

## STRUCTURE AND INNERVATION OF THE THIRD AXILLARY MUSCLE OF *MANDUCA* RELATIVE TO ITS ROLE IN TURNING FLIGHT

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### SUMMARY

The morphology, ultrastructure, innervation and physiology of the third axillary muscle in *Manduca sexta* were examined to investigate the role of this muscle in flight. The muscle consists of three parts: the upper bundle, which originates on the episternum, and the middle and lower bundles, which originate on the epimeron; all three parts insert on the tip of a projection from the third axillary sclerite. The middle bundle is composed of tonic fibres, and is innervated by a single slow axon, while the other two bundles consist of intermediate fibres and are each innervated by a single fast axon. The shape and position of the third axillary sclerite within the wing hinge are such that its primary function appears to be remotion of the wing. The length of the third axillary muscle determines the amount of remotion, independently of the degree of elevation or depression of the wing and independently of the amount of remotion of the contralateral wing. Electrophysiological recordings from the three parts of the muscle during tethered flight indicate that they may each function independently of each other and in different ways. The tonic (middle) bundle is capable of maintaining tension to hold the wings in the folded position at rest and is active when the wings are folded at the end of flight. The intermediate (upper and lower) bundles are activated phasically with impulses that may occur with various relationships to the timing of activation of a direct depressor, the subalar, or of several of the elevators. The findings are consistent with the hypothesis that the third axillary muscles on both sides are important in determining the asymmetric degrees of remotion observed in turning flight.

### INTRODUCTION

The wings of a flying insect move in a complicated path that may vary from stroke to stroke. The movements relative to the long axis of the body can be approximated at the wing tip by a distorted figure eight. In addition, the wing undergoes various deformations and torsions that differ in the upstroke and downstroke (Wootton, 1981; Zarnack, 1983; Brodskiy & Ivanov, 1983). Alterations in wing movements,

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angle of attack and deformation of the wing, particularly asymmetries of the two sides, result in turning (for a review, see Kammer, 1985). The mechanisms underlying the complicated wing movements produced during straight flight and the ways that the wings are controlled during turning are poorly understood.

Various cuticular specializations, including small pieces of rigid cuticle, bending cuticle and elastic ligaments, form the articulation between the thorax and the wing and constitute an important mechanism for controlling the path of the wing. Typically there are three or four separable axillary sclerites, the humeral and median plates, plus several sclerites that may be closely allied either with the wing or with the scutum. Because of the way that the notal and pleural wing processes act on the axillary sclerites, a 'least-effort' path of the wing may be mechanically determined, with the indirect flight muscles providing the power to move the wing automatically along a complicated path. Smaller muscles that insert directly on the various parts of the wingbase may then act to modify the interaction between the sclerites and the wing and thus alter the wing stroke to produce differences in pronation and supination, or promotion and remotion.

One of the most important of the direct muscles may be the muscle that inserts on the third axillary sclerite (in the mesothorax: M85 of Snodgrass, 1935; t-p 13, 14 of Matsuda, 1970). This muscle is thought to have a function in folding the wing against the abdomen at the end of flight (Snodgrass, 1935; Pringle, 1957, 1968; Lepidoptera, see Sharplin, 1963, 1964; stonefly and alder fly, see Ivanov, 1981). In addition, there is electrophysiological evidence that the third axillary muscle may also function during flight. In moths, recordings from a medial portion of the third axillary muscle indicate that it could function as a phasic wing remotor during turning flight, with the motor patterns showing complicated phase changes with regard to the main depressor muscle, the dorsal longitudinal (Kammer, 1971). In flying locusts, the third axillary muscle is excited rhythmically during straight flight, and imposed rolls result in small phase shifts and changes in number of spikes per wingbeat (Elson & Pflüger, 1986). Anatomical studies of locusts suggest that contractions of the mesothoracic third axillary muscle (M85) reduce pronation and increase the angle of attack (Pfau, 1977; Pfau & Nachtigall, 1981). Thus the increased excitation on the side rolled down would produce an appropriate compensatory increase in lift (Elson & Pflüger, 1986). In Diptera, changes in motor patterns associated with turning have been recorded from direct muscles (Heide, 1968, 1971, 1983), and these results together with morphological data suggest that the third axillary muscles influence stroke amplitude as well as wing retraction (Miyan & Ewing, 1985).

The morphology and innervation of the third axillary muscle is complicated in Lepidoptera. Nuesch (1953, 1957) observed in *Antheraea* that the muscle is composed of three parts, and that it is jointly innervated by two separate main nerve branches, IIN2 and IIN5. Subsequent descriptions of the structure and innervation in *Manduca* (Eaton, 1971, 1974; Rind, 1983a) were conflicting. To investigate the role of this muscle in flight, the present study was made to describe the fine structure and innervation pattern of the muscle, and the structure of the sclerite which it

controls. The results indicate that the third axillary muscle in *Manduca* is largely involved in controlling remotion, rather than supination, during flight.

#### MATERIALS AND METHODS

The experimental animals were adult hawk moths (*Manduca sexta*) raised from eggs provided by the Insect Physiology Laboratory, Department of Agriculture, Beltsville, MD.

Intracellular recordings were made from the third axillary and subalar muscles, using conventional techniques (Rheuben, 1972; Rheuben & Kammer, 1980), after exposing the muscles by removing the flexible exoskeleton that surrounds the subalar sclerite. For extracellular recording of muscle potentials during flight, a ground wire was placed in the abdomen, and fine insulated copper wires were placed through the hard exoskeleton near or at the origins of the appropriate muscles. The electrical signals were treated as previously described (Kammer, 1971), and data were recorded upon both a six-channel Brush chart recorder and a Hewlett-Packard four-channel FM tape recorder.

The major nerve branches were visualized with leuco-methylene blue (Stark, Smalley & Rowe, 1969) during gross dissection. Specimen preparation for thin-section electron microscopy and for freeze-fracture was as described by Rheuben & Reese (1978), and utilized a fixative containing 4% paraformaldehyde, 1% glutaraldehyde and  $0.2 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  in  $0.1 \text{ mol l}^{-1}$  phosphate buffer. Scanning micrographs of the external features of the wingbase were obtained by mounting the animal as for tethered flight, positioning the wings, injecting the fixative, and holding the wings in position until the muscles had hardened. The specimens were then trimmed and the scales removed. After dehydration and critical-point drying, the exoskeleton was coated with gold in a sputter coater and examined in the scanning electron microscope.

Other observations on the wingbase were made on anaesthetized and decapitated specimens mounted on a post as for flight experiments. Small amounts of the flexible cuticle were removed to show the relationships of the sclerites to the muscles beneath, and the wings could be manipulated to show the changes in angle of force that resulted from shifting the position of the point of insertion on the third axillary sclerite relative to the rest of the wingbase.

#### RESULTS

##### *Anatomy of the third axillary muscle*

The third axillary muscle in *Manduca* is composed of three parts and lies anterior and medial to the subalar muscle, originating partly on the episternum and partly on the epimeron; all three parts insert on the tip of the third axillary sclerite (Fig. 1A,C). Nuesch (1953) observed in *Antheraea polyphemus* that the more medial portion that originates on the episternum is divided into two parts, which he termed pd2a and pd2b, and the lateral portion is single, pd2c. Eaton (1974) described the



Fig. 1A,B. For legend see p. 378



medial portion in *Manduca* as two parts combined, a+b, and he viewed the lateral part, c, as one muscle. We find three distinct parts: one large, more dorsal and medial bundle originates on the episternum and *two* lateral bundles originate on the epimeron. It is likely that the medial bundle in *Manduca* corresponds either to Nuesch's pd2a alone, or to pd2a and pd2b combined, and that the lateral bundles would then have to be termed pd2c and pd2d. Examination of two specimens of *Antheraea polyphemus* showed that pd2a and pd2b are indeed quite separable in that

