cuticle of the frons was pulled forward exposing the FG, which was then removed using fine forceps. The frons was repositioned and the wound sealed with a dermatological adhesive (New Skin; Medtech Laboratories, Inc., Jackson, WY, USA). Sham-operated controls underwent the same procedure, but the FG was simply touched with the forceps. The operated animals were kept under CO<sub>2</sub> anesthesia until the glue dried.

As an alternative to the surgical removal of the FG, we blocked the contractions by filling the foreguts with a bolus of veterinary tissue adhesive (n-butyl cyanoacrylate; Vetbond, 3M). The adhesive was injected through the larva's mouth into the foregut using a blunt needle attached to a syringe. The adhesive immediately hardened and prevented foregut contractions.

### *Tracking the passage of the molting fluid*

We tracked the resorption of MF during the 4th–5th instar molt by injecting 100–150 µl of either 2% (w/v) carmine dye (Alum Lake; Fischer Scientific Company, Suwanee, GA, USA) or 5 mg ml<sup>-1</sup> fluorescein isothiocyanate (FITC)-inulin (Sigma, St Louis, MO, USA; Lorenz and Gruenstein, 1999) into the exuvial space of larvae. Carmine red is an inert, non-absorbed dye and inulin is an inert polysaccharide that has long been used to evaluate water balance and membrane permeability (O'Donnell and Maddrell, 1983; Smith, 1951). Both markers were dissolved in saline (Ephrussi and Beadle, 1936). A 100 µl Hamilton syringe equipped with a 33-gauge needle was used to make one or two shallow injections to deliver the solutions into the exuvial space. Larvae selected for injection were between 0 h and 14 h after the initiation of the molt (see Results), and any larvae suspected of having had their underlying epidermis mistakenly penetrated by the needle were discarded. Carmine-injected larvae were sacrificed 6–8 h later and the distribution of the carmine dye was determined. At least 10 h after larvae were injected with FITC-inulin, we extracted hemolymph from the larvae and inulin levels were

measured using spectrophotometry. The distribution of the FITC-inulin within the hemocoel was also determined by examining the larvae.

In order to analyze blood levels for the presence of FITC-inulin, the dorsal horns of ice-anesthetized, FITC-inulin-injected larvae were cut off and the hemolymph was collected in 1.5 ml centrifuge tubes containing a few crystals of phenothiocarbamide to prevent the oxidation of the hemolymph. The blood samples were vortexed for 15 s and then centrifuged for 1 min at 17 000 g. The blood was then transferred to cuvettes and its absorption at 490 nm was measured with a spectrophotometer (Biochrom Ultraspec II; Biochrom, Cambridge, UK).

In order to examine the distribution of the two dyes in internal tissues, the injected larvae were dissected and examined under a low-power stereomicroscope. Epifluorescence was used to visualize the FITC-inulin. To avoid contaminating the hemocoel with dye particles, an initial incision was made along the dorsal midline of the exuvia of ice-anesthetized larvae, and saline was used to flush away any dye remaining in the exuvial space near the incision. A second dorsal midline incision was then made in the underlying cuticle to expose the entire gut and hemocoel. The preparation was pinned open and the distribution of either the carmine or FITC-inulin inside the body cavity was determined. The movement of the FITC-inulin-labeled MF was also tracked in a group of larvae whose foreguts had been immobilized either by surgical removal of the FG or with Vetbond adhesive (see above). Photographs were taken with a Kappa DX30 C digital camera and Kappa ImageBase Control software. Identical acquisition settings were used throughout the experiments.

#### Monitoring foregut movement

Animals were anesthetized on ice before excising the head and thorax. The dorsal cuticle was cut down the midline, and the preparation transferred to a 35 mm plastic tissue culture dish lined with silicone elastomer (Sylgard; Dow Corning, Midland, MI, USA). Pinning the preparation open revealed the foregut, brain and FG (Fig. 1). To free the foregut from the rest of the larva, the extrinsic buccal and esophageal dilator muscles were cut as near as possible to their attachment points on the exoskeleton, often removing a small portion of the cuticle. The foregut was also cut free of its mouth attachments. All nerves exiting the brain were cut except for those connecting the brain to the FG. Once free from the body, the foregut, FG and brain were transferred and pinned to a separate Sylgard-lined dish. Preparations were bathed in physiological saline (140 mmol l<sup>-1</sup> NaCl; 5 mmol l<sup>-1</sup> KCl; 4 mmol l<sup>-1</sup> CaCl<sub>2</sub>; 28 mmol l<sup>-1</sup> dextrose; 5 mmol l<sup>-1</sup> Hepes; 2 mmol l<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O; 5 mmol l<sup>-1</sup> trehalose; modified from Trimmer and Weeks, 1989). Isolation of the foregut and FG has been shown to have little effect on the foregut motor pattern generated by the FG (Miles and Booker, 1994).

Larvae were dissected as above and the activity of their foreguts was observed using a dissecting microscope. In some instances, the amplitude of the foregut contractions was

measured using a movement transducer constructed from a piezoelectric phonograph cartridge. One end of a 2 cm-long piece of 75 µm-diameter tungsten wire was etched to a fine point and the end was glued to the stylus of the phonograph cartridge. The free end was bent to form a hook and attached to the intrinsic foregut constrictor muscles. A micromanipulator was used to position the movement transducer at a 90° angle to the long axis of the foregut, and the lateral edge of the constrictor muscles was hooked as illustrated in Fig. 1. In some experiments, the recurrent nerve was cut near the FG, and muscle contractions were elicited with exogenous stimulation delivered *via* a suction electrode. To elicit contractions, 500 ms trains of 5 ms pulses were delivered at 40 Hz using a Grass S48 Stimulator (Astro-Med, West Warwick, RI, USA). The output of the movement transducer was amplified using a differential amplifier (A-M Systems, Carlsborg, WA, USA). The signals were stored on a VHS cassette tape, and real-time playback was conducted using a high-speed chart recorder (MT95000; Astro-Med). The transducer output was calibrated by measuring the displacement of the end of the wire using an ocular micrometer and converting the changes in recorded voltage to microns of movement. The response characteristics of the transducer were tested and a linear relationship was found between the voltage output and the range of movement transducer deflections. The values are reported as means ± S.E.M. Significant differences between the contraction amplitude means were determined by analysis of variance (ANOVA) using the Games–Howell test for differences (significance level=0.05; pair-wise *post-hoc* comparisons).

#### Electrophysiology

The isolated foregut was prepared as described above. In order to stabilize the foreguts of intermolt larvae for intracellular recordings from the muscles, a piece of Sylgard was cut to the approximate diameter and length of the foregut and was placed inside the foregut lumen. This arrangement greatly diminished the overall amplitude of the foregut contractions. Extracellular recordings of nerve and foregut muscle activity were conducted with custom-made suction electrodes. Intracellular electrodes of 20–25 MΩ were suspended loosely from the headstage of the amplifier (Neuroprobe 1600; A-M Systems) in order to impale the foregut. Signals were stored and replayed as above. In some experiments, muscle responses were elicited with exogenous stimulation (10 ms pulses) delivered to the cut recurrent nerve *via* a suction electrode. To verify that the EJPs of the foregut muscle were generated in response to nerve activity, the recurrent nerve was ejected and, without moving the suction electrode, the response of the muscle to the stimulus was monitored.

We used the following approach to record intracellularly from the neurons of the FG. Once the foregut, brain and FG were removed from the body, the buccal region was cut free from the esophagus. To stabilize the FG, the buccal region of the foregut was pinned to the Sylgard dish. The brain was

flipped anteriorly and pinned ventral side up to the Sylgard dish. The anterior edge of the esophagus was securely pinned into a shallow indentation carved out of the Sylgard. This arrangement permitted the esophageal portion of the foregut to contract while the FG remained stable. In most cases, the sheath surrounding the FG was permeabilized by placing a crystal of pronase (Sigma) on the surface of the ganglion for a few seconds before being washed away with saline. The intracellular recordings were made with 20–25 M $\Omega$  borosilicate electrodes filled with 3 mol l<sup>-1</sup> KCl. Motorneurons were identified with the concurrent recordings from the foregut musculature and based on previous descriptions (Miles and Booker, 1994). The signals were amplified, recorded and replayed as above.

