

Structure of the Forewing Stretch Receptor Axon in Immature and Mature Adult Locusts

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ABSTRACT

During the first 2 weeks following imaginal ecdysis, the wingbeat frequency of *Locusta migratoria* doubles, and the activity of the forewing stretch receptor (fSR), in response to wing elevation, increases. We examined the three-dimensional structure of the centrally projecting axon of the fSR during adult maturation to determine if there are changes in the branching geometry.

We found that changes occur in the mesothoracic projection (IISR Meso). Here, there was a significant increase in the volume of the projection from $2.3 \times 10^4 \pm 0.2 \times 10^4 \mu\text{m}^3$ in immature locusts to $6.0 \times 10^4 \pm 1.2 \times 10^4 \mu\text{m}^3$ in mature locusts. There were also significant increases in the total length, the number of branch points, the number of axonal swellings, and the diameters of first- and second-order branches of the projection. No significant changes were observed in the prothoracic projection (IISR Pro), and the only significant change observed in IISR Meta was negative allometric growth relative to IISR Meso.

These results demonstrate that during adult maturation, growth of the fSR axon is heteromorphic between different ganglionic projections and that there is a potential increase in the connectivity of IISR Meso to other flight neurons in the mesothoracic ganglion. We suggest that this may be a mechanism for maintaining the efficacy of afferent input to flight interneurons that are also growing during maturation. © 1996 Wiley-Liss, Inc.

Indexing terms: maturation, three-dimensional reconstruction, afferent, motor pattern, insect

Motor pattern development has been described in many invertebrate and vertebrate systems, and one of the underlying similarities is the transition, either gradual or punctuated, from an irregular, unpatterned oscillation to a complex, phasic rhythm (*Xenopus*, Sillar et al., 1991; chick, Bekoff et al., 1987, 1989; leech, Jellies et al., 1992; locust, Kutsch, 1974a). This transition may be dependent on modifications in the afferent input or may be developmentally programmed. In the chick, the motor pattern for hatching switches to one for walking as a result of modifications in the postural afferent input (Bekoff et al., 1987, 1989). In *Xenopus*, however, postembryonic development of the swimming circuit appears to be centrally mediated and is concurrent with the incorporation of descending serotonergic inputs into the spinal cord (Sillar et al., 1992).

Studies of vertebrate and invertebrate systems have shown that changes in the morphology of sensory afferents, resulting from developmental growth or competition with other afferents, occur in parallel with, and may be necessary for, changes in connectivity to postsynaptic elements. Monocular deprivation in kittens results in a decrease in the responsiveness of cortical neurons postsynaptic to geniculocortical afferents serving the deprived eye (Wiesel

and Hubel, 1963). More recently, it has been shown that restructuring of the deprived geniculocortical afferents, i.e., a decrease in growth relative to non-deprived afferents, can occur within 1 week (Antonini and Stryker, 1993). Afferents that serve ventral filiform hairs on the prosternum of *Locusta migratoria* undergo activity-mediated reorganization during postembryonic development resulting in a change in the connectivity to postsynaptic interneurons (Pflüger et al., 1994). In both of these studies, competition between bilaterally homologous afferents may also be involved in redefining axonal morphology. In the flight system of mature adult *L. migratoria*, removal of a hindwing proprioceptor, the tegula, results in an initial decrease in the flight rhythm frequency (Büschges et al., 1992a). Over the next 2 weeks the wingbeat frequency increases to near pre-ablation levels, and this may be mediated by sprouting of the metathoracic branches of the forewing tegula afferents, resulting in new synapses forming with interneurons that normally receive input from the hindwing tegulae

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(Büschges et al., 1992b). This implies that there is competition for postsynaptic sites between the metathoracic branches of the forewing and hindwing tegulae. In intact animals, the hindwing tegula afferents outcompete forewing tegula afferents, and this competition is removed by hindwing tegula ablation.

Flight-like motor patterns of the locust can be expressed as early as the first larval instar, but the fully mature pattern is not evident until 2 weeks after the imaginal moult (Stevenson and Kutsch, 1988). Recently, we have shown that there are modifications of phasic sensory feedback from the forewing stretch receptor (fSR) during adult maturation (Gray and Robertson, 1994).

The wing hinge stretch receptors are ideal for examining the role of afferent input in motor pattern function because they exist as single cells at the base of each wing and they connect to many components of the flight system (Reye and Pearson, 1987). Each responds to wing elevation with an increased firing rate (Wilson and Gettrup, 1963; Pfau et al., 1989; Gray and Robertson, 1994). The fSR makes monosynaptic connections to flight motor neurons (Burrows, 1975) and interneurons (Reye and Pearson, 1987). It has also been shown that the fSR can entrain the flight rhythm to match that of an artificially applied stimulus (Reye and Pearson, 1988). Thus, the SR plays a role in modulating the mature flight rhythm.

Central projections of the fSR have characteristic patterns within the thoracic ganglia. These were first described by Bentlage and Huber (unpublished, cited in Huber, 1975) and later described in detail by Altman and Tyrer (1977a,b). Although embryonic growth of the fSR has been documented (Heathcote, 1981), there has been no detailed description of the morphology during adult maturation. The goal of the present study was to describe the structure of the centrally projecting axon of the fSR at different times during adult maturation. During this time the entire ventral nerve cord is growing (Sbrenna, 1971), and the wingbeat frequency is increasing exponentially to a maintained level (Kutsch, 1974a). We were particularly interested in determining if there is growth of the fSR during maturation and, if so, if it can be temporally correlated to the increase in the motor pattern frequency.

MATERIALS AND METHODS

Adult male locusts were selected from a crowded colony maintained at the Department of Biology at Queen's University (28°C; 16:8 light:dark photoperiod). Animals were selected at one of four stages; fifth-instar nymph ($n = 3$), immature (0–3 days past the imaginal moult, $n = 7$), day 7 ($n = 5$), and mature (at least 14 days past the imaginal moult, $n = 9$).