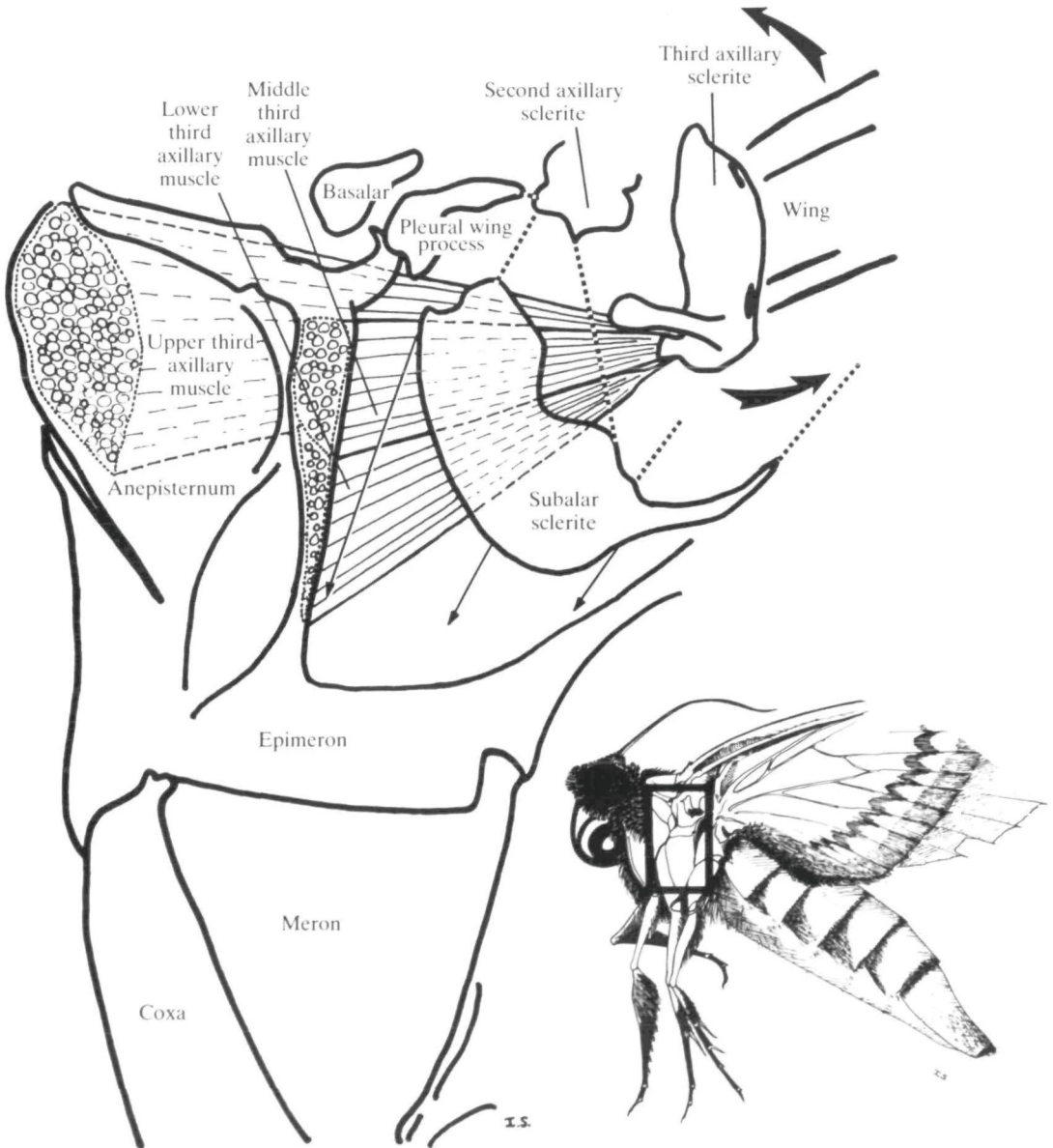


Fig. 1C. For legend see p. 378

species by virtue of clearly delineated regions of origin on the episternum. However, pd2c in that species is also divided into two fascicles originating on the epimeron, making a total of four fascicles and not three. To avoid introducing the implication that the divisions of the third axillary muscle in *Manduca* can be identified homologically with the muscles in *Antheraea*, we have called them the upper, middle and lower portions of the third axillary muscle with reference to the dorsal surface. The upper third axillary originates on the dorsal part of the episternum anterior to the origin of muscle pv6, and the middle and lower portions originate on the epimeron, caudal to the pleural ridge (Fig. 1A,C). The lower muscle is distinctive in inserting on the third axillary sclerite *via* a slender apodeme.

### Ultrastructure

The middle third axillary is a thin, flat muscle, translucent or white in colour. It is typically tonic in structure, with sparse, small mitochondria, 10–12 thin filaments around each thick filament, and with filaments not grouped into well-defined fibrils (Fig. 2). The basal lamina is thick, three times that of subalar or dorsal longitudinal fibres (typical phasic muscles). The membrane of middle third axillary fibres is characterized by clefts and infoldings instead of the paired round openings to T-tubules that are a feature of phasic muscle membranes in insects.

Freeze-fracture views of middle third axillary fibres show chains of small particles in the cytoplasmic leaflet at the borders of the clefts, while the remainder of the border of a cleft is relatively particle-free (Fig. 2B). Thin-section views of the comparable region adjacent to the openings of the clefts reveal an especially electron-dense fuzzy material just under the membrane, forming a structure like a hemidesmosome (Fig. 2A). Similar chains of particles have been seen adjacent to clefts in both tonic and phasic muscles of crayfish (Eastwood, Franzini-Armstrong &

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Fig. 1. Lateral view of the left side of the mesothorax and wingbase. (A) Scanning electron micrograph of the surface cuticular structures seen when the wing is fixed in the fully up and back (i.e. fully remoted) position. In this position, the third axillary muscles would be at their shortest. The tip of the longer projection of the third axillary sclerite and the 'knee' that it forms by articulating with the fourth axillary sclerite are marked with an asterisk. The curved arrow indicates the path that the knee follows during depression of the wing. The numbers refer to the corresponding axillary sclerites, only portions of which can be seen from this angle.  $\times 25.4$ . (B) The change in location and angle of the knee when the wings have been lowered and fixed in a down and back position (wings clipped off to expose the structures shown).  $\times 23.6$ . The angle of photography is approximately the same for the two micrographs. (C) Diagram identifying the superficial cuticular structures shown in A, and illustrating the origins (dotted lines) and the point of insertion of the three parts of the third axillary muscle. The figure is drawn as if the flexible cuticle and the fourth axillary sclerite had been removed. The position and direction of the subalar muscle, which lies superficial to the third axillary muscles, is indicated by the three long arrows. The ligaments supporting the subalar sclerite and connecting the ventral portion of the second axillary sclerite to the pleural wing process are shown as dashed lines. The figure was drawn with the wings fully remoted and up, as in A. The curved arrows indicate the expected movements of the wing and the sclerite if the wing were to be promoted, or pulled anteriorly. The boxed area in the inset illustrates the orientation and location of the region shown in the scanning micrograph and the accompanying diagram.

Peracchia, 1982) and in the retractor unguis muscle of locust (Newman & Duce, 1982) which has a mixture of fibre types according to Usherwood (1967). They are not, however, seen in the surface membranes of the phasic flight muscles in *Manduca*. In freeze-fractures of the middle third axillary muscle, we have also observed the clusters of parallel bands of particles ('scratches') described by Newman & Duce (1982) and Eastwood *et al.* (1982) in the surface membrane between the clefts (Fig. 3). Again, we have not seen these scratches in the other muscle fibre types. Although it is not possible to assign a specific function to these particle arrays, it is reasonable to keep the characteristic differences in mind when comparing the membrane properties of different types of muscle fibres. A similar association between a particle specialization and muscle fibre type has been made in vertebrate muscle. Orthogonal square arrays of large particles are present in large numbers on the sarcolemma of fast-twitch muscle fibres in the rat, and are rarely present on the membrane of slow-twitch fibres (Ellisman, Rash, Staehelin & Porter, 1976). The square arrays are also present in other tissue types, particularly glial cells, and it has been suggested that they may be functional in an ATP-dependent active transport mechanism; their distribution is affected by cold, protein synthesis inhibitors and seizure-inducing substances such as alumina (Anders & Brightman, 1982; Hatton & Ellisman, 1984). It may be that the ultrastructural differences that we observe between the membranes of tonic and phasic muscle fibres can ultimately be correlated with a physiological difference in function.

Tracheation in middle third axillary muscle fibres is relatively sparse, and close association with the muscle fibre membrane occurs *via* branches of tracheoles that lie within the clefts.

The upper and lower third axillary muscles are pink and have the fine structure of the 'intermediate' type of arthropod muscle fibre, with a high ratio of thin to thick filaments (10–12 thin around each thick), a relatively long sarcomere of about 6  $\mu\text{m}$ , but with an extensive sarcoplasmic reticulum, and with mitochondria present in numbers between those typical for tonic and phasic muscles (Fig. 4). [See Rheuben & Kammer (1980) for a description of phasic muscles from *Manduca* in thin section.] The cross-sectional diameters of fibres from the upper and lower third axillary are intermediate between those of the tonic middle third axillary and a phasic muscle, the subalar, as are the basal lamina thicknesses (Table 1). The freeze-fracture image of the sarcolemma is quite similar to that of tonic muscle fibres (Fig. 4B).