To analyze the bursting properties of the recurrent nerve, recordings were re-digitized using Clampex 8.1 (Axon Instruments, Inc., Union City, CA, USA) at 10 kHz and imported into Matlab 6.1 (The MathWorks, Inc., Natick, MA, USA). The individual signals were digitally rectified, filtered at 30 Hz with a 5-pole, Butterworth, zero phase-shift, digital low-pass filter, then fit with a curve generated from the first few components of the Fourier transform of the signal. We found that including the fundamental (burst rate), plus 5–7 harmonics produced a fit that adequately captured the shape of the bursts. The same analysis parameters were used for all recordings.

After the means for the baseline and maximum amplitude level were assigned to each rectified trace, the amplitude of the trace was divided into quartiles. All activity that crossed the first quartile above baseline was recorded. The onset and termination of the burst were assigned when the rectified trace dropped below the first quartile. The burst duration was calculated as the time between onset and termination points of the burst. The burst amplitude was the mean maximum amplitude of the rectified trace. Burst duty cycle is defined as the proportion of the cycle that is dedicated to the burst. We also calculated the mean number of spikes during the interburst period that crossed the first quartile (interburst count cycle<sup>-1</sup>) as well as the ratio between the mean amplitude of the interburst activity over the mean burst amplitude.

#### *FM1-43 staining and synaptotagmin immunocytochemistry*

The activity-dependent fluorescent dye FM1-43 (Molecular Probes, Eugene, OR, USA) was used to label foregut nerve terminals of molting and intermolt larvae. Because FM1-43 marks only those cells that have undergone a cycle of exocytosis and endocytosis, it can be used to monitor synaptic activity (Betz and Bewick, 1992; Cochilla et al., 1999; Consoulas and Levine, 1998; Consoulas et al., 1999). The methods used here were modified from Kuromi and Kidokuro (1999). The basic dissection used in these experiments was similar to that outlined above. While the synaptic terminals on all foregut muscles loaded FM1-43, the terminals on the single layer extrinsic muscles, the esophageal dilators (ED), were more amenable to imaging and analysis. Foregut preparations from both intermolt and early-molt larvae (<16 h after HCS)

were initially incubated for 15 min in normal physiological saline containing 10  $\mu$ mol l<sup>-1</sup> FM1-43. This allowed the loading of FM1-43 through endogenous activity. To prevent the release of the captured dye, the preparations were rinsed three times for 30 s in Ca<sup>2+</sup>-free saline (Ca<sup>2+</sup> was replaced by 20 mmol l<sup>-1</sup> MgCl<sub>2</sub>, NaCl was lowered to 124 mmol l<sup>-1</sup>, and 0.5 mmol l<sup>-1</sup> EGTA was added) and were then viewed after an additional 15 min incubation in Ca<sup>2+</sup>-free saline. The preparations that failed to load the FM1-43 under endogenous activity were then induced to take up FM1-43 by incubating them in high K<sup>+</sup> saline (KCl was increased to 90 mmol l<sup>-1</sup> and NaCl was lowered to 55 mmol l<sup>-1</sup>) containing 10  $\mu$ mol l<sup>-1</sup> FM1-43. After a 15 min incubation in high K<sup>+</sup> saline, the larvae were rinsed and examined in Ca<sup>2+</sup>-free saline as described above. Larvae were viewed under a 40 $\times$ , 0.8 NA water-immersion lens using a Nikon Eclipse 600-FN epifluorescent microscope (Nikon, Melville, NY, USA) equipped with a 100 W mercury lamp. The images were captured with a CCD SPOT2 camera (Diagnostic Instruments, Sterling Heights, MI, USA) with identical acquisition settings throughout each experiment. Using PhotoShop 7 (Adobe Systems, Inc., San Jose, CA, USA), the amount of FM1-43 taken up by the nerve terminals was estimated by measuring the mean luminosity of individual FM1-43 fluorescent puncta, from which the mean background level for each image was subtracted. The density of FM1-43-labeled puncta was calculated by counting the number of fluorescent puncta per 20  $\mu$ m<sup>2</sup>. Significant differences between the means (both puncta luminosity and density) were determined by ANOVA using the Games–Howell test for differences ( $P=0.05$ ; pair-wise *post-hoc* comparisons).

We also imaged the synaptic terminals on foregut muscles with an antiserum raised against *Drosophila melanogaster* synaptotagmin (DSYT2; Littleton et al., 1993; a generous gift of J. T. Littleton and H. J. Bellen). The muscles were fixed in 4% paraformaldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer, pH 7.4 (PBS) for 1 h at room temperature. After rinsing in PBS containing 1% Triton-X-100 (PBSX) overnight, the preparations were blocked for 2 h in 10% normal goat serum and incubated at 4°C overnight in primary antiserum (1:1000) made in PBSX. After washing in PBSX and PBS for 2 h, the tissues were incubated in Cy3-conjugated secondary antiserum overnight at 4°C. The preparations were then rinsed in PBSX and PBS, dehydrated in ethanol and cleared in methyl salicylate.

## Results

### *Removal of frontal ganglion blocks molting fluid resorption*

The activity of the foregut of *Manduca sexta* is controlled solely by the rhythmic output of the frontal ganglion (FG), a small ganglion of approximately 35 neurons that lies anterior to the brain on the dorsal surface of the foregut (Fig. 1; Miles and Booker, 1994). In order to determine whether the foregut plays a role in the resorption of the MF prior to ecdysis, we examined molting larvae whose FG had been removed approximately 24 h prior to the onset of the molt. Removing FG from larvae has been shown to immediately and



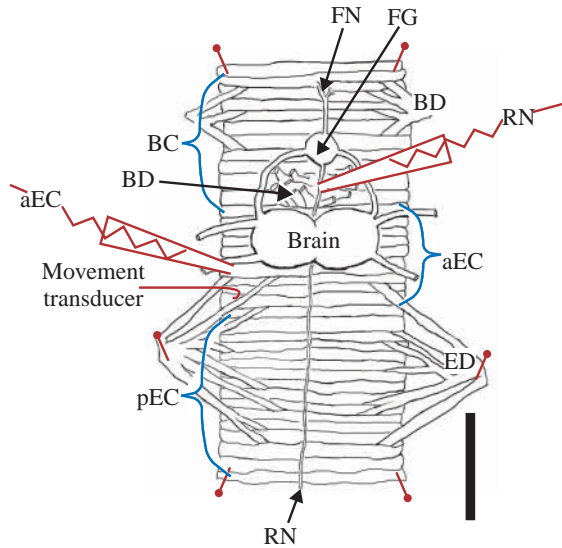


Fig. 1. Diagram of the foregut preparation. BD, buccal dilator; BC, buccal constrictor; ED, esophageal dilator; aEC, anterior esophageal constrictor; pEC, posterior esophageal constrictor; FN, frontal nerve; FG, frontal ganglion; RN, recurrent nerve. Examples of suction electrode and movement transducer placements are illustrated in red. Note that the muscles have been cut from their cuticular attachments and that the branching patterns of the nerves have been omitted from the drawing. Scale bar, 500  $\mu$ m.

irreversibly stop foregut peristalsis (Miles and Booker, 1994). As expected, lesioning the FG impaired the ability of larvae to ingest their food (Miles and Booker, 2000). However, since the movements of the mouthparts are controlled by neurons of the subesophageal ganglion, removal of the FG did not affect the ability of larvae to bite their food (Rohrbacher, 1994). As a result, the lesioned animals continued to feed and grow, although at a slower rate compared with sham-operated controls. We found that 24/28 of the lesioned larvae initiated the molt cycle (onset of HCS), although typically one day later

than sham-operated larvae. After initiating the molt cycle, the lesioned animals appeared healthy and progressed through the molt at a normal rate. As expected, all of the sham and unoperated control larvae showed signs of MF resorption (the presence of air bubbles in the head capsule) about 21 h after HCS. However, none of the lesioned larvae ( $N=24$ ) showed signs of MF resorption. Consequently, while 91% (10/11) of the sham-operated larvae successfully completed ecdysis, only 8% (2/24) of the lesioned larvae managed to completely shed their exuviae. In most cases, there was a tendency for the exuviae of the lesioned larvae to adhere to the newly formed cuticle and tear during ecdysis. Those larvae that did not completely remove their exuviae lost weight and died two or three days after their failed ecdysis attempt.