Central afferents of the fSR were filled *in vivo* using 0.2–0.4 M CoCl_2 . The higher concentration was necessary to fill the fifth-instar and immature fSR afferents completely, whereas the lower concentration prevented parallel filling of adjacent motor axons in day 7 and mature afferents. The afferents were filled by placing the cut end of nerve 1D₂ (named according to Campbell, 1961) into a vaseline well containing the CoCl_2 (Fig. 1A) for 24–72 hours at 4°C in a humid container. Fifth-instar and immature axons took longer to fill than day 7 and mature axons because of the smaller diameter of the main axons. Projections were considered to be filled completely if the most distal terminal of IISR Meta, that is, the posterior portion of the branch that extends into the first three, fused abdominal neuromeres, was clearly visible.

After the allotted filling time, the three thoracic ganglia (prothoracic, mesothoracic, and metathoracic) were dissected out, developed in approximately 5 ml of saline to which 3–4 drops of ammonium sulphide had been added, fixed in acetic acid:alcohol (4:1), and silver intensified using the methods of Bacon and Altman (1977). The ganglia were cleared in methyl salicylate and whole mounted onto glass slides with Permount. The coverslips were raised slightly with spacers so as not to distort the Z-axis of the filled afferents.

Three-dimensional reconstructions of the afferents were created using the Eutectic[®] Neuron Tracing System attached to a Leitz Diaplan compound microscope with a 25× objective lens (see Fig. 2). The central projections of the fSR were named after the scheme of Altman and Tyrer (1977a), that is, IISR Pro refers to the prothoracic projection, IISR Meso refers to the mesothoracic projection, and IISR Meta refers to the metathoracic projection, where IISR denotes the stretch receptor from the second (meso-) thoracic segment. Quantitative measurements included 1) the volume of the axon branch within a particular ganglion (the volume was measured starting from the first branch point in the prothoracic and mesothoracic projections and between the first and last branch point along the main axon of the mesothoracic projection; arrows in Fig. 1B–D); 2) the total length (i.e., summed length of all branch segments) of the projection within the same regions defined for volume (as in measurement 1, above); 3) the number of branch points and swellings within each projection (we define swellings as segments of the axon that have a larger diameter than adjacent segments and are not more than twice as long as the diameter with the enlarged segment; these were clearly resolved with the magnification used; arrows in Fig. 2B); 4) the maximum branch order and the mean diameters of first, second, and third-order branches within each projection.

Quantitative data (Figs. 4–6) are displayed as box plots representing the median and error bars representing the 5th and 95th percentiles. Significant differences were assessed using a one-way ANOVA and Student-Newman-Keuls multiple comparison test. Samples were considered significantly different at $P < .05$. To assess possible allometry of growth of IISR Pro and IISR Meta relative to IISR Meso, the log of the data were fitted with a model II regression. Slopes and r^2 values were determined for the projection volume and projection length of IISR Pro and IISR Meta.

RESULTS

Structure of the fSR axon

Altman and Tyrer (1977a) provide a detailed description of the morphology of central fSR projections in mature locusts. Briefly, the fSR bears a single axon that projects ipsilaterally into all three thoracic ganglia (Fig. 1). The prothoracic projection (IISR Pro) enters the prothoracic ganglion from prothoracic nerve 6 (Pro N6) extending anteriorly and laterally along the dorsal neuropil. The mesothoracic projection (IISR Meso) enters the mesothoracic ganglion from mesothoracic nerve 1 (Meso N1) and extends initially posteriorly and medially among the dorsal neuropil. Shortly after giving off a lateral branch (Fig. 3A), IISR Meso turns posteriorly and runs along the dorsal midline to enter the meso–metathoracic connective. There it enters the metathoracic ganglion and runs posteriorly along the midline initially in the dorsal neuropil, giving off short branches along its length. About midway along the