### *Innervation*

The innervation of the three bundles of the third axillary muscle in Lepidoptera is complex, at least historically. Nuesch (1957), studying *Antheraea*, and Eaton (1974) and Rind (1983a), studying *Manduca*, reported from methylene blue studies that branches from two main nerve trunks, IIN2a and IIN5c, enter the third axillary muscle [IIN5 is IIN6 in Eaton's (1974) terminology]. Rind (1983a) concluded from intracellular cobalt fills and from twitches elicited by stimulation of individual motor neurones that the more medial and dorsal, 'upper', portion (pd2a plus pd2b) is

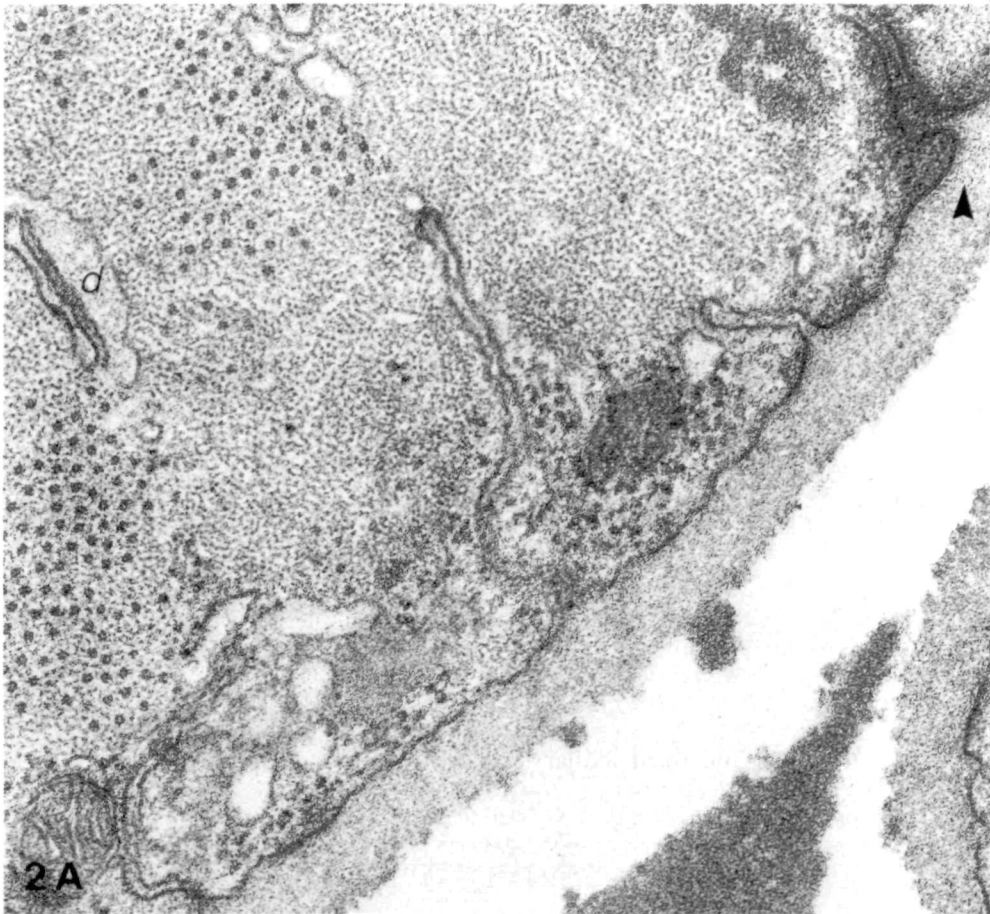






Fig. 3. Freeze-fracture view of the cytoplasmic leaflet of a muscle fibre from middle third axillary muscle, showing the cluster of parallel bands termed scratches. So far the scratches have only been observed in the membranes from the tonic middle third axillary fibres.  $\times 66\,000$ .

innervated by one axon and the lateral part, pd2c ('middle' and 'lower' in our terminology), by another, both *via* nerve IIN5a. She found no physiological evidence for innervation *via* nerve IIN2a, nor did she report that the lateral portion of the muscle was divided into two parts.

Fig. 2. Structure of the tonic muscle fibres of the middle third axillary muscle. (A) The muscle is shown in cross-section, illustrating the narrow clefts, thick basal lamina, scarcity of mitochondria and arrangement of the thick and thin filaments that are characteristic of insect tonic muscle fibres. In some regions the section passes through the I-band (only thin filaments) and in some regions also includes profiles of the thick filaments (A-band). Near the openings to clefts, there is an increase in electron-dense material both within the cytoplasm and in the basal lamina (arrowhead). *d*, dyad;  $\times 80\,500$ . (B) Freeze-fracture view of the surface of a tonic middle third axillary muscle fibre, cytoplasmic leaflet. The borders of the clefts are relatively particle-free, except for short chains of particles. The surface lacks the regularly paired T-tubules that are characteristic of phasic insect muscle fibres.  $\times 58\,900$ .

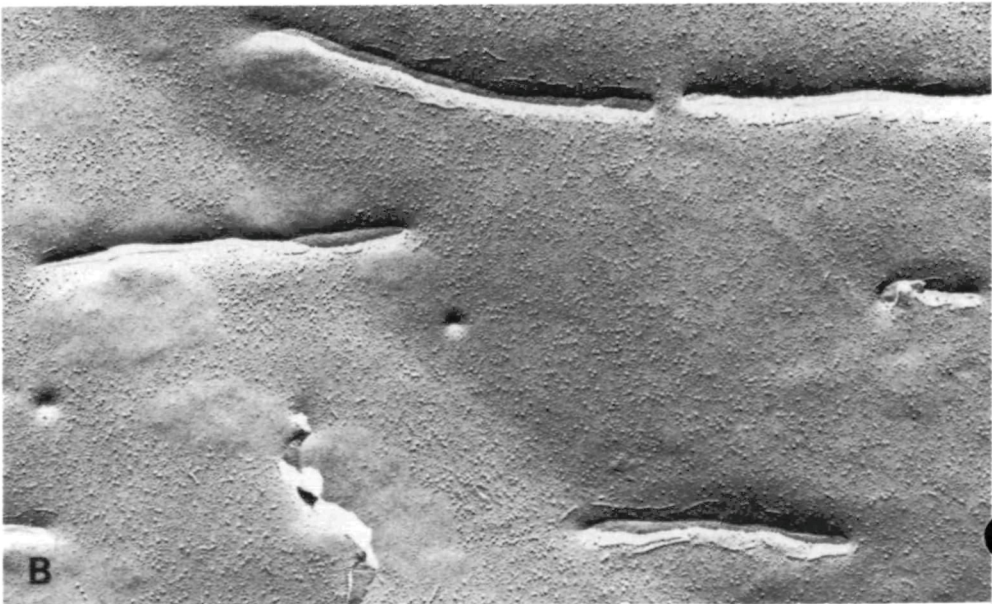
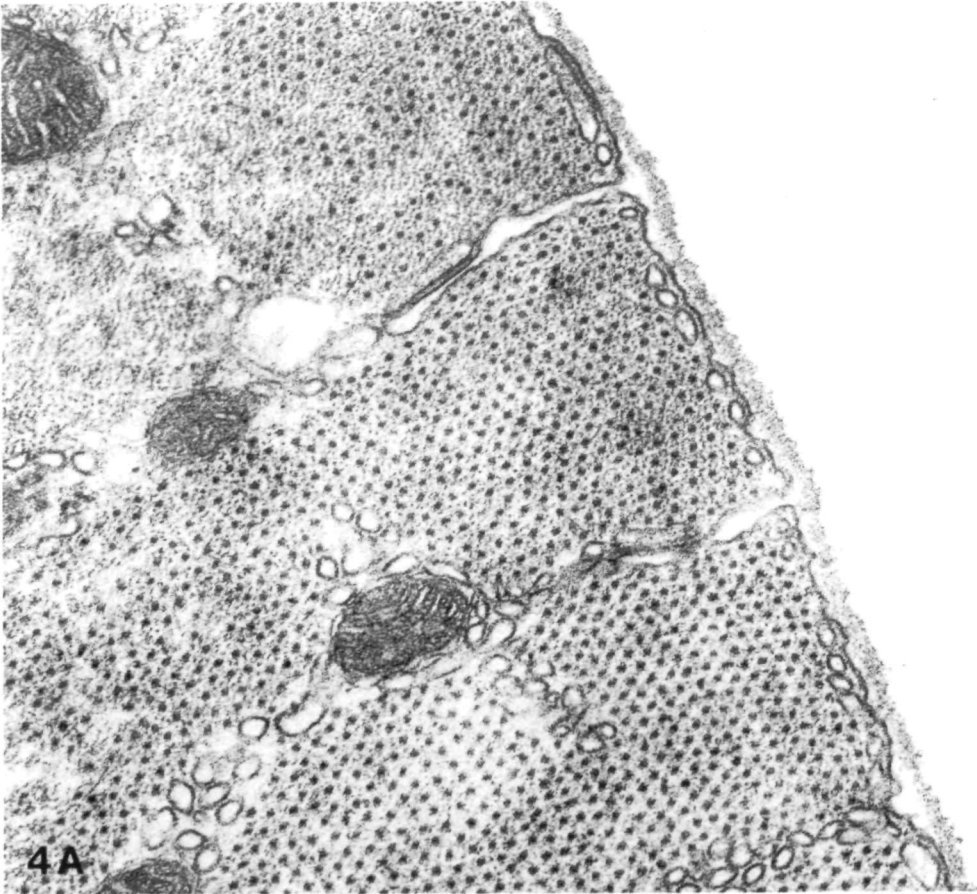




Table 1. *Characteristics of muscle fibres*

Muscle	Fibre widths* ( $\mu\text{m}$ )	Basal lamina thickness ( $\mu\text{m}$ )	Fibre type
Upper third axillary	$25.3 \pm 5.9$ N = 25	$0.063 \pm 0.023$ N = 120	Intermediate
Middle third axillary	$17.5 \pm 4.3$ N = 73	$0.118 \pm 0.031$ N = 125	Tonic
Lower third axillary	$20.9 \pm 4.0$ N = 39	$0.086 \pm 0.029$ N = 62	Intermediate
Subalar	$35.7 \pm 5.5$ N = 11	$0.039 \pm 0.015$ N = 42	Phasic

\*Measurements of polygonal shapes taken across short diameter and not corrected for tissue shrinkage. Average diameter would be proportionately larger for each.