#### *The migration of the molting fluid during the final larval molt*

The results outlined above suggested that during a larval-larval molt, foregut motor activity was required for both the resorption of MF and the successful completion of ecdysis. To track the movements of the MF during the larval-larval molt, we labeled the MF with either carmine red or FITC-inulin. Dissection and examination of early-molt larvae whose pharate, 5th instar mouthparts had not begun to tan (up to 15 h after HCS) revealed that both carmine dye and FITC-inulin remained in the exuvial space. Neither carmine nor FITC-inulin was found associated with any region of the body cavity, including the gut ( $N=7$  and  $N=10$ ; respectively, Fig. 2A). We also failed to detect FITC-inulin in the hemolymph samples of these early-molt larvae ( $N=6$ ). By 16–18 h after HCS, while most of the carmine dye remained in the exuvial space, only trace amounts were detected in the foregut ( $N=4$ ). Once larvae reached the late-molt stage ( $\geq 18$  h after HCS), the lumen of the foregut and midgut were heavily labeled in 94% (16/17) of the carmine-injected larvae and all of the FITC-inulin-injected larvae ( $N=6$ ) (Fig. 2B). We did not detect labeled-MF in the hemolymph of FITC-inulin-injected larvae ( $N=3$ ).

These results suggested that the foregut served as the

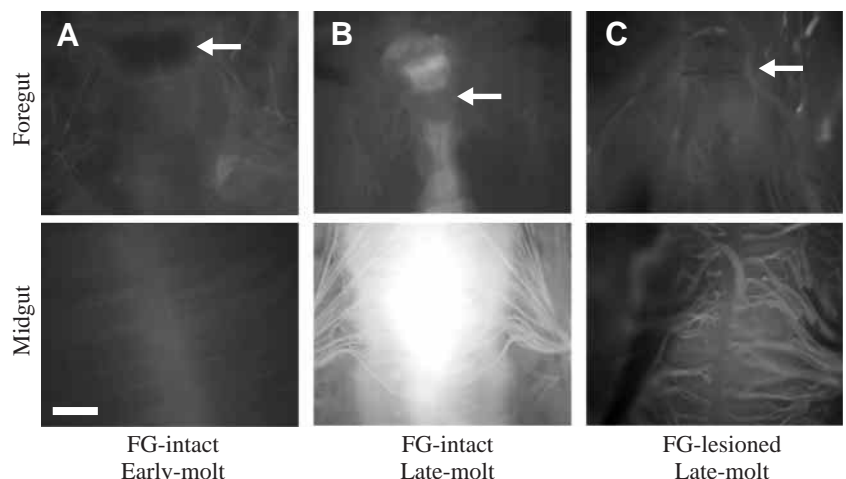
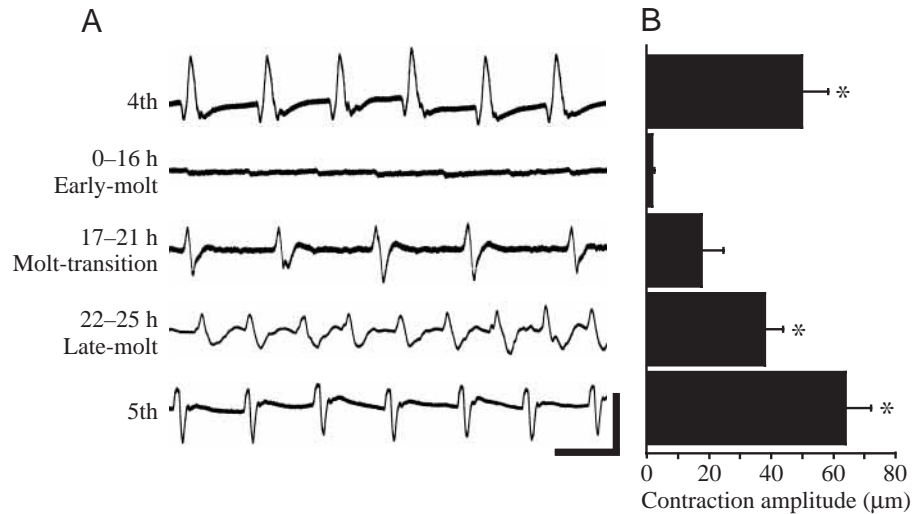


Fig. 2. FITC-inulin distribution in molting larvae. FITC-inulin was injected into the molting fluid (MF) of molting larvae. All larvae were exposed to the dye for 10 h before they were dissected. Foreguts are shown in the upper panels, and midguts are shown in the lower panels. (A) An early-molt larva [13–16 h after head capsule slippage (HCS)] with low levels of fluorescent material in its digestive tract. (B) We observed high levels of FITC-inulin in the foregut and midgut of the late-molt larva (22–25 h after HCS). The robust contractions of the foregut had resumed by the late-molt stage. (C) A late-molt stage larva (22–25 h after HCS) whose foregut was silenced by lesioning the frontal ganglion (FG) shows only low levels of fluorescent material in its gut. All larvae were photographed with the same camera exposure and microscope settings. Arrows indicate the right edge of the brain. Scale bar, 500  $\mu$ m.

Fig. 3. The contractions of the foregut were monitored using a movement transducer attached to anterior esophageal constrictor (aEC) muscles. (A) Output of the movement transducer recorded during the intermolt 4th and 5th instars, as well as during the molt between the two larval stages. Molting stages are given in hours after head capsule slippage (HCS). There were no significant differences in the mean period of contraction between any larval stages. Scale bars, 30  $\mu\text{m}$  and 2 s. (B) A movement transducer was used to determine the mean maximum amplitude of movement ( $\pm$  S.E.M.) for each stage. The asterisk represents significant difference from early-molt values ( $P < 0.05$ ,  $N = 9-37$ ).



primary route for the removal of MF prior to ecdysis. However, it seemed plausible that alternative routes such as transepidermal resorption and resorption of MF through the hindgut might also contribute to its removal. In an attempt to test these possibilities, we tracked the movement of FITC-inulin-labeled MF in larvae whose foreguts were inactivated either by the surgical removal of the FG or by the injection of a surgical adhesive into the lumen of the foregut (see Materials and methods). At no point did we detect FITC-inulin in any area of the digestive tract of larvae with inactive foreguts ( $N = 6$ ; Fig. 2C). We also failed to detect any signs of dye in the hemolymph of late-molt larvae either following the removal of the FG ( $N = 6$ ) or blockage of the foregut with adhesive ( $N = 5$ ). Furthermore, we did not find any evidence of the passage of FITC-inulin through the newly formed integument or accumulation of fluorescent particles around pores in the developing pharate 5th instar cuticle in larvae with either active or inactivated foreguts.

#### Foregut activity during the molt

We examined larvae to determine whether foregut motility was modulated during the molt to control the movements of MF into and out of the exuvial space. The larval foregut is a muscular tube divided into the buccal and esophageal regions, each region consisting of a set of circumferential constrictor and extrinsic dilator muscles (Fig. 1). During the intermolt, feeding, 4th instar stage, the normal pattern of foregut activity consisted of regular alternating rhythmic contractions of the buccal and esophageal regions, resulting in robust posteriorly directed peristaltic contractions with a mean period of  $3.2 \pm 0.4$  s ( $N = 12$ ). By attaching movement transducers to the various muscle groups that form the buccal and esophageal regions, we measured the amplitude of their contractions. Because the general activity patterns of the different muscle groups were not significantly different, only the results for the anterior esophageal constrictors (aEC) are reported (Fig. 3) in order to simplify our presentation.