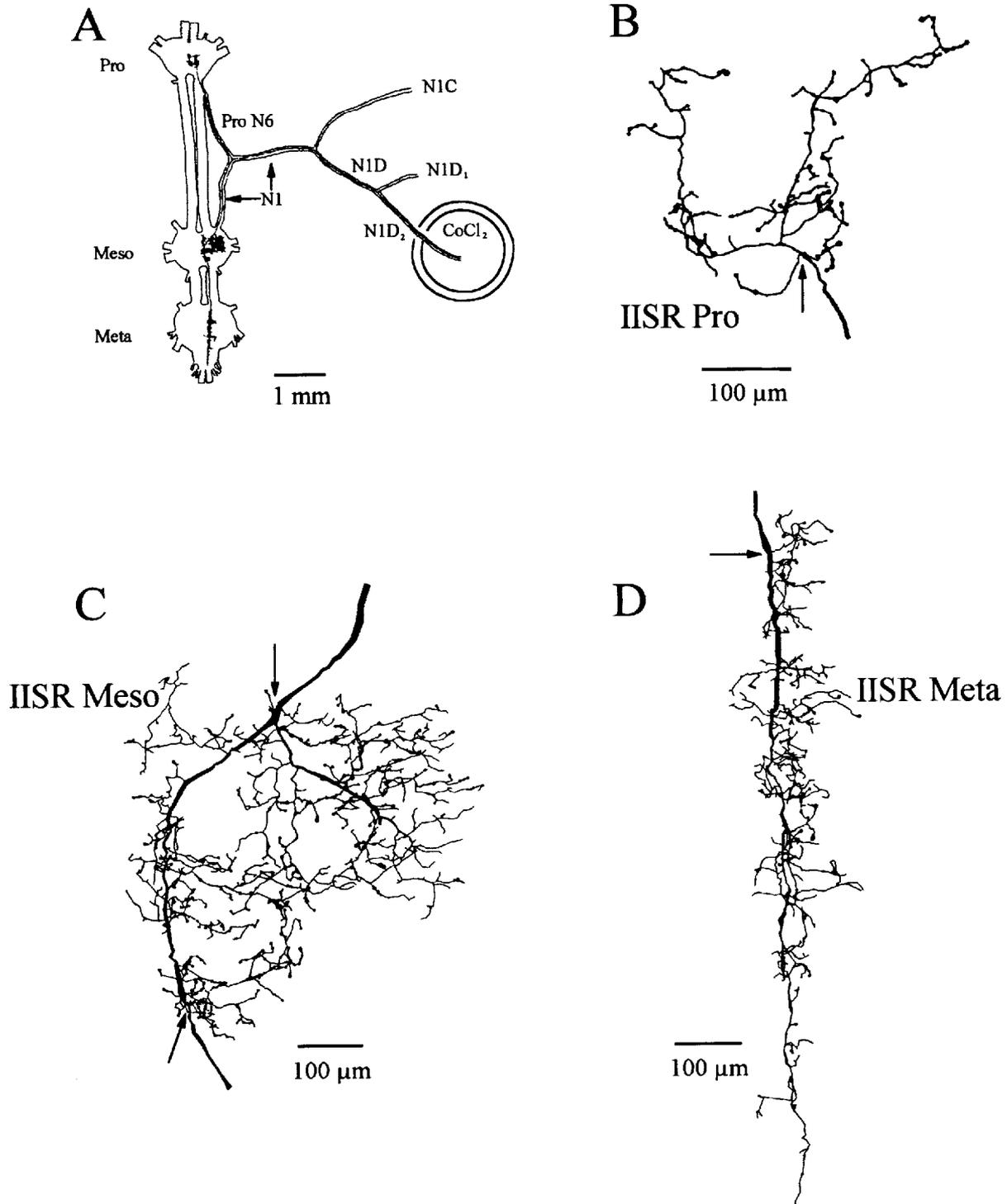


Fig. 1. Diagram of setup for filling central projections (A) and representative drawings of right prothoracic (IISR Pro) (B), mesothoracic (IISR Meso) (C), and metathoracic (IISR Meta) (D) projections from a mature animal. Labelling of nerves in A is from Campbell (1961). Pro, prothoracic ganglion; Meso, mesothoracic ganglion; meta, metathoracic ganglion.

antero-posterior axis of the ganglion the main trunk of IISR Meta dips ventrally and extends further posterior to terminate in the fused first three abdominal neuromeres.

racic ganglion. Arrows in B and D indicate the first branch point of IISR Pro and IISR Meta, respectively. Top and bottom arrows in C indicate first and last branch points, respectively, in IISR Meso (see Results in text). Dorsal planar view with anterior at the top.

The gross morphological characteristics of all three projections are conserved during maturation (examples of IISR Meso are shown in Fig. 3A–D). The only noticeable differ-

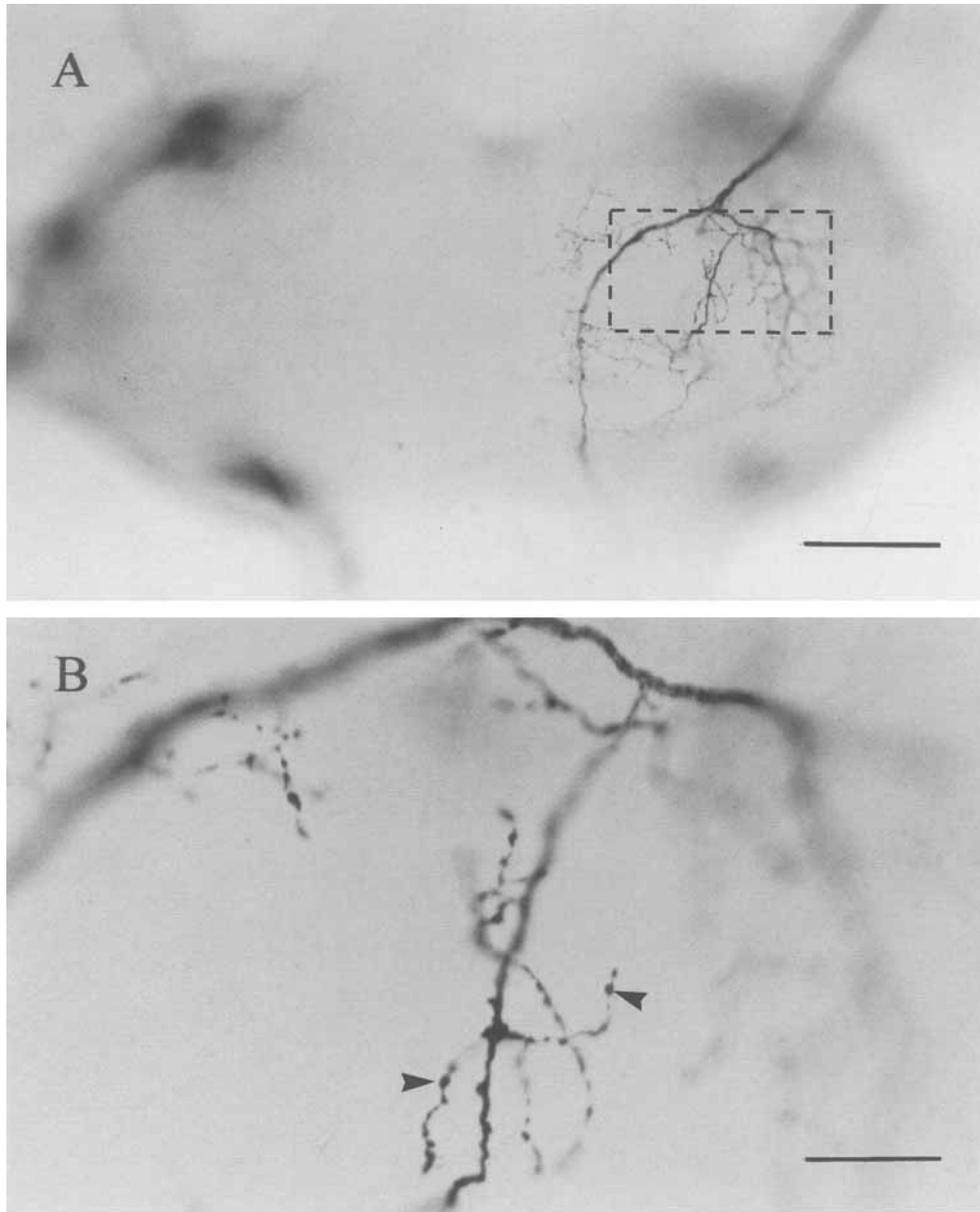


Fig. 2. Photomicrographs of a mature IISR Meso filled with 0.02 M CoCl_2 . **A:** Whole projection of IISR Meso using a $\times 6.3$ objective lens. The dashed line indicates the region enlarged in B. Scale bar = 200 μm . **B:** Same fill using a $\times 25$ objective lens. Samples of axonal swellings are indicated by arrows. Scale bar = 40 μm .