Intracellular recordings from the fibres of the three bundles of the third axillary muscle in *Manduca*, combined with axon counts in the various nerve branches as described below, lead us to conclude that the fibres of the upper and lower third axillary muscles are indeed each innervated by a separate fast axon, but that one of the separable lateral portions, the middle third axillary, is innervated by a slow axon. Spontaneous activity in the central nervous system (CNS) frequently provided slow excitatory junction potentials (EJPs) in fibres of the middle third axillary (see fig. 1 in Rheuben & Kammer, 1983, reprinted as fig. 1 in Rheuben, 1985), but not in fibres of the other bundles or in the adjacent subalar muscle. Small stimulating electrodes, inserted in each of the muscle bundles separately or placed to stimulate all three simultaneously with a higher voltage, elicited only slow EJPs in the middle bundle, and fast EJPs in the upper and lower bundles (Fig. 5), regardless of stimulus strength. Methylene blue stains, summarized diagrammatically in Fig. 6, clearly showed the two nerve branches, IIN2a and IIN5c, as previously described by Nuesch, Eaton and Rind as having branches which enter the third axillary muscle. We find two regions where branches could allow possible anastomosis and exchange of axons between nerves IIN2a and IIN5c (Fig. 6). The most proximal connection appears as a network of finely branched, beaded axons. This network could be neurosecretory judging from its appearance or, if the innervation resembles that found in *Antheraea*, it may be the remainder of the dual innervation of another muscle, pd3, which appears to be degenerating in the adult *Manduca*. (The presence

Fig. 4. Characteristics of the intermediate type of muscle fibre. (A) A slightly oblique cross-section of a portion of an upper third axillary fibre. The plane of section includes regions of both the A- and I-bands. These fibres have relatively more mitochondria than the tonic fibres, a substantially increased amount of sarcoplasmic reticulum, and a thinner basal lamina. The relatively high ratio of thin to thick filaments, which is partially indicated here in the upper part of the figure, is similar to that of the tonic fibres.  $\times 61\,000$ . (B) Freeze-fracture image of the cytoplasmic leaflet from a lower third axillary fibre. No substantial differences were noticed between the membranes of the upper and lower third axillary fibres.  $\times 36\,000$ .

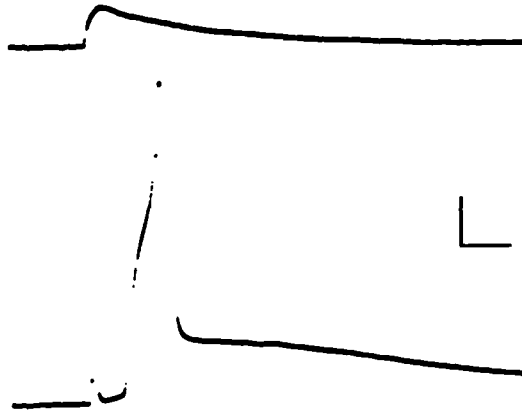


Fig. 5. Intracellular recordings from middle third axillary (top trace) and lower third axillary (bottom trace) muscle fibres showing the characteristic amplitude and time course of the two types of excitatory postsynaptic potentials. The time course of the lower third axillary synaptic potential is obscured at the end of the falling phase of the active membrane response by movement artifact from the twitch. Calibration bars for the upper trace are 5 mV and 20 ms, and for the bottom are 10 mV and 5 ms. The records were obtained separately.

and appearance of both the network and this latter muscle differed from specimen to specimen, making interpretation uncertain. Muscle pd3 in *Antheraea* is a much more substantial structure than in *Manduca*.) The second connection is a branch from IIN2a which crosses over and is attached briefly to IIN5c and proceeds towards the middle third axillary (Fig. 6). Serial thick sections showed that IIN2a prior to the crossover point contains one large axon. Thin sections of IIN2a, subsequent to the crossover point, where it enters the middle third axillary also show only one axon (Fig. 7). Sections of IIN5c prior to the region of proximity to IIN2a contain two large axons; this nerve subsequently divides into two branches with one axon each. Both main nerve branches in the region of the third axillary muscles also contain an auxiliary population of very small axons which lie *outside* the glial sheath that surrounds the motor axons. These small axons are considered to be neurosecretory in function, and were observed to have active zones and clusters of clear- and dense-cored vesicles close to where the nerve enters the muscle (Rheuben & Kammer, 1983).

Although it is not possible to rule out additional contributions by very small axons running with the neurosecretory axons in the neural lamella of the motor nerve, the available evidence is strong that each of the bundles is innervated by a single motor axon. The tonic middle third axillary is innervated by a slow axon from nerve IIN2a, and the intermediate type muscles (upper and lower) are each innervated by a single fast axon arriving from nerve IIN5c, presumably corresponding to the ones identified by Rind (1983a). The slow innervation would not have been readily detected by Rind (1983a), who looked only for twitches in response to stimulation of the individual motor neurones.

*Structure of the third axillary sclerite relative to its role in positioning the wing*

The third axillary sclerite in *Manduca* consists of an irregularly shaped body (Figs 1C, 8, 10), which provides an articulation for the two branches of the anal wing vein at the posterior edge of the wing, and two slender projections. The shorter of these projections emerges at the posterior part of the sclerite body, at a very slight angle to it, and forms at its round tip the point of insertion of all three third axillary muscles (Figs 1C, 8B, 10A). The longer projection arises from the body of the sclerite in the same region, but in a plane nearly perpendicular to that formed by the body and the first projection (Fig. 1C). This slender projection articulates with the rod-like fourth axillary sclerite to form a flexible 'knee'. The fourth axillary sclerite is

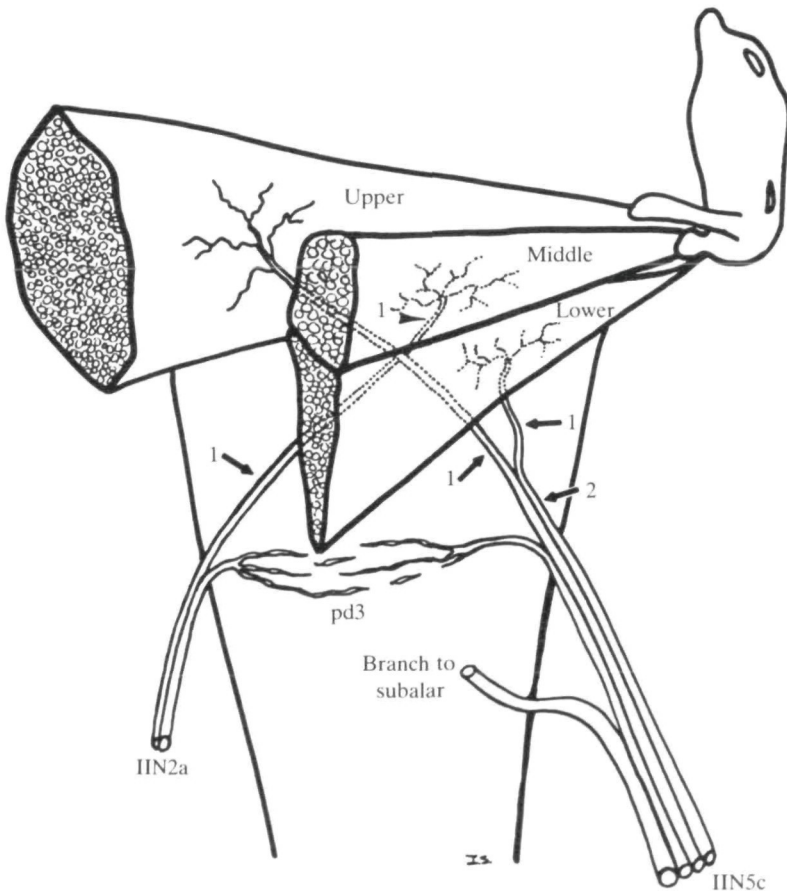


Fig. 6. Diagram of the innervation of the third axillary muscle. The view is of the left lateral side of the animal; anterior is to the left, and the subalar muscle has been removed. The numbers by the arrows refer to the number of large axons surrounded by a solid glial wrapping observed in a cross-section of the nerve at that point (excluding the very small neurosecretory axons that accompany the peripheral nerves, and which are not usually well wrapped by glia). The arrowhead indicates the point at which the section shown in Fig. 7 was taken. The label pd3 indicates the region where vestiges of muscle pd3 are observed relative to the nerve branches.