In intermolt 4th instar larvae, the mean amplitude of aEC

contractions was  $49.8 \pm 8.4$   $\mu\text{m}$  ( $N = 11$ ). Following the onset of the larval-larval molt, there was a sharp decline in foregut motility (Fig. 3). In 71% (90/127) of the early-molt larvae we examined (0–16 h after HCS), there were no visible signs of foregut motility. The 37 early-molt stage larvae with active foreguts exhibited weak peristalsis or weak, uncoordinated contractions of the buccal and/or esophageal regions. These observations were confirmed using the movement transducer. The mean amplitude of aEC contractions for early-molt larvae measured only  $1.7 \pm 0.6$   $\mu\text{m}$  ( $N = 47$ ), a 30-fold decline relative to intermolt larvae (Fig. 3). Despite the dramatic reduction in foregut activity, in those larvae where the peristaltic rhythm was discernable, the mean period of the aEC contractions was  $3.3 \pm 0.9$  s and was not significantly different from the 4th instar value ( $P > 0.05$ ,  $N = 20$ ).

The molt-related decline in foregut motility had already occurred by the time larvae had slipped their head capsules. In an effort to define the onset of foregut modulation more accurately, we undertook a systematic examination of the foreguts of late-molt 4th instar larvae. Unfortunately, larvae exhibit few developmental markers before the onset of HCS. However, we did find a correlation between the response of the larva to stimulation of its mouthparts and foregut activity. During the majority of the 4th instar, 100% of the larvae responded to tactile stimulation of their mouthparts with a bite response. However, approximately 4 h prior to head capsule slippage, larvae stop feeding and fail to bite in response to stimulation of their mouthparts. When the foreguts of the non-responsive, late 4th instar larvae were examined, 86% had foreguts that were either inactive or exhibited only weak contractions ( $N = 37$ ). These results suggest that the robust peristalsis of the foregut is disrupted about 4 h prior to onset of HCS.

The results of our FITC-inulin and carmine dye experiments suggested that the labeled MF began to enter the foregut between 16 h and 18 h after HCS. An examination of the foreguts of animals at this stage revealed a correlation between the timing of MF entry into the foregut and an increase in

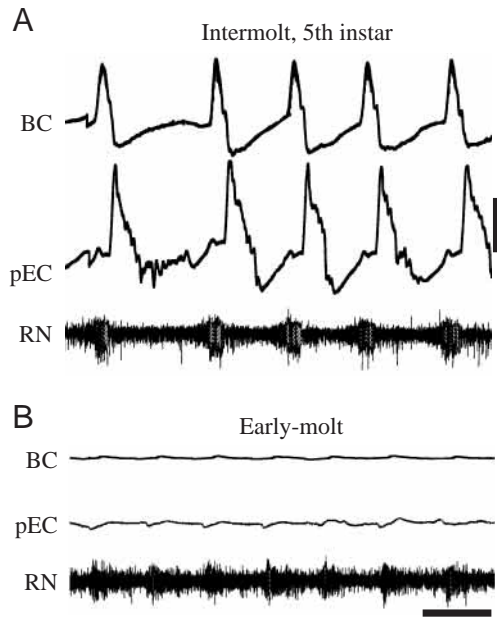


Fig. 4. Activity recorded from the recurrent nerve (RN) paired with alternating contractions of the buccal constrictor (BC) and posterior esophageal constrictor (pEC) muscle groups. (A) Recordings of muscle movement and nerve activity from an intermolt 5th instar larva. (B) Muscle movement and nerve activity recorded from an early-molt larva (0–16 h after head capsule slippage). Although the foregut contractions of the early-molt larva are of low amplitude, the RN shows its characteristic bursting activity and the foregut muscle contracts in a pattern similar to the 5th instar peristalsis. Scale bars, 50  $\mu\text{m}$  and 3 s.

foregut motility. Between 16 h and 18 h after HCS, approximately 75% (25/33) of the foreguts examined were peristaltically active. Compared with the early-molt larvae (0–16 h after HCS), the contraction amplitude of the aEC increased 10-fold to  $17.6 \pm 6.9 \mu\text{m}$  ( $N=11$ ). As the molt progressed, there was a further increase in foregut contraction activity. By the time air bubbles first appeared inside the old head capsule ( $\geq 21$  h after HCS), 97% (31/32) of the foreguts exhibited strong peristaltic contractions and the mean amplitude of the aEC contractions was similar to the levels observed in intermolt 4th instar larvae (Fig. 3B). In late-molt larvae, the period of the foregut contractions was  $3.5 \pm 0.5$  s, a value similar to that observed for intermolt and early-molt larvae ( $P > 0.05$ ,  $N=9$ ).

#### *The rhythmic activity generated by the frontal ganglion is unaltered during the molt*

We set out to determine the cellular mechanisms responsible for the modulation of foregut activity during a larval–larval molt. One possibility was that changes in the motor activity generated by the FG might be responsible for the disruption of foregut peristalsis during the molt. To test this hypothesis, we compared the pattern of activity of the two nerves that exit the FG – the frontal nerve (FN) and the recurrent nerve (RN) – in early-molt and intermolt stage larvae. The FN projects

Table 1. Rhythmic activity recorded from the recurrent nerves of intermolt and early-molt larvae

Parameter	Intermolt ( $N=8$ )	Early-molt ( $N=8$ )
Period (s)	$3.2 \pm 0.5$	$3.1 \pm 0.4$
Burst amp. (mV)	$3.5 \pm 0.5$	$2.3 \pm 0.5$
Burst duration (s)	$0.85 \pm 0.1$	$0.92 \pm 0.1$
Burst duty (%)	$29.9 \pm 4.8$	$29.5 \pm 4.3$
Interburst count cycle <sup>-1</sup>	$0.17 \pm 0.8$	$0.23 \pm 0.10$
Interburst burst <sup>-1</sup> amp.	$0.16 \pm 0.06$	$0.18 \pm 0.07$

Values shown are means  $\pm$  S.E.M.

Burst duty is the percentage of the total cycle dedicated to the burst. Interburst count cycle<sup>-1</sup> is the mean number of spikes outside of the burst that we recorded per cycle. Interburst burst<sup>-1</sup> amp. is the ratio of the mean maximum amplitude of the interburst activity over the maximum amplitude of the burst (see Materials and methods for further explanation). The means for the molting and intermolt larvae were not significantly different for any of the variables tested ( $P > 0.05$ , unpaired  $t$ -test).

anteriorly and innervates the muscles of the buccal region, while the RN projects to esophageal muscles (Fig. 1). In intermolt animals, the bursts of activity in these two nerves correspond to the motor pattern that generates foregut peristalsis (Miles and Booker, 1994). Our recordings revealed no obvious differences in the pattern of activity recorded from the FN and RN in intermolt and early-molt stage larvae (Fig. 4; Table 1).

Intracellular recordings from FG motorneurons revealed that the motor pattern generated by the FG was not altered during the molt. We surveyed FG motorneurons in both early-molt (0–16 h after HCS;  $N=17$ ) and intermolt 5th instar larvae ( $N=52$ ) and recorded from all four classes (esophageal and buccal constrictors and dilators) present in the FG (Miles and Booker, 1994). We failed to detect any significant differences in the patterns of activity of the FG motorneurons that could account for the decline in foregut motility observed during the early stages of the larval–larval molt. For example, comparison of the bursting properties of posterior esophageal constrictor motorneurons (pEC MN), both amplitude and frequency characteristics, did not reveal significant differences between early-molt and intermolt larvae (Table 2). Fig. 5 gives examples of activity recorded intracellularly from pEC MN as well as concurrent extracellular recordings using fine-tipped suction electrodes from the anterior and posterior esophageal constrictor (aEC and pEC) groups of foregut muscles. During the early-molt stage, the foregut was either inactive or displayed low amplitude contractions (Figs 3, 4), yet the pattern of activity recorded from the FG MNs in early molt larvae was not significantly different from that recorded from intermolt larvae (Fig. 5; Table 2).

Despite the large drop in foregut contraction amplitude that occurs during the molt, the pattern of EJPs recorded from the foregut musculature of molting larvae was not significantly different compared with intermolt larvae (Figs 5, 6A; Table 3).