ences observed were an increase in the overall size of IISR Meso and an expansion of its lateral branch into the lateral neuropil of the mesothoracic ganglion. Bridging fibres, branches that extend between the medial and lateral branches of IISR Pro and IISR Meso (see Altman and Tyrer, 1977a), were present in all stages examined.

IISR pro. In fifth-instar and immature stages, the medial branch of IISR Pro consistently extended farther anteriorly than the lateral branch. The general structure was similar in day 7 and mature stages; however, in both cases the lateral branch often extended as far as the medial branch (one of five cases in day 7 and four of nine cases in mature, Fig. 1B). The presence of bridging fibres extending

between the lateral and medial branches was variable both within a given stage and between all four stages examined.

IISR meso. The geometry of IISR Meso was consistent in all stages examined (Fig. 1C). The only noticeable differences within specific branches are those mentioned above.

IISR meta. The general structure and extent of branching of IISR Meta were similar in all stages examined (Fig. 1D).

Absolute and relative growth of the fSR axon

The volume and total projection length were measured to determine how the fSR axon grows during maturation. The

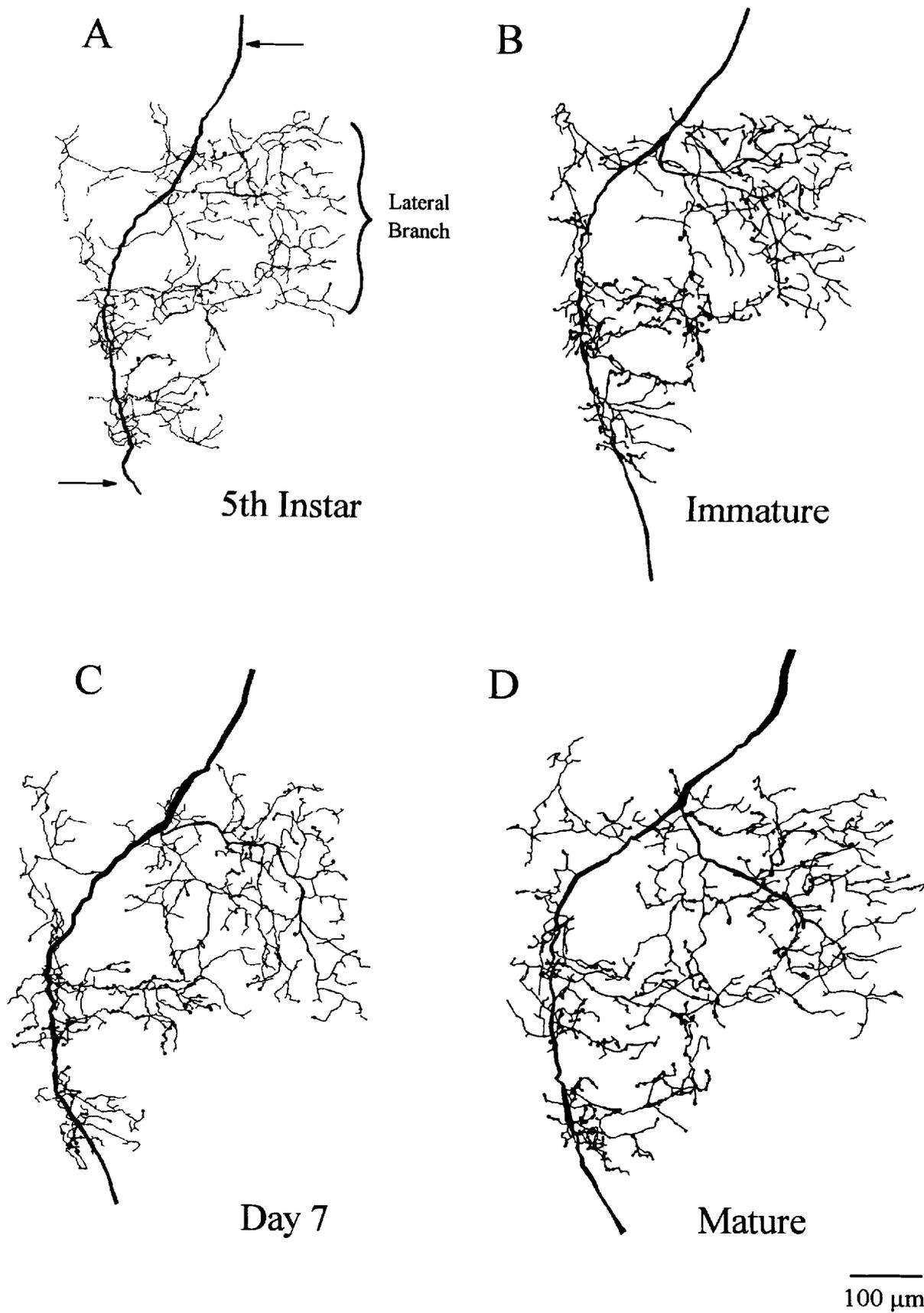
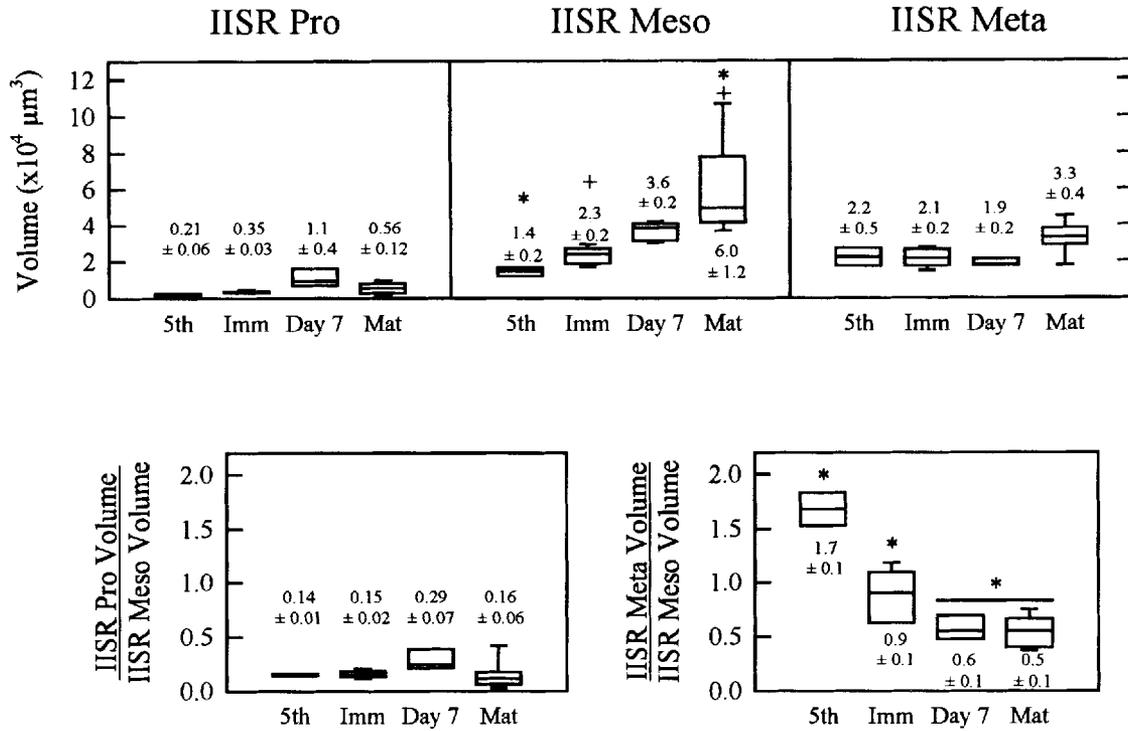