Fig. 7. Cross-section of the nerve innervating the middle third axillary muscle. There is one large motor axon (asterisk) within the main glial sheath. The location of a very small, presumably neurosecretory axon (identified at higher magnification) is indicated by the arrow.  $\times 2160$ .

braced against (or is considered by some insect morphologists to be continuous with) the posterior notal wing process. The knee fits into a depression in the subalar sclerite when the wing is fully depressed (Fig. 1B). The process from the third axillary sclerite and the fourth axillary sclerite are separated by loose flexible cuticle, and the angle between them is very changeable. The angle is increased when the wing is promoted (pulled craniad), in either the up or down position (Fig. 9). The plane in which this angle lies is determined by wing position. When the wing is elevated, the knee swings anterolateral and upwards; when the wing is depressed, the knee swings posteromedial and downwards and fits into a depression in the subalar sclerite. When the wing is folded to the rest position horizontally over the abdomen, there is a corresponding  $90^\circ$  change in the angle of the body of the sclerite (Fig. 10).

The articulation between the wing and the third axillary sclerite is very flexible; the position of the sclerite can influence to some extent the geometric angle of attack of the wing as a whole, but the posterior surface of the wing is free to be affected by the pressure of the airflow over the wing or by interaction with the ipsilateral hindwing. The third axillary sclerite articulates with two branches of the anal vein. The region of the wing supported by the anal vein is separated from the rest of the wing by the anal furrow (nomenclature after Madden, 1944; called the claval furrow by Wootton, 1981), which runs the length of the wing and forms a line of flexion. In *Manduca* males the forewing and the hindwing are connected by a frenulum which projects from the hindwing and is engaged by a frenulum hook on the under surface of the forewing. The region in which the two wings are caused to overlap can include all or part of the flexible portion of the forewing posterior to the anal furrow, and is therefore also the region directly influenced by the third axillary sclerite.

When the wing is folded in the rest position or when it is extended and horizontal the origin of the upper third axillary muscle is such that the fibres are lying almost parallel with the notum, with the anterior end slightly lower. The origins of the middle and lower portions cause their angles of pull to be even more ventral and lateral. The portion of the third axillary sclerite on which the three muscle units insert lies posterior and medial to the fulcrum about which the wing rotates. The primary effect of the third axillary muscle appears to be remotion, or flexing of the wing caudally, and this effect can occur with the wing in any elevated or depressed position. If the wing remains in one plane, simple remotion or promotion occurs as a pivoting of the wing and the more distal sclerites around a projection formed by the

first axillary sclerite and part of the second (see Fig. 8B). During elevation and depression, the wing hinges along a line considerably medial to this point, in the region of the medial notal wing process. During a wingbeat cycle, the point of the third axillary sclerite onto which the muscle inserts describes an arc. As the wing is remoted, the plane of the arc is shifted medially, closer to the fulcrum; consequently, the arc is smaller and requires a shorter length of muscle to perform it. This particularly ingenious mechanical relationship allows either a constant degree of remotion at any part of the wing stroke with a constant muscle length or a variable path by altering tension and muscle length at the top and bottom of the stroke. Because the actions of the right and left third axillary muscles are mechanically independent, remotion and stroke path can be different on the two sides. The muscles are well positioned to control direction of flight.

Contraction of one or more of the components of the third axillary muscle might have effects on wing position other than remotion. These effects are likely to be dependent both upon the up or down position of the wing at that moment and upon the extent to which simple remotion is opposed by the basalar or other muscles. For example, if the wing is depressed but starting the upstroke, contraction of the third axillary muscle might be able to cause supination of the wing by retarding the ascent of the posterior part of the wing. Alternatively, the plane of the process on which the muscle inserts is such that its contraction could aid in elevation as well, once the wing is above the horizontal. This interpretation is supported by observations on the motion of the sclerite and the action of the exposed muscles in moths that are spontaneously holding their wings elevated and somewhat remoted during small-amplitude flight or shivering. It can be seen that the point of insertion of the third axillary muscle and the process forming the knee are oscillating in a plane approximately  $45^\circ$  from the vertical, and the lower third axillary muscle is in a particularly good position to act with the elevators in pulling the wing up and back, although all three parts could probably have that effect. The extent to which the third axillary functions as either an elevator or a supinator is probably subsidiary to its role as a remotor, and is strongly influenced by the amount that simple remotion is countered by the action of the antagonistic basalar, and by the actions of other muscles controlling wing position.

In addition to muscular control, several mechanical interactions among components of the wing hinge may be important in obtaining promotion and remotion or supination and pronation. One such interaction involves the third and fourth axillary sclerites and the subalar sclerite. The relationship of the knee to the depression in the subalar sclerite is designed with very close tolerance. At the bottom of the downstroke, if there is no opposing muscle contraction, the tip of the third axillary sclerite at the knee, which is braced by the fourth axillary sclerite and its articulation with the posterior notal wing process, can come into contact with the subalar sclerite. This contact with the subalar sclerite can limit the downward excursion of the posterior part of the wing, but not the anterior part. In specimens in which the wings are depressed manually, this mechanical arrangement causes pronation of the wing. Two tethered flight experiments, in which paint was placed either on the knee or within the hollow in the subalar sclerite, indicated that under most conditions the





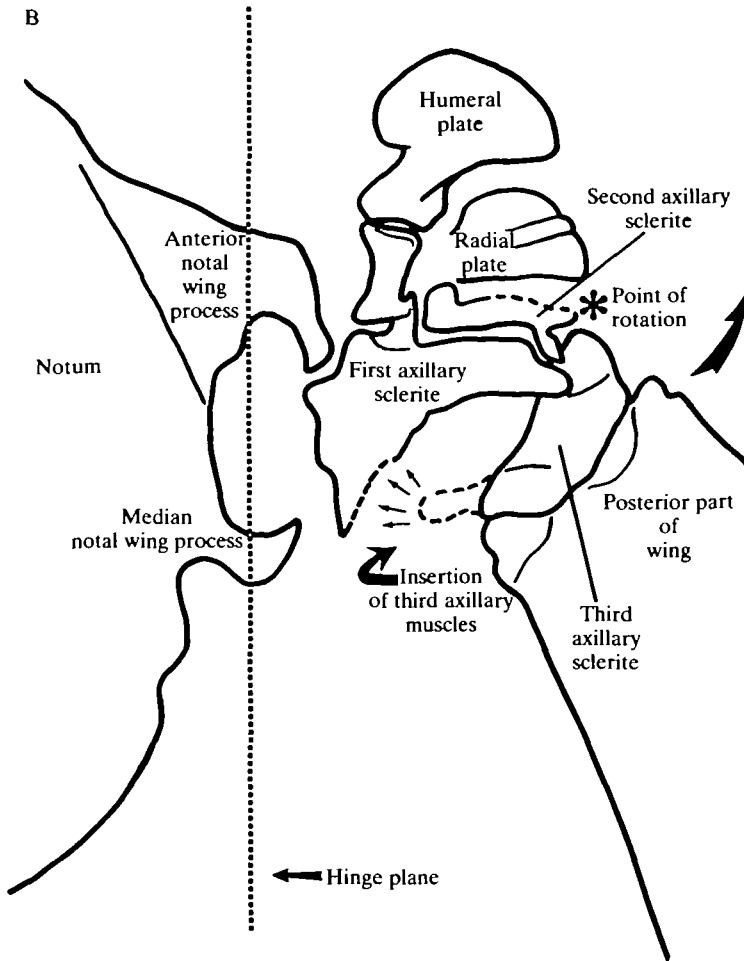
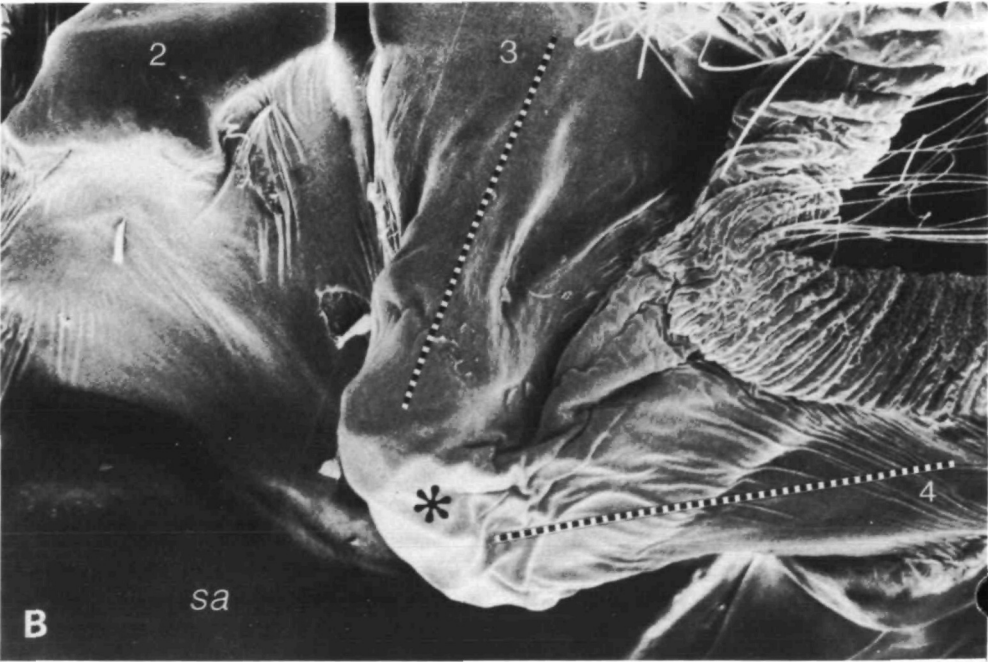
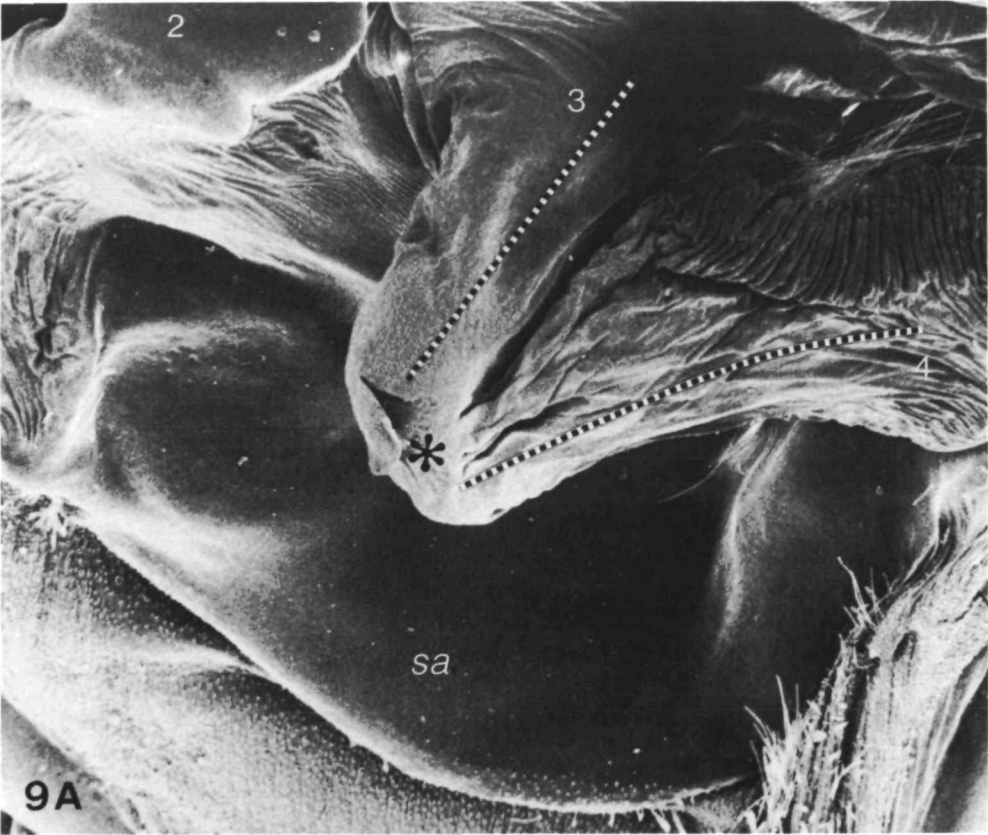