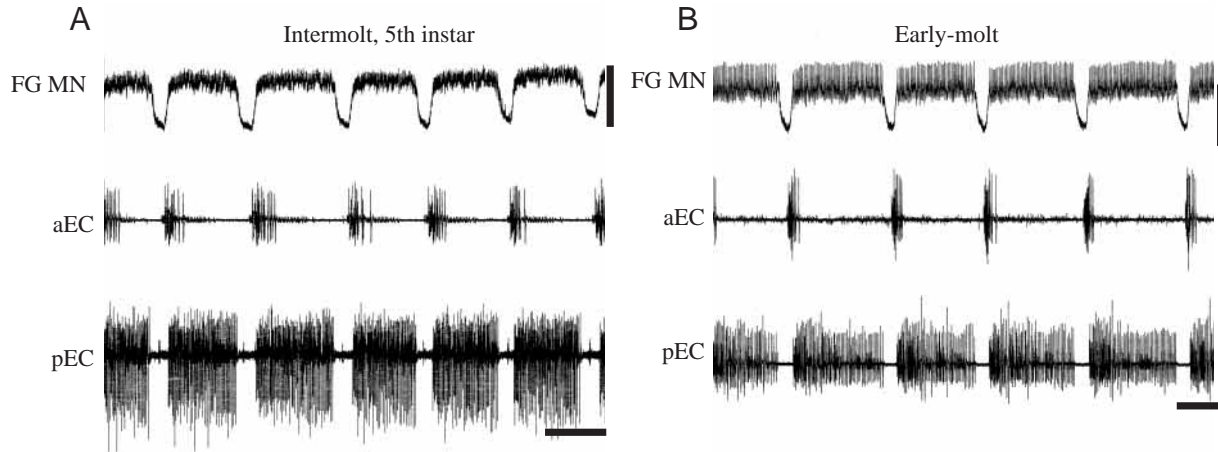


Fig. 5. Recordings from the posterior esophageal constrictor motorneurons (pEC MNs) located in the frontal ganglion and concurrent extracellular suction electrode recordings from the foregut musculature from anterior and posterior esophageal constrictor (aEC, pEC) muscles. (A) Examples of intracellular recordings from a pEC MN in an intermolt 5th instar larva. (B) Recording from an early-molt stage larva. Despite the dramatic decrease in foregut motility during the early-molt, the pattern of activity recorded appears similar to that observed in intermolt animals. Scale bars, 25 mV and 2 s.

Table 2. Activity recorded from the posterior esophageal constrictor (pEC) motorneurons of the frontal ganglia of intermolt and early-molt larvae

Parameter	Intermolt (N)	Early-molt (N)
Period (s)	3.2±0.4 (12)	4.3±0.7 (5)
$V_r$ (mV)	-37.3±2.6 (7)	-35.8±2.8 (4)
Burst amp. (mV)	20.6±1.1 (11)	16.1±3.0 (5)
Spike amp. (mV)	7.6±0.8 (9)	9.6±1.2 (5)
Burst duty (%)	77.6±3.5 (12)	76.4±4.0 (5)
Spikes burst <sup>-1</sup>	53.0±10.4 (9)	53.5±9.4 (5)
Spike freq. (Hz)	20.2±6.1 (4)	18.5±5.3 (3)

Values shown are means ± s.e.m. *N* is given in parentheses.

Burst duty is the percentage of the total cycle dedicated to the burst.  $V_r$  is the membrane potential recorded at rest. The means for the molting and intermolt larvae were not significantly different for any of the variables tested ( $P>0.05$ , unpaired *t*-test).

We observed no differences in the burst duty cycle (percentage of the total cycle dedicated to the burst), the number of spikes per second, the number of spikes per burst or the number of spikes in the burst onset (first half of the burst). In addition, the cycle period was not significantly different between the early-molt and intermolt larvae.

#### *Molting larvae have a significantly lower foregut EJP amplitude*

The results outlined above suggest that the output of the FG is not modulated during the early-molt stage and therefore could not be responsible for the molt-related changes in the contractile properties of the foregut musculature. We next examined whether the decline in foregut motility observed during the early-molt resulted from a decline in the

Table 3. Activity recorded extracellularly from the anterior esophageal constrictor (aEC) foregut muscles of intermolt and early-molt larvae

Parameter	Intermolt (N)	Early-molt (N)
Period (s)	3.2±0.2 (65)	2.9±0.1 (108)
Burst duty (%)	29.1±2.7 (34)	34.1±2.7 (48)
Spikes burst <sup>-1</sup>	19.5±1.4 (30)	19.5±1.8 (39)
Spike freq. (Hz)	37.6±3.8 (30)	30.2±2.9 (38)
Spikes onset (%)	71.1±2.9 (15)	66.4±2.5 (21)

Values shown are means ± s.e.m. *N* is given in parentheses.

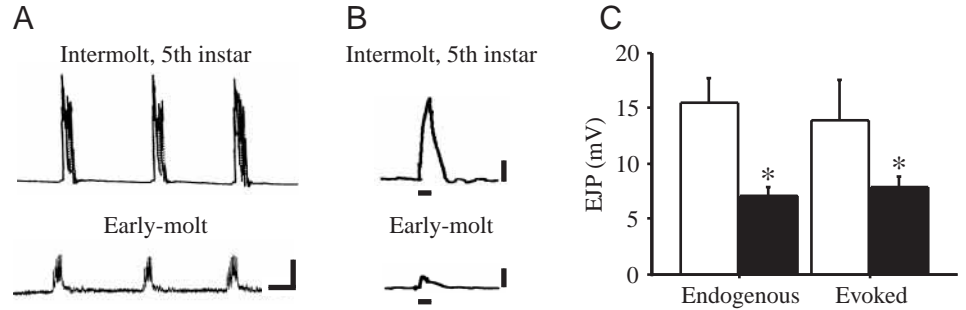
Burst duty is the percentage of the total cycle dedicated to the burst. Spikes onset is the percentage of spikes in the first half of the burst. The means for the molting and intermolt larvae were not significantly different for any of the variables tested ( $P>0.05$ , unpaired *t*-test).

responsiveness of the muscle. The mean resting membrane potential of the aEC muscles was not significantly different between the early-molt and the intermolt larvae ( $-37.0±0.6$  mV and  $-36.3±0.9$  mV, respectively). However, the amplitude of the EJPs recorded intracellularly from early-molt larvae was significantly lower than that of the intermolt larvae (Fig. 6). This is illustrated by the responses of the aEC muscles to either endogenous FG activity or exogenous stimulation of the RN. When the FG was left intact, the mean EJP amplitude recorded from intermolt larvae was almost twice that of the early-molt larvae. Similarly, when EJPs were elicited by exogenous stimulation of the RN, the mean EJP amplitude recorded from the aECs of intermolt larvae was again significantly greater than the mean recorded from the early-molt larvae.

Consistent with the results outlined above, we found that upon entering the molt there was a dramatic decline in the



Fig. 6. Intracellular recordings from anterior esophageal constrictor (aEC) muscles reveal that early-molt larvae exhibit a sharp decline in excitatory junctional potential (EJP) amplitude compared with intermolt larvae. (A) Response of foregut muscle to endogenous frontal ganglion (FG) activity. Scale bars, 5 mV and 1 s. (B) Examples of foregut EJPs elicited by a 10 ms stimulation pulse delivered to the recurrent nerve and recorded from the aEC muscles. Scale bars, 10 ms and 5 mV for the intermolt larva and 2.5 mV for the early-molt larva. (C) The mean EJP amplitudes ( $\pm$  s.e.m.) recorded from intermolt larvae (open bars) and early-molt larvae (filled bars). The asterisk represents significant difference from intermolt values ( $P=0.05$ , unpaired  $t$ -test).



amplitude of the foregut contractions elicited in response to exogenous stimulation of the RN (Fig. 7). All the foreguts of the intermolt larvae responded to RN stimulation with a mean contraction of  $51.1 \pm 17.9 \mu\text{m}$  ( $N=6$ ). By contrast, stimulation of the RN failed to yield a detectable contraction in 31% (13/42) of the foreguts isolated from early-molt stage larvae. Of the early-molt larvae that did respond, the mean amplitude of the contraction was only  $3.4 \pm 0.7 \mu\text{m}$  ( $N=29$ ). Between 16 h and 18 h after HCS, we observed a return of the robust contractions of the foregut. In these late-molt stage larvae, exogenous stimulation of the RN elicited a contraction that was not significantly different from the foreguts of intermolt larvae.