Fig. 3. Representative drawings of IISR Meso from fifth-instar (A), immature (B), day 7 (C), and mature (D) animals. Note the overall increase in projection size, particularly in the thickness of the main axon trunk (demarcated in A by arrows) and extent of lateral branching (lateral branch in A).

A



B

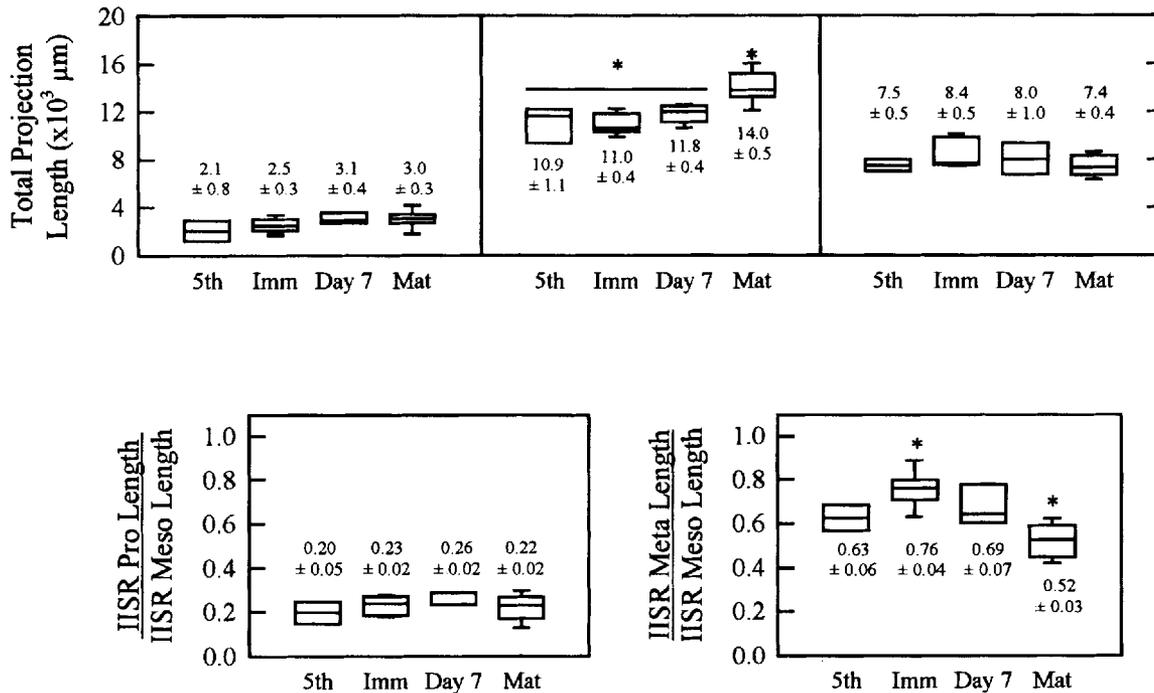


Fig. 4. Box plots of absolute and relative dimensions of all three fSR projections during maturation. The volume (A, top row) and total projection length (B, top row) increase in IISR Meso only. The volume (A, bottom row) and total projection length (B, bottom row) of IISR Pro increases isometrically relative to IISR Meso, whereas that of IISR

Meta increases negatively allometrically. * and + indicate significant differences at $P < .05$. The bar above 5th, Imm and Day 7 in B indicates that there are no significant differences between these stages but that each is significantly different from the mature total projection length. Numbers are means \pm SEM.