Fig. 8. Dorsal view of the right wingbase, with the wings down and back. (A) Scanning electron micrograph showing the relationship of the axillary sclerites to the notum and to the posterior part of the wing. The anterior part of the wing, sloping sharply forward, is not shown well from this angle, which shows the wing base from above and slightly to the rear.  $\times 28.5$ . (B) Outline drawing of the same wingbase, distinguishing the borders of the sclerites (hard cuticle) from the soft flexible cuticle; the borders are not as readily seen in a scanning preparation as in the living animal. The projection of the third axillary sclerite onto which the muscles insert lies just beneath the wrinkled soft cuticle, and is indicated here by a dotted line. The cluster of arrows indicates the direction in which the third axillary muscles pull. The longer projection of the third axillary sclerite, which articulates with the fourth axillary sclerite, is not shown on this diagram. The approximate plane along which the wing apparently hinges when it is elevated and depressed is indicated by a dashed line. The centre of rotation, which remains relatively motionless when the wing is promoted and remoted in a horizontal plane, is indicated by the asterisk. In this view, the wing is fully remoted, and promotion would occur with the wing being pulled forward as indicated by the curved arrow.

Two sclerites did not actually come into contact with each other, but on occasion, during vigorous flight or struggling, paint was transferred to the other sclerite. The extent to which this might occur normally during flight has not been determined.



Other mechanical interactions involve several ligaments connecting the subalar sclerite to adjacent structures. Two small ligaments run from the subalar sclerite to the ventral portion of the second axillary sclerite (Sharplin, 1963, and present observations on *Manduca*); contraction of the subalar muscle exerts a force through these ligaments onto the second axillary sclerite to cause remotion. The subalar sclerite is also connected with a third ligament to the posterior notal wing process and rather loosely *via* a fourth ligament to the fourth axillary sclerite. No really clear ligamentous connection, other than that provided by the flexible cuticle, was observed between the subalar and the third axillary sclerite. The various loose connections provided by the regions of flexible cuticle probably have no role in the mechanical operation of this region of the wing joint other than providing a site for bending. The second axillary sclerite is tethered to the pleural wing process by a very short ligament, which may contribute stability to an important fulcrum for wing movement.

#### *Electrophysiological recordings during tethered flight*

It is clear from the above analysis of the cuticular structures and from the differing fibre composition and innervation of the three parts of the third axillary muscle, that their actions are likely to be complicated and, because of their differing angles of origin, to be different from each other. Kammer (1971), recording from the upper third axillary muscle, found that the motor input to that muscle bundle varied from single impulses to bursts, and from firing in phase with the subalar muscle during wing depression to firing both with the subalar and with an elevator muscle. This variation in the motor pattern appeared during attempts to turn during stationary flight.

To see if there was any evidence for differences in function among the three parts of the third axillary, we recorded from the middle and lower third axillary muscles as well as the upper portion. A fine copper wire was inserted just inside the common region of origin of the middle and lower parts of the third axillary muscle on the shelf of the epimeron. In that location extracellular muscle potentials from both parts of the muscle could be observed. (These are very small muscle units, and it was observed that wires inserted into either or both of these muscle units would pick up currents from both. Comparing intracellular and extracellular recordings of spontaneously generated potentials allowed us to ascribe the small, slow EJPs to the middle third axillary.) A second wire was inserted into the upper third axillary either at its origin on the episternum, or more caudally by passing through muscle pv6 at the episternum just anterior to the pleural suture. Other wires were inserted in the

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Fig. 9. Comparison of the angle formed by the third and fourth axillary sclerites when the wing is up and remoted (A,  $\times 56$ ), and up and promoted (B,  $\times 54$ ). Lateral views of the left wingbase. The angle between the sclerites is increased when the wing is pulled forward. The positions of the articulating projection from the third axillary sclerite and of the fourth axillary sclerite are indicated by dashed lines, since they are partly obscured by the folds of flexible cuticle that lie over them. The articulation, or knee, is indicated by the asterisk. *sa*, subalar sclerite; the numbers refer to the portions of the corresponding axillary sclerites visible in this view.



subalar, the basalar or an elevator muscle to assess the type of flight and for reference. Positions of electrode tips were verified after the experiment by dissection of the fixed specimen.

In non-flying moths only small-amplitude slow EJPs were recorded when a wire was placed in the middle third axillary muscle. As had previously been observed intracellularly, there was no indication of any secondary active membrane response in the fibres of this bundle, even when the slow EJPs summated at short latencies, and there was no evidence for innervation by another type of motor neurone. In both intracellular and extracellular recordings there was frequently a steady input of slow potentials to the muscle even in an apparently quiescent moth. Blowing on the wing or moving it slightly caused an increase in the frequency of these potentials as the moth resisted the movement (Fig. 11A). Additional tactile stimulation both increased the frequency of the slow unit and recruited others (Fig. 11B). At the onset of flight, the frequency of the slow potentials also increased. However, when the adjacent lower third axillary became active, its large action potentials obscured the smaller slow EJPs and it was impossible to determine the function of the middle bundle during flight (Fig. 12).