#### *FM1-43 staining reveals a deficit in presynaptic activity of molting larvae*

The results presented above suggested that during the early-molt, the decline in foregut motility was achieved by a reduction in the efficacy of the synapses onto the foregut musculature. To confirm this, we used the styryl dye FM1-43 to label the foregut synaptic terminals of intermolt larvae and the early-molt larvae (Fig. 8). Because FM1-43 is taken up primarily by synapses that undergo vesicle release and recycling, it serves as a marker of synaptic activity (Cochilla et al., 1999).

The FG-intact foregut preparations were exposed to FM1-43 in normal saline, allowing the endogenous bursting of the FG to drive synaptic activity and FM1-43 uptake (see Materials and methods). Following exposure of intermolt larvae to FM1-43, we observed bright fluorescent puncta on the surface of foregut musculature (Fig. 8A). We observed the rapid unloading of the FM1-43 from the synaptic terminals of the intermolt larvae following their exposure to high  $\text{K}^+$  saline (Fig. 8B). By contrast, the terminals on the foreguts of early-molt larvae failed to load following exposure to FM1-43 in normal saline (Fig. 8C). However, we observed a pattern of labeling comparable with that observed for intermolt larvae following the exposure of the early-molt foreguts to FM1-43 in high  $\text{K}^+$  saline (Fig. 8D). The return of the robust contractions of the foreguts late in the larval molt (>18 h after HCS) was correlated with a dramatic increase in FM1-43 loading (Fig. 8E). Like the intermolt larvae, exposure to high

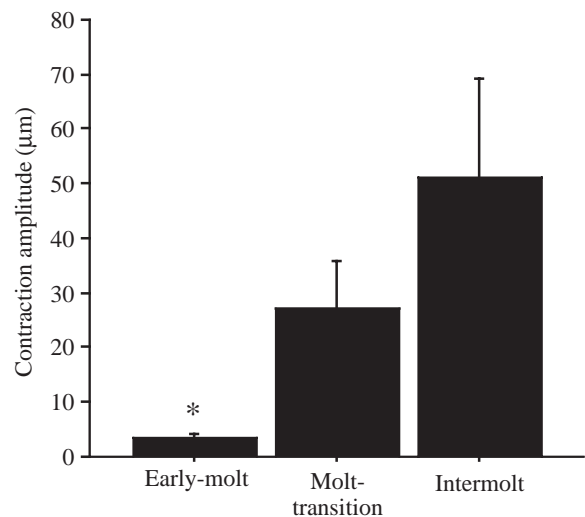


Fig. 7. Foregut contractions elicited by stimulation delivered to the recurrent nerve (RN) are significantly lower in early-molt larvae than in both late-molt and intermolt stage larvae. RNs were stimulated with 5 ms pulses delivered at  $40 \text{ pulses s}^{-1}$  for 500 ms duration trains. The asterisk represents significant difference from both the molt-transition and intermolt larvae ( $P<0.05$ ). Values are means  $\pm$  s.e.m.

$\text{K}^+$  saline was sufficient to unload the FM1-43 dye (Fig. 8F). At all the stages examined, the observed pattern and density of FM1-43 staining on the foregut was similar to that observed following synaptotagmin immunocytochemistry (Davis et al., 2001; Littleton et al., 1993; Fig. 8G,H). Synaptotagmin protein is highly localized to synaptic boutons, and anti-synaptotagmin immunocytochemistry produces a characteristic punctate pattern of staining similar to the FM1-43-labeled foreguts. This similarity suggests that the FM1-43 labeling specifically labeled the synaptic terminals on the foregut musculature.

To quantify the level of FM1-43 labeling, we calculated the density of FM1-43-loaded puncta by imaging a neuromuscular junction and counting all the fluorescent puncta that fell within a  $20 \mu\text{m}^2$  area (Fig. 9A). In late-molt and intermolt larvae, we counted 7–9 fluorescent puncta per  $20 \mu\text{m}^2$ . By comparison, the density of labeled puncta on the foregut of early-molt larvae

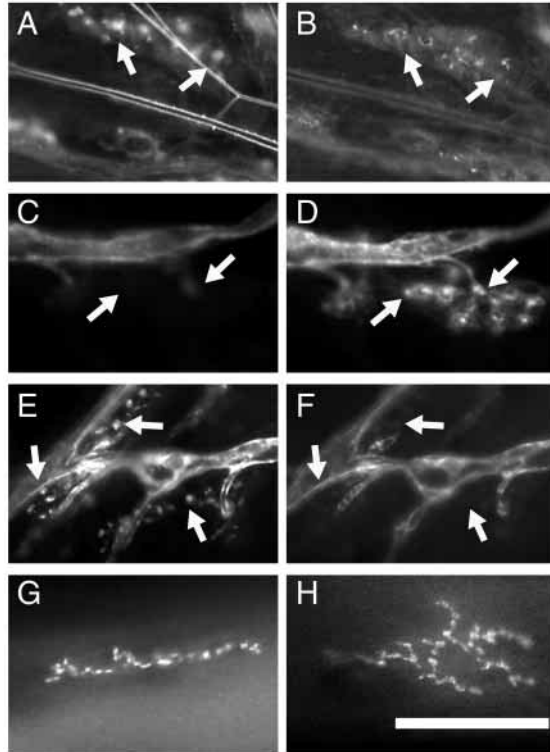


Fig. 8. Molt-related changes in FM1-43 loading of nerve terminals located on foregut esophageal dilator muscles of 4th instar intermolt (A,B), early-molt (C,D) and late-molt (E,F) stage larvae. (A) Endogenous activity was sufficient to load FM1-43 into the foregut synaptic terminals of the 4th instar intermolt larvae. (B) The FM1-43 unloaded when the labeled foreguts were incubated in a high  $K^+$  solution. (C) Endogenous activity failed to load the terminals of the early-molt stage larvae. (D) Exposing early-molt larvae to high  $K^+$  with FM1-43 resulted in the loading of FM1-43 into the synaptic terminals on the foregut. (E) Endogenous frontal ganglion (FG) activity was sufficient to cause FM1-43 dye labeling of neuronal terminals of late-molt larvae (>21 h after head capsule slippage) that had begun to swallow MF. (F) The terminals of the late-molt stage larvae unload the dye when the tissue is incubated in high  $K^+$  saline. (G,H) Two examples of synaptotagmin immunoreactivity, a marker of neuronal presynaptic terminals, present on the esophageal dilator muscles. Arrows indicate equivalent positions. Scale bar, 50  $\mu\text{m}$ .

was <25% of the values observed for intermolt and late-molt stage larvae. When high  $K^+$  saline was used to stimulate synaptic activity, the level of FM1-43 loading on the foreguts of the early-molt larvae was similar to that observed in both intermolt and late-molt stage larvae.

While there was a decline in the density of FM1-43-labeled terminals, there was no significant difference in the relative luminosity of the labeled puncta found in early-molt stage larvae compared with intermolt and late-molt stage larvae (Fig. 9B). This suggests that, while fewer terminals on the foreguts of early-molt larvae loaded with FM1-43, those that did load did so at a level comparable with that observed for larvae with active foreguts. Each of the three larval stages did show significant increases in the amount of FM1-43 uptake over their

endogenously loaded levels when loaded with high  $K^+$  saline. Furthermore, when the FM1-43-loaded preparations were stimulated with high  $K^+$  saline alone, all stages exhibited significant drops in the level of FM1-43 luminosity, indicating that the dye was released from the synaptic terminals.