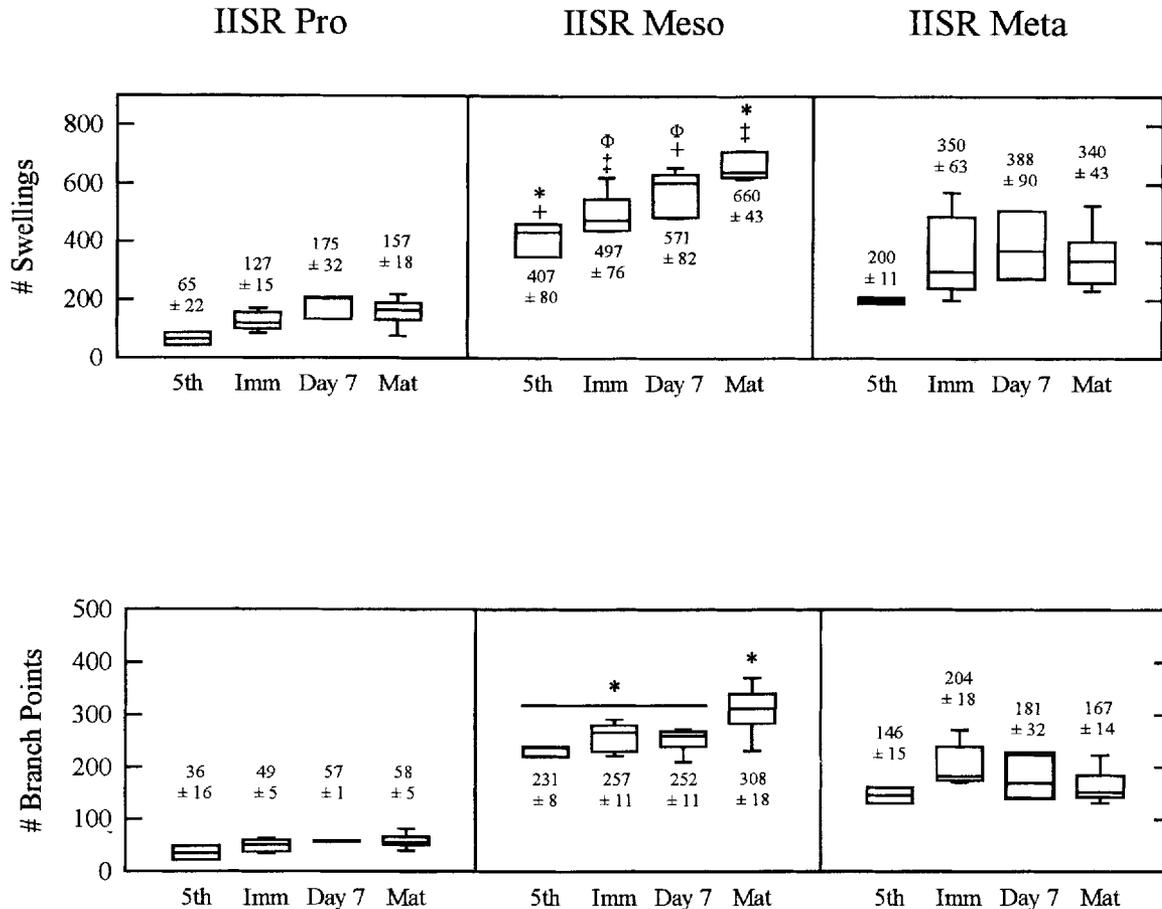


Fig. 5. Box plots of the number of swellings (top row) and number of branch points (bottom row) in all three projections. In both cases a significant increase was observed in IISR Meso only. *, +, ‡, and φ indicate significant differences at $P < .05$. Numbers are means \pm SEM.

volume and total length of IISR Pro and IISR Meta were also standardized to those of IISR Meso to determine if growth was uniform throughout the axon. Figure 4A shows that there was a significant increase in the volume (top row) of IISR Meso only. No significant changes were observed in IISR Pro or IISR Meta. Within the axon, growth, relative to IISR Meso, appeared isometric for IISR Pro and negatively allometric for IISR Meta (Fig. 4A bottom row). However, fitting the log of the data with a model II regression revealed that the volume of IISR Pro versus that of IISR Meso had a slope of 0.11 ($r^2 = 0.09$), whereas a similar fit of IISR Meta versus IISR Meso had a slope of 0.54 ($r^2 = 0.66$). Similar changes were observed for the total projection length. The only significant increase was in IISR Meso after day 7 (Fig. 4B, top row). A model II regression of the log of the data revealed that the length of IISR Pro versus IISR meso had a slope of 0.23 ($r^2 = .21$), and that of IISR Meta versus IISR Meso had a slope of 0.62 ($r^2 = 0.01$). This suggests that the changes in the volume and projection length of IISR Pro and the projection length of IISR Meta are not correlated to that of IISR Meso, whereas the volume of IISR Meta grows negatively allometrically to that of IISR Meso. Thus, during maturation, growth of the fSR axon is confined to IISR Meso. Indeed, the size of IISR Meta declines relative to IISR Meso during maturation. Its

volume is more than 1.5 times that of IISR Meso in the fifth instar but only 0.5 times at maturity.

Branching parameters

The stereotypic organization of the fSR axon suggests that the structure of the projections within their respective ganglia may reflect certain aspects of their connectivity to other components of the flight system. Altman and Tyrer (1977a) suggest that the "blebby" appearance of the projections is an indication of the presence of synapses to motoneurons and interneurons. Although Peters et al. (1985) claim that output synapses are not restricted to these axonal swellings, the swellings invariably contain output synapses. Thus a measure of the number of axonal swellings during development is reflective of the number of synapses. During adult maturation there is a significant increase in the number of swellings from immature to mature IISR Meso only (Fig. 5, top row). Significant differences were not observed in IISR Pro or IISR Meta.

The number of branch points counted in each projection is an indicator of its complexity. Again, significant differences were observed only in IISR Meso after day 7 (Fig. 5, bottom row). Another significant change that we observed in IISR Meso was an increase in the mean diameter of first- and second-order branches (Fig. 6).