The activity of the upper third axillary muscle during flight and turning has been described previously (Kammer, 1971). This muscle was characterized by very changeable motor patterns, and the variety has again been observed in the experiments described here. The muscle potentials ranged from unpatterned at a relatively high frequency (about five per wingbeat), singles evenly spaced at twice the wingbeat frequency (Fig. 13, top), singles to a long burst with the subalar muscle potentials (Fig. 13), a burst with the subalar and a single spike with the elevator potentials (Fig. 13), a burst with the elevator (not illustrated), or bursts with both elevators and depressors at twice the wingbeat frequency (Fig. 12). The most common pattern was a burst occurring at approximately the same time as the subalar was activated (Fig. 13, bottom, Fig. 14A), but various phases relative to the firing of the subalar were observed (see also fig. 12 in Kammer, 1971). The muscle may also

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Fig. 10. Dorsal view of the right wingbase, with anterior being towards the top of the figure and lateral to the right, comparing the positions of the third axillary sclerite when the wing is down and back (A) and when it is folded in the rest position (wings horizontal and lying over the abdomen) (B). In assuming the rest position there is a marked change in the relationship of the third axillary sclerite with the first. Numbers refer to the associated axillary sclerites; A  $\times 67$ , B  $\times 47$ . For reference, the same spot on the cranio-lateral edge of the third axillary sclerite is marked with an asterisk in both A and B. The sclerite both rotates and slides further under the first axillary sclerite when the wings are folded. The arrow indicates the position under the flexible cuticle of the projection of the third axillary sclerite onto which the muscles insert. In comparing A and B, note also that the wing ( $w$ ) in the rest position appears to be folded along a line just anterior to the point of articulation with the third axillary sclerite. This is a region of the wing cuticle which is particularly flexible and would allow the posterior part of the wing to assume a different plane from the anterior part. Compare also with Fig. 8B, where only the posterior flap has been drawn, and Fig. 8A, where the fold appears to be a relatively deep crevasse. In the live animal, however, in the wings-folded position, the posterior flap is supported by the metathoracic wing and remains coplanar with the rest.

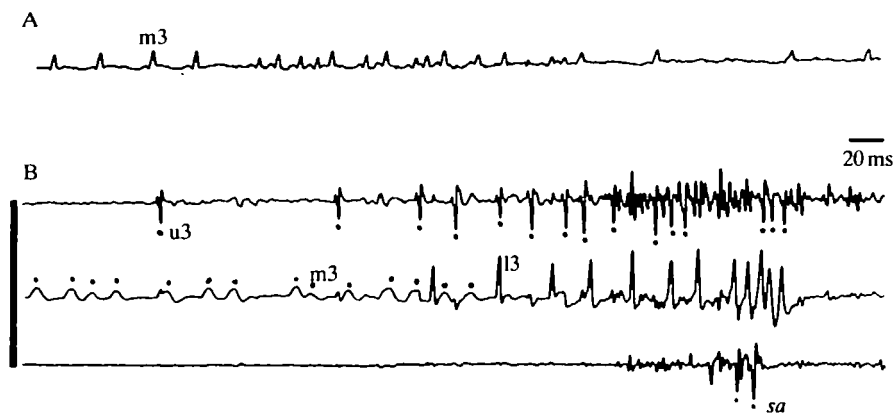


Fig. 11. Electromyograms from the third axillary muscle in non-flying moths. (A) Variable frequency of slow excitatory junction potentials from the middle unit (m3). As demonstrated in a subsequent part of the record (not shown), the electrode in m3 also detected signals from the adjacent lower third axillary unit, which was not active at this time. Other electrodes in the upper third axillary unit, the subalar muscle and an elevator muscle detected no concurrent activity (traces not shown). (B) Response to tactile stimulation in a different preparation, with the wings remaining folded. The three units of the third axillary muscle do not fire synchronously, but all increase their firing frequency in response to the stimulus (u3, upper unit; m3, middle unit; l3, lower unit; sa, subalar muscle recorded differentially).

be entirely 'quiet' even though the subalar and the lower third axillary are active (Fig. 14).

The lower third axillary, if it is being activated, usually fires with or slightly later than the subalar muscle (Figs 13, 14). Burst length ranged from a single spike to five or six spikes. The bursts were most often relatively long, with the burst preceding and following the time of activation of the subalar (Fig. 14B). Phase changes relative to the subalar were also observed (Fig. 14). In addition, as was observed in the upper third axillary, the muscle might also be excited in phase with the subalar and by additional spikes in phase with an elevator (Fig. 15). Thus, during flight, both intermediate units of the third axillary muscle were usually synergistic with a direct depressor and/or remoter, the subalar, although both could fire with the elevator muscles.

Examination of motor patterns recorded simultaneously from both the bundles indicates that, even though there were some general similarities in the observed types of phase relationships, the upper and lower portions of the third axillary frequently performed different functions since they were not being excited by the same motor patterns at the same time. Each of these intermediate units exhibited a variety of phase relationships with respect to the subalar muscle.

Visual observations of the moth during tethered flight in conjunction with the motor patterns again supported the contention that the activity of the third axillary muscle is important in turning (Kammer, 1971). Patterns of spikes in both upper and lower bundles changed when the amount of remotion on one side relative to the other side appeared to change.



Frequently the upper and lower parts of the third axillary muscle, the subalar and the basalar muscles were firing approximately in synchrony with each other and out of phase with an indirect elevator. On occasion there were marked changes in the number of spikes per burst in the basalar and changes in its phase relative to the activation of the subalar. In at least one animal the basalar, in addition to showing bursts of potentials in synchrony with the subalar, was activated by one or two spikes in phase with the elevator. Marked changes in the motor pattern to the basalar (either an increase or a decrease in the numbers of spikes per burst) were frequently accompanied by a change in the pattern to the subalar or the upper and lower third axillary muscle. These observations, along with the data presented in Kammer (1971), support the hypothesis that the balance between promotion and remotion may be achieved by the relative amounts of activation of the basalar *versus* the subalar and third axillary muscles. More conclusive evidence can only be obtained with the simultaneous monitoring of wing position and motor activity.

The third axillary sclerite and muscle have been implicated in the folding of the wing into the rest position in *Lepidoptera* (Sharplin, 1963, 1964). The recordings of the motor patterns from the tethered moths were examined to see if the electrical

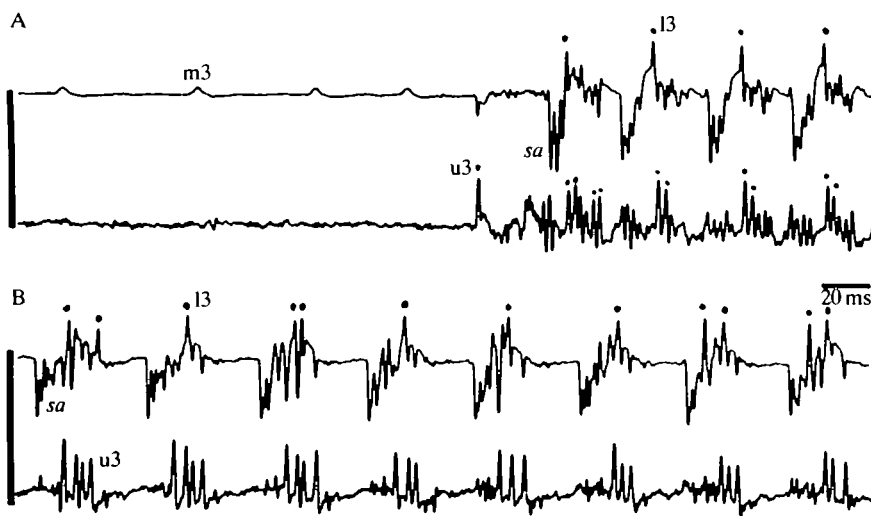


Fig. 12. Electromyograms showing the onset and continuation of flight. Two samples from a continuous record are shown. The upper trace represents the signals recorded differentially from one wire that detected potentials from both the middle (m3) and lower (l3) parts of the third axillary muscle (positive-going potentials) and a second electrode in the subalar muscle (sa) (negative-going potentials). The lower trace was recorded from a single electrode in the upper unit (u3) of the third axillary muscle. During strong flight or during times when differentially recorded muscles are being activated synchronously, the identity of individual spikes is not clear but it could be sorted out during the experiment by temporarily grounding one lead. (A) At the onset of flight, the upper third axillary unit was activated at twice the wingbeat frequency. Excitation of the subalar muscle indicates the beginning of the downstroke; activity in the upper third axillary muscle preceded it. (B) Continuation of flight. The upper and lower units are excited synchronously during the latter part of the downstroke, following the activation of the subalar muscle.



Fig. 13. Rapid variability in the motor patterns from the upper (u3) unit of the third axillary muscle during flight (continuous record of two traces). Note the changing phase relationships of u3 with the subalar muscle (*sa*). Activity in the lower third axillary unit (13) is relatively constant in this example; however, because of pick-up of the upper third axillary potentials on that lead (small downward deflections on the uppermost trace), signals are less clear during periods of overlapping intense activity from the upper third axillary as shown on the second set of traces. The recordings were made as described for Fig. 12.

activity in the three muscle bundles supported this hypothesis. The normal flight pattern consisted of alternating bursts of spikes from the elevators and the depressors. In some cases just prior to the cessation of flight, the duration of the wingbeat cycle was lengthened somewhat, but the alternating phase relationship of the elevators and depressors was maintained. The records were examined from the point of view of determining whether the units of the third axillary were active after the last burst in the normal alternating sequence had occurred. In nine out of ten records in which the moth stopped flying spontaneously, the last activity consisted of either a short burst of spikes from the upper and/or lower third axillary, or a train of small potentials of slowly decreasing frequency from the tonic middle third axillary muscle. We conclude that, although there may be additional ways that the wings can be folded, the third axillary muscle is frequently used in this capacity.