The results generated using FM1-43 suggest that with the onset of the molt there is a sharp decline in the release and/or recycling of vesicles from the synaptic terminals found on the foregut musculature. However, we cannot eliminate the possibility that modulation of the properties of the musculature also contributes to the decline in foregut motility during the early stages of the molt. At this point, we have established that the contractile properties of the foregut muscle do not appear to be altered following the onset of the molt. The amplitude of the foregut contractions elicited by the application of either 10  $\text{mmol l}^{-1}$  caffeine (to release intracellular calcium) or depolarization with high  $K^+$  saline was similar for foreguts isolated from intermolt and early-molt stage larvae (data not shown). These results suggest that the muscle contractile apparatus is not disrupted during the molt. Alternatively, the molt-related decrease in foregut motility could result from modulation of the receptors on the foregut muscle. We attempted to address this question with iontophoresis of glutamate and carbachol (an acetylcholine agonist) on to the foregut constrictors. Unfortunately, neither of these treatments elicited a response (data not shown). However, it is important to note that, at this point, the foregut neuromuscular junctions are uncharacterized in both their anatomy and neurotransmitter. As a result, it is not possible at this time to determine whether there was a postsynaptic contribution to the sharp decline in foregut motility observed during the early-molt.

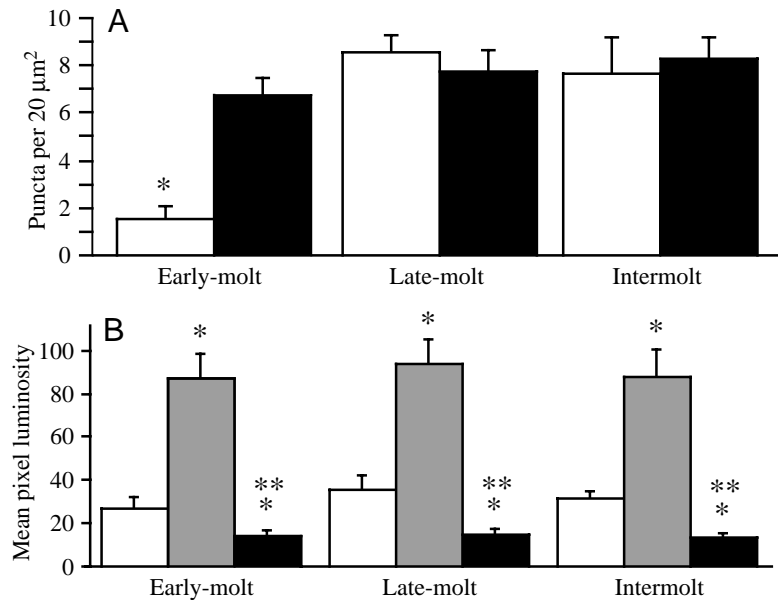
## Discussion

### *Foregut control of molting fluid resorption*

The successful completion of a molt requires the coordination of a number of molt-related motor patterns. The initiation of the molt is marked by the cessation of feeding and a search for a safe place to undergo the molt (Reynolds, 1980). While the pre-ecdysis and ecdysis motor patterns, which together first loosen then remove the old cuticle, are among the last molt-related motor patterns displayed (Copenhaver and Truman, 1982; Miles and Weeks, 1991; Žitňan and Adams, 2000), the data presented above reveal that foregut activity is modulated during a larval-larval molt to control the movement of the MF.

A characteristic of an intermolt larva is the constant, robust peristalsis of its foregut, regardless of when or what it has eaten (Miles and Booker, 1994). However, we found that foregut contractile activity is modulated as larvae pass through the molt cycle. Of the early-molt larvae (0–16 h after HCS) we examined, 71% had quiescent foreguts. In those early-molt larvae that did display foregut peristalsis, the mean contraction amplitude was only 5% of the mean observed in intermolt larvae. The lack of external markers of larvae in the hours prior

Fig. 9. Molt-related differences in the loading of FM1-43. (A) Endogenous activity (open bars) produces fewer FM1-43-labeled synaptic terminals on the foreguts of early-molt larvae compared with late-molt and intermolt larval stages. Equal densities of labeled synapses were found when high  $K^+$  saline (filled bars) was used to stimulate FM1-43 loading. The mean number of fluorescent puncta per  $20\mu m^2$  area ( $\pm$  S.E.M.) is given. The asterisk represents significant difference from the high  $K^+$  saline-loaded level ( $P < 0.05$ ,  $N = 6-15$ ). (B) FM1-43 was loaded into synapses by endogenous frontal ganglion activity (open bars), by stimulation with high  $K^+$  saline (grey bars) and unloaded with stimulation with high  $K^+$  saline (black bars). For each treatment, there were no significant differences between any of the larval stages in the luminosity of the FM1-43 labeled puncta ( $P > 0.05$ ). For all groups, high  $K^+$  saline resulted in a significant increase in mean fluorescent luminosity ( $\pm$  S.E.M.) compared with endogenous-load conditions. All of the terminals unloaded FM1-43 when incubated in high  $K^+$  saline. \*Significant change from endogenous levels ( $P < 0.05$ ); \*\*significant change from high  $K^+$  saline-load levels ( $P < 0.05$ ).  $N = 7-20$ .



to HCS made it difficult to determine the exact timing of the loss of foregut motility. Using bite-response as a marker, we estimated that foregut peristalsis was suspended about 4 h prior to HCS and the first appearance of MF in the exuvial space. During the intermolt larval stages, the ongoing peristalsis of the foregut powers ingestion (Miles and Booker, 1994). As 4th instar larvae initiate apolysis and begin the molt, the old head capsule slips over the mouthparts of the pharate 5th instar larva, placing the 5th instar mouthparts in direct contact with the MF. Thus, we would predict that the failure to suspend foregut peristalsis following the initiation of the molt would result in the premature ingestion of the MF.

During the molt between the 4th and 5th larval stages, the first sign of MF resorption is the appearance of air bubbles in the head capsule approximately 21 h after HCS. The onset of MF resorption was correlated with a significant increase in foregut motility. Between 16 h and 18 h after HCS, a few hours before the first appearance of air in the exuvial space, we observed a 10-fold increase in the mean amplitude of the foregut contractions. These data suggested that the reinitiation of foregut peristalsis occurred at the proper time to control the resorption of MF prior to the onset of ecdysis. This possibility was tested using two different approaches. During the larval stage, the FG is solely responsible for generating the movements of the foregut (Miles and Booker, 1994). The removal of the FG results in the complete loss of foregut activity. While lesioned larvae entered the molt, they failed to resorb their MF prior to ecdysis. We also observed that 90% of the lesioned larvae failed to shed their old cuticle successfully and died within 48 h of their attempted ecdysis. These results reveal that the proper timing of MF resorption is critical for the successful completion of a larval-larval molt. Blocking MF resorption increased the amount of time the old cuticle was exposed to MF. This may have produced excessive thinning and weakening of the old cuticle, resulting in the

tearing we observed as the larva attempted ecdysis. These results also argue against the transepidermal resorption of MF. If larvae had resorbed MF through their epidermis, as suggested by Wigglesworth (1933, 1972), Lensky et al. (1970) and Passonneau and Williams (1953), then silencing foregut activity would, at best, only partially block MF resorption. We found that all of the lesioned larvae initiated ecdysis with an excess of MF in the exuvial space. We failed to detect any indications of MF resorption in lesioned larvae prior to the onset of ecdysis.

We also used the dyes FITC-inulin and carmine red to track the movements of the MF during the molt. During the early-molt stage there was little or no evidence of the movement of the dyes from the exuvial space. The dramatic increase in foregut motility observed 16–18 h after HCS was correlated with the movement of the labeled MF from the exuvial space into the anterior gut (Fig. 2). Inhibiting foregut motility by either removing the FG or filling the gut lumen with adhesive prevented movement of the labeled MF into the gut. In late-molt stage larvae whose foreguts had been silenced, we failed to detect signs of the accumulation of FITC-inulin or carmine red particles around pores or channels in the epidermis of the molting larvae, as described by Lensky et al. (1970) for the pupal-adult molt of *Cecropia*. Indeed, there has been some discussion as to whether the osmotic drive thought to power the movement of the MF across the epidermis would be sufficient to remove the MF within the small window of time at the end of the molt cycle (Reynolds and Samuels, 1996). However, while we believe that the gut serves as the primary conduit for the bulk removal of MF, we cannot rule out the possibility that small molecules and/or water from the MF may enter the hemocoel *via* transepidermal resorption.