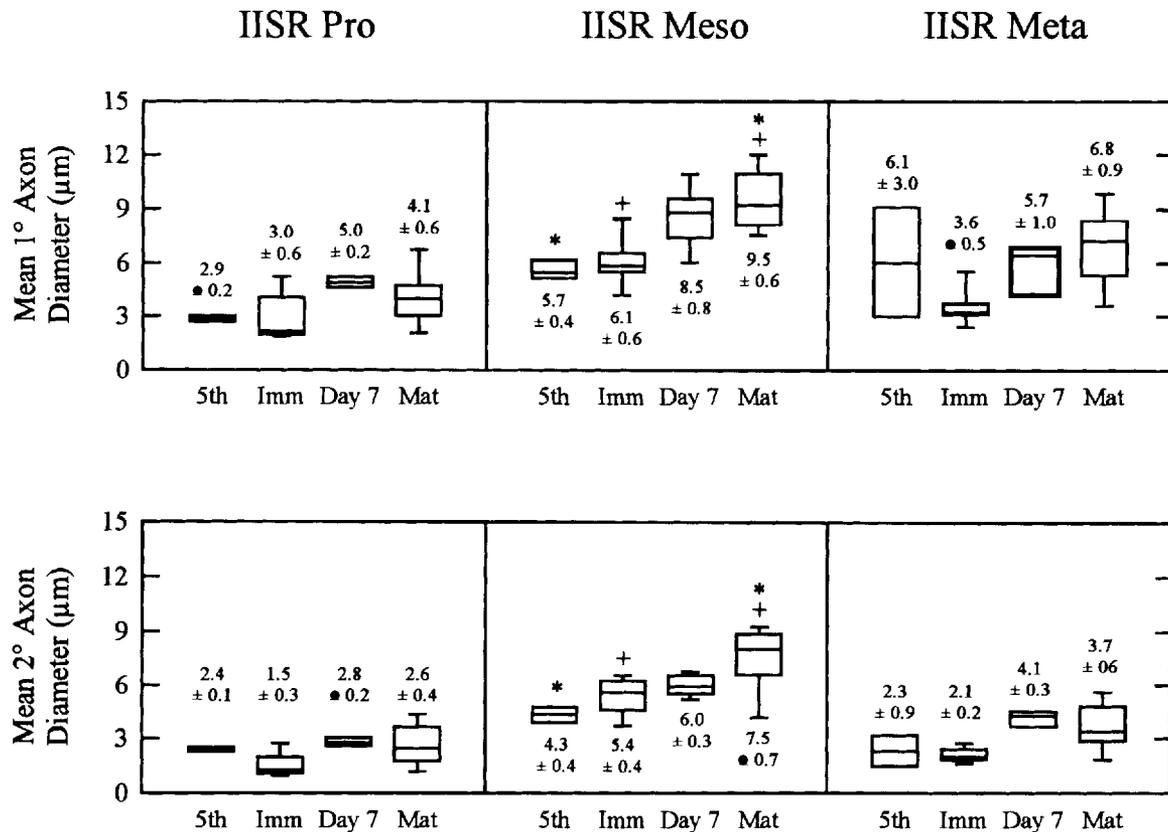


Fig. 6. The mean diameter of primary (top row) and secondary (bottom row) axons increases in IISR Meso only. * and + indicate significant differences at $P < .05$. Numbers are mean \pm SEM.

DISCUSSION

General structure of the fSR axon

Bentlage and Huber (unpublished, cited in Huber, 1975) first described the central branching projections of the fSR in *L. migratoria* as a large multi-branched axon that runs along the dorsal neuropil of the mesothoracic ganglion. Later, Altman and Tyrer (1977a) described the detailed branching structure of the fSR in all three thoracic ganglia. Although variations in the central arborizations of the fSR exist, for example, the position along the anteroposterior axis that IISR Meta dips toward the ventral region of the ganglion (see Altman and Tyrer, 1977b), the general structure is remarkably consistent between animals and, as demonstrated in this study, between stages of adult maturation. Differences between the results from this study and those of Altman and Tyrer (1977a) include the presence of a loop line between IISR Pro and IISR Meso and the division of the lateral branch of IISR Meso into medio-lateral and lateral subbranches. We observed a loop line that connects IISR Pro and IISR Meso through the pro-meso connective in only one of our preparations. This is not surprising, however, since Altman and Tyrer (1977a) described it in only five of more than 100 preparations from three Orthopteran species. Further, we found that the division of the lateral branch into medio-lateral and lateral subbranches was inconsistent. In about 50% of the preparations, a separate branch extended from the main lateral trunk or even from the main axon itself; however, it was not distinct

in all preparations. The variation in the presence of the medio-lateral branch was not correlated with developmental age.

Growth of the fSR axon during adult maturation

Most of the growth of the fSR (i.e., the increase of the projection volume and the total projection length) occurs in IISR Meso, and the volume of IISR meta grows negatively allometrically relative to that of IISR Meso. The increase in axon diameter of first- and second-order branches of IISR Meso is consistent with an increase in diameter of the main axon in nerve 1 (Altman et al., 1978; Gray and Robertson, 1994). Changes in the number of swellings and the number of branches, also confined to IISR Meso, occur after day 7, suggesting that the fSR increases its connectivity to other neurons within the mesothoracic ganglion in the latter half of maturation. Peters et al. (1985) have shown that although output synapses of the fSR are not restricted to axonal swellings, the swellings invariably contain output synapses. This is also the case for the dorsal fields of spiking local interneurons in the metathoracic ganglion (Watson and Burrows, 1985). Thus, a measure of the number of axonal swellings on the fSR is an index of the number of output synapses. Taken together, these results demonstrate that growth and potential changes in connectivity of the fSR occur within specific regions of the central arborization.

It has been shown that formation of the fSR in *Schistocerca gregaria* begins in the embryo and that the main branches of the mesothoracic projection are present at hatching (Heathcote, 1981). However, only the main branches of the locust SR are present at hatching, and the present findings demonstrate that growth also occurs in the adult.