#### DISCUSSION

The main objective of this study was to characterize differences in the morphology, innervation and excitation of the three units of the third axillary muscle as a basis for understanding their roles in flight. The main function of all three units appears to be remotion, either folding the wing at the end of flight or altering the path of the wing during flight. The electrical recordings show that the three parts of the third axillary muscle may act synchronously, but they are under no constraints to do so. The physiological data support the morphological observations, indicating that each bundle has a separate innervation and can be excited independently. The middle, tonic unit appears to have a postural function, maintaining wing position in a

non-flying moth. Because of the small size of its junction potentials, we were unable to ascertain if this unit is excited during flight. Differences in the origin of the upper and lower bundles of the third axillary muscles, and differences in their motor patterns recorded concurrently suggest that these two units may have somewhat different actions during flight. However, without an accurate monitor of wing position during flight, it is not possible to ascertain specifically how the three bundles function in regulating wing movement during the different phases of the wing stroke.

The available data permit some suggestions about the function of the third axillary muscle as a whole and some comparisons with the role of this muscle in other insects. The morphology of the wingbase and the observations of the movements that the third axillary sclerite can execute support the hypothesis that the sclerite and its



Fig. 14. The marked differences occurring between the activation of the upper and lower parts of the third axillary muscle. (A) The upper third axillary (u3) is active in phase with the subalar muscle (sa) and the lower unit (l3) lags the subalar somewhat. In B, the upper unit is completely silent and the lower unit is activated with a longer burst of spikes which begins before activation of the subalar. The two samples are from the same experiment. The top trace represents the signals from a single electrode detecting the upper unit of the third axillary muscle and much smaller potentials from distant muscles including elevators and depressors. Middle trace, single electrode in the lower unit. Bottom trace, differential recording from two parts of the subalar in A and with only one lead active in B. The slow change in the baseline is movement artifact.



Fig. 15. The lower third axillary unit (l3) during this flight was excited both with a depressor muscle, the subalar (sa), and with elevator muscles (dv2 and dv1b). The middle trace represents the differential recording from electrodes in the subalar and dv2; the other two traces are each single-ended recordings from the muscles indicated.

associated muscle bundles function largely in remoting the wing. Hawkmoths are capable of rapid changes in the relative amounts of promotion and remotion on the right and left sides; this capability presumably contributes to their manoeuvrability during flight. Visual observations, using stroboscopic illumination of the wings during tethered flight, also suggest that the path of the wing can be altered by differing amounts of remotion at the top or at the bottom of the stroke, thus possibly altering the direction of the aerodynamic forces produced. Remotion may be maintained for many wingbeats during sustained attempts to turn (*Manduca*, visual observations; Kammer & Nachtigall, 1973). The motor patterns from the three portions of the third axillary are consistent with its function in controlling remotion under such a variety of circumstances.

The motor patterns from the upper third axillary, showing excitation in phase with both elevators and depressors, suggest that remotion can occur during both the upstroke and the downstroke, and that it may be maintained over many wingbeat cycles. The presence of a muscle unit containing tonic muscle fibres suggests that a certain degree of remotion might also be maintained throughout the entire wingstroke, although we were unable to record electrophysiological evidence of its control through the slow motor axon after flight had commenced. Rind (1983b) observed that an optomotor interneurone, D1, produced excitatory synaptic potentials in both the motor neurone to the lower third axillary muscle (second pleuroaxillary) and the motor neurone to the subalar muscle, but none was observed in three recordings from the motor neurone to the upper third axillary. This finding is consistent with the motor patterns described here, in that the subalar and lower third axillary units were most often seen to be excited essentially in synchrony, albeit with bursts of varying numbers of spikes and with differing phase relationships. Furthermore, the upper third axillary was also activated out of phase with them suggesting that it has significant input from interneurones different from D1. The subalar muscle appears to be able to aid in remotion by virtue of the two ligaments

that connect it to the second axillary sclerite. Both it and the third axillary muscles are opposed in this action by the basalar muscles; however, the subalar and the basalar are also viewed as wing depressors. If the magnitudes of the opposing forces in the horizontal plane from the basalars and from the subalar and third axillary are essentially equal, straight depression of the wing will be the net result. One aspect of the control of remotion could be provided by the interneurone D1 in response to visual stimuli if it functions to add to the spikes per burst of both subalar and lower third axillary or shifts the timing of their occurrence relative to the activation of the basalar so that they are no longer in phase, allowing differing amounts of remotion or promotion to occur. Indications of changes in the input to the basalar concurrent with changes in the input to the third axillary units were obtained in the present study but, again, studies are needed in which the precise wing position is monitored simultaneously with the muscle activity to verify that this is a reasonable interpretation. It will also be necessary for this type of analysis to compare the contraction times (duration of tension) produced under the appropriate conditions of motor input for each of the muscles involved. The units of the third axillary are either tonic or intermediate, while those of the subalar are phasic (Rheuben, 1985), and the various units of the basalar have not been examined. Differences in duration of tension from different muscle fibre types could have important implications in the control of wing position during different phases of the wing stroke even if neuronal activation is essentially synchronous.

It is unlikely that the third axillary muscle of hawk moths plays a significant role in regulation of supination, in contrast to its role in the forewing of locusts (Pfau, 1977; Pfau & Nachtigall, 1981). In moths, the structure of the wing and wingbase and the orientation of the third axillary muscle differ from those described for locusts. The moth forewing is more rigid, with no capability to form a Z-fold. There is a line of flexion in the posterior part of the wing, and the third axillary sclerite articulates loosely with the flap posterior to this line. In the flight position, the posterior part of the forewing flap can overlap the anterior part of the hindwing and may be, at least in males, partly held in this relationship by the frenulum. (In the male the frenulum and frenulum hook are well-developed. In the female the bristles forming the frenulum are seen only to interact with other bristles on the forewing and may be speculated to have only a sensory function.) Because the connection between the posterior part of the forewing and the third axillary sclerite is so flexible, the position of this flap could readily be influenced by the position of the hindwing, either by direct mechanical means or indirectly through the channelling of the airflow between the two wings.

The possible contribution of the construction of the wing hinge to supination on the upstroke has not been explored experimentally. Flash photographs of moths in tethered flight (unpublished) show there is a marked difference between the upstroke and the downstroke with regard to the twist of the wing; as in other insects, pronation on the downstroke probably results from contraction of the basalar muscles. The third axillary muscle may make a small contribution to wing twist by retarding the upward movement of the posterior part of the wing at the beginning of

the upstroke and by holding the posterior part of the wing elevated at the start of the downstroke. This suggestion is based on anatomical relationships observed by manipulating the wing and on the phase relationships between the excitation of the third axillary and other muscles; it is unlikely that supination or pronation will be major actions because of the looseness of the articulation between the posterior wing flap and the sclerite.

Although homologies are apparent across insect orders, comparisons of the wingbase structures and the muscles controlling wing movements reveal specializations. The third axillary muscle of locusts consists of two similar units that produce a tonic force on the third axillary sclerite and phasic modulation of the force (Elson & Pflüger, 1986). Contraction of the third axillary muscle appears to decrease pronation (Pfau, 1977; Pfau & Nachtigall, 1981), and patterns of excitation suggest that it is involved in compensating for an imposed roll (Elson & Pflüger, 1986). Locusts can change the relative angles of the anterior and posterior regions of the forewing in a controlled fashion not possible in moths. Locusts, in contrast to moths, make banked turns and appear to make little use of changes in remotion during flight.

Diptera have still another set of specializations. In addition to automatic changes in the angle of attack because of the construction of the wing hinge, pronation and supination can be influenced by direct muscles that insert on the first, third and fourth axillary sclerites (Miyan & Ewing, 1985). In contrast to locusts and moths, the fourth axillary sclerite is well-developed and supplied with a set of antagonistic muscles (Heide, 1971; Miyan & Ewing, 1985). Morphological observations suggest that in flies the muscles of the first, third and fourth axillary sclerites influence wing remotion during flight. The third axillary and other direct muscles may also increase the amplitude of the downstroke (Miyan & Ewing, 1985); in flies (and in locusts) the direction of the axillary muscles is much more vertical relative to the horizontal axis of the animal than in moths. These suggestions based on anatomy are supported by electrical recordings from direct muscles (Heide, 1971, 1975, 1983). Because the direct muscles do not exhibit the morphological specializations of the asynchronous flight muscles, they probably function as tonic muscles relative to the wingstroke, although they may be excited in phase with the wingbeat and exhibit an increment in tension after each spike (Heide, 1983).

In summary: muscular control of remotion during flight in locusts seems to be less important than control of supination; in moths it is dependent on a single, but multiunit, axillary muscle and two pleural muscles (basalar and subalar); it is complex in Diptera, in which it is dependent on several axillary muscles inserting on three axillary sclerites, plus some pleural muscles. Although we can guess at the aerodynamic consequences of changes in remotion, we know of no experimental analysis of this aspect of insect flight. Asymmetrical remotion may contribute significantly to the rapid turns apparent in the flight of hawk moths and flies.

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