It appears that gut is the major route used for the removal of MF during all three life stages of *Manduca*. Prior to adult eclosion, MF is also resorbed *via* the foregut (Miles and



Booker, 1998). The silencing of the foregut by the surgical removal of the FG blocks the resorption of the MF prior to the onset of the adult ecdysis motor program. During the larval–pupal molt, it appears that the hindgut is the primary route used for the resorption of MF (Cornell and Pan, 1983). This conclusion is based on tracking the movement of dyed MF. An examination of the foregut of pharate pupae provides an explanation for why *Manduca* switches to the hindgut to resorb its MF. In *Manduca*, the onset of metamorphosis is accompanied by the loss of motility and histolysis of the foregut musculature (C. I. Miles and J. E. Bestman, unpublished observations). At this point, we have no information on the mechanisms involved in the modulation of hindgut motility during the larval–pupal molt.

#### *Mechanism of foregut modulation during the molt*

In many ways, the disruption of foregut motility during a larval–larval molt in *Manduca* appears similar to what is observed following the emergence of larvae in the parasitic wasp *Cotesia congregata* (Miles and Booker, 2000). *Cotesia* inject their eggs into the hemocoel of *Manduca* larvae, where the parasites grow and develop with little effect on the behavior or development of their host. However, once the *Cotesia* larvae emerge prior to pupating, there is a dramatic reduction in *Manduca*'s foregut motility, resulting in a decline in its ability to ingest food (Adamo, 1998; Miles and Booker, 2000). While both the emergence of the parasite and the onset of a larval–larval molt trigger a decrease in foregut motility, the mechanisms responsible for the modulation appear to differ. Current evidence suggests that the emergence of *Cotesia* triggers a surge in hemolymph octopamine levels, which disrupts the firing patterns of the FG neurons (Miles and Booker, 2000). However, the changes in contraction force occurring during the larval–larval molt were not due to simple disruption of the rhythmic output of the FG. We also failed to detect changes in motorneuron firing frequency or spike number typically associated with the modulation of muscle contraction force (Morris and Hooper, 1997; Enoka and Fuglevand, 2001). Also, our recordings of the kinetics of the muscle EJP activity revealed no differences between early-molt and intermolt stage larvae (Tables 1–3).

It appears that the molt-related decline in foregut motility was, in part, a result of a decline in the efficacy of the synaptic terminals located on the foregut musculature. With the initiation of the molt, there was a 50% reduction in the amplitude of the EJPs recorded from the foregut muscles (Fig. 6). In an attempt to discriminate between a pre- or postsynaptic mechanism for the decrease in foregut activity, we employed the activity-dependent dye FM1-43. This dye is selectively taken up into synaptic vesicles during the process of vesicle fusion and recycling. FM1-43 has also been used to estimate the relative level of synaptic release at a particular terminal (Betz and Bewick, 1992). When endogenous activity was used to load the FM1-43 dye, we observed an 80% reduction in the density of labeled terminals on the foreguts of early-molt as compared with the late-molt and intermolt stage

larvae (Figs 8, 9). However, when labeled terminals were detected, the mean luminosity of the individual fluorescent puncta on the foreguts of the early-molt and intermolt stage larvae was similar. These data indicated that, upon entering the molt, the activity of the majority of the synapses on the foregut musculature was downregulated. The drop in FM1-43 loading could result from a decline in the probability of vesicle release/recycling or a reduction in vesicle pool size.

The inability of the terminals on the foregut of early-molt larvae to load with FM1-43 may be explained by a number of mechanisms. It is possible that the changes in synaptic activity that occur during the molt are the result of a disruption of the machinery required for vesicle release or in a depletion of the vesicle pools. It is also possible that calcium entry into the synaptic terminal is modulated. In an effort to distinguish between these possibilities, we incubated the foreguts in high  $K^+$  saline. We found that, following exposure to high  $K^+$  saline, the density of labeled terminals on the foreguts of early-molt and intermolt larvae were similar. Since high  $K^+$  saline is thought to trigger the exocytosis of all the available vesicles, this result suggests that the terminals on the foregut are modulated in a manner that does not involve changes in vesicle pool size. This indicates that the mechanism favored would be one where the vesicle pool is available but the efficiency of the exocytosis is reduced.

In the production of a contraction, an often overlooked factor is the transformation of neural activity by the muscles into movement (Hooper and Weaver, 2000). Following the onset of a larval–larval molt, there is a 50% decline in the mean amplitude of the EJP recorded from the foregut muscle fibers, which results in a >90% decrement in the amplitude of the foregut contractions (Figs 3, 6). This outcome illustrates the non-linearity that is often observed between the amplitude of the neural input and the resulting muscle contraction in invertebrate muscle (Hoyle, 1983). This non-linearity has the potential to increase the dynamic range of the response of the motor system to modulators. Furthermore, the molt-related modulation of *Manduca*'s foregut motor system is yet another example of how the pattern of motorneuron activity is not necessarily a good predictor of the response of the motor system (Hooper and Weaver, 2000). During the larval molt, it is not the output of the FG that is the primary determinant of the pattern of musculature contraction but the state of the modulation of the synaptic terminals.

The modulation of foregut activity during the molt is a common theme among arthropods (Sehnal, 1985). A number of different species swallow fluids or air before or during ecdysis in order to split the old cuticle (Grieve, 1937; Passano, 1960; Dall and Smith, 1978; Reynolds, 1980; Richardson and Baker, 1996; Chung et al., 1999; Phlippen et al., 2000). Soon after emergence, locusts, blowflies and crickets swallow air to aid in the expansion of their appendages (Carlson and O'Gara, 1983; Cottrell, 1962; Hughes, 1980). During much of adult development, *Manduca*'s foregut is silent (Miles and Booker, 1998). However, prior to the emergence of the adult, the foregut is activated to swallow MF. Once the resorption of MF is complete, the foregut falls silent



until after the adult moth has completed ecdysis. At that time, the foregut is activated a second time to fill the crop with air to aid in the process of wing inflation. Silencing the foregut by lesioning the FG prior to the onset of adult development blocks both the resorption of MF and wing inflation.

Like pre-ecdysis and ecdysis, the modulation of the foregut motor system can be added to the series of behaviors that must be properly coordinated to assure the successful completion of a molt. The accessibility of *Manduca*'s endocrine and nervous system has allowed us to accumulate a wealth of information on the role that hormones play in coordinating the timing of pre-ecdysis and ecdysis behaviors that occur at the end of the molt (Reynolds, 1980; Ewer et al., 1997; Gammie and Truman, 1997; Weeks et al., 1997; Žitňan et al., 1999; Consoulas et al., 2000). The successful completion of a molt requires that the activation of foregut motor activity be coordinated relative to the other molt-related events. Given this requirement, it seems reasonable that the modulation of foregut activity during the molt would also be under endocrine control. This view is supported in several crustacean species. Increases in blood levels of neuropeptides have been correlated with the onset of the foregut motor program to swallow water, which aids in rupturing the carapace prior to ecdysis (Chung et al., 1999; Phlippen et al., 2000). Similarly, MF swallowing by the eclosing adults of *Manduca* appears to be triggered by eclosion hormone (Miles and Booker, 1998). It appears that during the larval-larval molts, neurohormones and/or neuromodulators play a role in modulating foregut motility. Our preliminary results suggest that an uncharacterized blood-borne protein triggers the decline in foregut motility observed during the early-molt stage (Bestman et al., 1997). We have also found that application of the nonapeptide crustacean cardioactive peptide triggers the precocious reinitiation of foregut peristalsis during the larval-larval molt (J. E. Bestman, unpublished results). Thus, the analysis of the mechanisms involved in the modulation of the foregut motor pattern during a larval-larval molt could provide a model for how different motor systems are coordinated.

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