Functional implications of morphological changes

An increase in the connectivity of the fSR to mesothoracic components of the flight system, suggested by the heteromorphic growth and the increase in the number of swellings, could serve to strengthen existing synapses and thus increase the efficacy of fSR input onto the flight CPG. Increases in connectivity that are associated with developmentally mediated afferent growth have been described in the locust and other animals.

Afferents from ventral filiform hairs on the prosternum of *L. migratoria* are reorganized during development such that the ipsilateral branch of a given axon is lost and the contralateral branch grows (Pflüger et al., 1994). This restructuring is, in part, dependent on the activity of the sensory hair and the contralateral growth is accompanied by an increase in its connectivity to interneurons in the same region of neuropil. In the cat, monocular deprivation experiments have shown that geniculocortical afferents serving the deprived eye are restructured within 1 week, whereas axons serving the non-deprived eye grow normally (Antonini and Stryker, 1993). Physiological effects of monocular deprivation include a decrease in the responsiveness of cortical neurons postsynaptic to geniculocortical afferents serving the deprived eye (Wiesel and Hubel, 1963). Thus, it is clear that neuronal activity can have a significant effect on axonal morphology, which, in turn, is expressed as a change in connectivity to postsynaptic elements.

We have previously shown that the activity of the fSR increases during adult maturation (Gray and Robertson, 1994), and the current study demonstrates heteromorphic changes in the axonal architecture during the same time. It remains to be determined if the morphological changes are related to fSR activity. This would necessitate the inactivation of the fSR in immature animals, perhaps by severing the connective tissue strand that spans the corresponding cuticular components, and examining the axonal morphology 2 weeks later.

Although growth of axonal arbors and increases in the number of swellings may reflect increases in connectivity to postsynaptic elements and an enhanced postsynaptic response, changes in other morphological parameters may, conversely, result in a decrease in synaptic efficacy. Theophilidis (1988) has shown that high-frequency stimulation of the metathoracic slow extensor tibiae (SETi) axon of the long-horned grasshopper, *Decticus albifrons*, results in conduction failure at branch points. Although conduction failure may be a result of changes in the electrical properties of the spikes rather than the morphology of the axon, it occurs predominantly at branch points. Thus, a measure of the number of branch points may reflect the probability of conduction failure. In this context, the increase in the number of branch points of IISR Meso during maturation would indicate an increase in the possibility of conduction failure, and subsequent decrease in synaptic efficacy, within the projection.

Given these seemingly contradictory results, the question remains as to whether growth of the fSR, during adult maturation, will result in changes in synaptic efficacy. Therefore, it is necessary to look at other components of the flight system during maturation. Gee and Robertson (1994) have recently demonstrated that dendritic processes from interneuron 302, a locust flight interneuron that receives input from the fSR, also grows during adult maturation. They predict that growth of these processes should result in a decrease in the amplitude of fSR-evoked excitatory postsynaptic potentials (EPSPs) owing to a decrease in postsynaptic input resistance. However, EPSP amplitudes did not change significantly during maturation, suggesting that some mechanism exists that compensates for a possible decrease in input resistance. Growth of goldfish retinal ganglion cells is accompanied by maintenance of postsynaptic PSP amplitude (Bloomfield and Hitchcock, 1991), possibly owing to an increase in the number of synaptic contacts on large axonal arbors (Hitchcock, 1993). In the cercal sensory system of the cricket, rearrangement of synapses between sensory afferents and interneurons results in a constant output to the CNS (Chiba et al., 1992). From these studies it is clear that changes in afferent morphology can effect changes in connectivity to postsynaptic neurons and thus modify (in these cases maintain) the postsynaptic response. The constancy of fSR-evoked EPSP amplitude in interneuron 302 (Gee and Robertson, 1994) may, therefore, be partly mediated by growth and the increase in the number of swellings in IISR Meso described here.

Maturation changes in fSR morphology may involve processes other than simple growth. Competition for postsynaptic inputs may exist between fully mature afferents. There is competition between the metathoracic branches of the forewing and hindwing tegulae that is removed when the hindwing tegula is ablated, at which time the wingbeat frequency decreases by about 18% (Büschges et al., 1992a,b). The subsequent sprouting of the metathoracic projection of the forewing tegula 2 weeks after hindwing tegula ablation is correlated to a concomitant increase in wingbeat frequency to near pre-ablation levels, suggesting that recovery is mediated by a modification of remaining afferents. The negative allometric growth of IISR Meta relative to IISR Meso may, therefore, reflect competition between IISR Meta and the metathoracic projection of the hindwing stretch receptor (IISR Meta). To test this possibility it is necessary to reconstruct IISR Meta during maturation and then reexamine the mature IISR Meta in animals that have had the hindwing stretch receptor ablated immediately after imaginal ecdysis. Because the fSR sends a single axon into the thoracic ganglia, it would be possible to make detailed comparisons between intact and ablated animals. An increase in the projection volume or total projection length would indicate that there is competition between IISR Meta and IISR Meta for synaptic sites on metathoracic interneurons or motoneurons.

Findings from earlier studies suggest that phasic sensory feedback is not involved in maturation of the locust flight motor pattern (Kutsch, 1974b; Stevenson and Kutsch, 1988). Kutsch and Stevenson (1984) describe changes in the motor pattern during development as involving a distinct switch from an irregular larval pattern to a more rhythmical, phasic adult pattern. However, given the recent findings that during adult maturation there is increased activity of the fSR (Gray and Robertson, 1994), allometric growth of afferent fSR projections and potential increases

in connectivity to flight interneurons (described in this paper), and growth of flight interneurons (Gee and Robertson, 1994), it is clear that developmental processes continue beyond adult emergence. If the morphological changes described here provide a mechanism for maintaining fSR input to the flight CPG, then it is evident that the adult flight motor pattern is not simply a static, preprogrammed oscillatory network, but rather a dynamic system that reacts to extrinsic influences. Indeed, the results of this investigation help to counteract a misconception that the insect nervous system is fully developed before ecdysis to the adult. Therefore, previously established ideas that insect motor patterns are fixed early in development may need to be reevaluated.

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