NEURAL REGULATION OF PUPARIATION IN TSETSE LARVAE

BY JAN ŽĎÁREK[1,2] AND DAVID L. DENLINGER[1,3,*]

[1]*International Centre of Insect Physiology and Ecology, PO Box 30772, Nairobi, Kenya,*
[2]*Insect Chemical Ecology Unit, Institute of Organic Chemistry and Biochemistry,*
Czechoslovak Academy of Sciences, U Šalamounky 41, 15800 Prague 5,
Czechoslovakia, and [3]*Department of Entomology, Ohio State University,*
1735 Neil Avenue, Columbus, OH 43210, USA

Summary

A neural mechanism coordinates pupariation behavior and tanning in the tsetse larva. At parturition, the mature larva has already received sufficient ecdysteroid to commit the epidermal cells to metamorphosis but, before sclerotization and tanning of the cuticle can begin, the larva must first select a pupariation site and then proceed through a stereotypic sequence of pupariation behavior that culminates in the formation of a smooth, ovoid puparium. Both pupariation behavior and tanning are inhibited by the central nervous system (CNS) during the wandering phase. This central inhibition is maintained by sensory input originating in the extreme posterior region of the body. At the transition from wandering to pupariation, the posterior signal that induces inhibition of pupariation behavior is removed and the larva begins the contractions associated with pupariation, but the CNS inhibition of tanning persists. At this point, separation of the body into two halves by ligation or nerve transection prevents tanning of the anterior half (containing the CNS), whereas the denervated integument of the posterior half tans completely. Transection of nerves to the midline of the body produces larvae with a tanning pattern that ends abruptly along a sagittal plane, implying that the central control of this process is uncoupled between the left and right regions of the CNS. A few minutes later, when the final shape of the puparium is completed, the CNS inhibition is lifted and the tanning process begins. At this time, separation of the body into two halves by ligation or nerve transection has no inhibitory effects on either part. Exogenous ecdysteroids fail to release the CNS inhibition, and hemolymph containing the pupariation factors from *Sarcophaga bullata* have no accelerating effects on tsetse pupariation. These results imply that regulation of metamorphosis in the insect integument is not the exclusive domain of blood-borne hormones.

Introduction

The onset of metamorphosis in the higher Diptera is marked by formation of the puparium. The third-instar larva stops feeding, wanders for a certain period, and then

---

becomes immobile, retracts the anterior segments and contracts the body into a smooth, ovoid shape. This process of pupariation (puparium formation) is followed by tanning, a biochemical event that renders the cuticle hard and dark. Thereafter, the larva molts to the pupal state and differentiates into an adult fly within the hard shell formed by the larval cuticle. Pupariation in flies provided some of the first evidence that insect metamorphosis is regulated by hormones (Fraenkel, 1934). Fly larvae ligated precritically (i.e. before release of a factor from the anterior region of the body), usually pupariate only in the front half of the body. Fly ganglia are fused into a ventral nerve mass that lies directly behind the brain so that ligation at the mid-body renders the hind part devoid of any central innervation or hormonal supply from the associated glands. The isolated hind part can be stimulated to pupariate by the injection of active hemolymph from the front half of the body. It was this response in *Calliphora erythrocephala* that served as the bioassay for the chemical identification of molting hormone (ecdysone and 20-hydroxyecdysone) (Butenandt and Karlson, 1954). The pupariation response thus contributed to the development of the classic scheme for regulation of metamorphosis by demonstrating the role of ecdysteroids in initiating this process. It is now clear that ecdysteroids do not act alone. Although 20-hydroxyecdysone commits the cells to metamorphosis, the final orchestration of pupariation is accomplished by a suite of neurohormones that are responsible for specific aspects of pupariation such as immobilization, retraction of the anterior segments and tanning (see review by Žďárek, 1985).

However, certain results in the fly literature cannot be readily explained. In *Sarcophaga bullata* (Ratnasiri and Fraenkel, 1974*a,b*) and *Calliphora erythrocephala* (Price, 1970) a significant portion of post-critically ligated larvae pupariate in the hind half but fail to do so in the front half containing the brain and ring gland, and in *Glossina morsitans* (Langley, 1967; Whitehead, 1974) and *Laspesia archippivora* (Chang, 1972) this is the dominant response. In this study, we reinvestigate this curious phenomenon in the tsetse fly *Glossina morsitans*. We experimentally separated nervous and hormonal action by a new technique of non-invasive nerve severance and provide the first direct evidence that the nervous system contributes to the coordination of pupariation.

Material and methods

Larvae used for our experiments were taken from the ICIPE colony of *Glossina morsitans* Westwood, which was maintained at 25 °C with a daily 12 h photophase. Aborted larvae of the third (last) instar used in the experiments were expressed from the female by gently squeezing the female's abdomen. Larvae were ligated posterior to the CNS with silk thread at different times during the third instar. Nerves innervating the posterior part of the body were severed by compressing the body with a blunt blade. Dissection revealed that this non-invasive technique severed the nerves and other underlying tissues. To inflict injury on the integument but not the underlying nerves and other tissues we used a hot wire to cauterize the girth of the larvae or a pair of sharp forceps (Dumont no. 5) to destroy the epidermis mechanically. A solution of 20-hydroxyecdysone (Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia) dissolved in 1 µl of 10 % ethanol was injected into larvae through a

finely drawn glass capillary. Capillaries were also used for transfer of hemolymph between larvae of the tsetse fly and the flesh fly *Sarcophaga bullata* (Parker). Flesh fly larvae were reared on beef liver as described by Denlinger (1972). Prior to injection, the tsetse larvae were immobilized on ice for 30 min.

Results

The tsetse life cycle differs significantly from that of most flies in one important way. While most species of flies have a free-living larval stage, larval development in the tsetse is completed within the body of the female (Denlinger and Ma, 1974; Tobe and Langley, 1978). The female ovulates a single egg at a time and that egg is retained within her uterus. The egg hatches there and the larva feeds on nutriment supplied from the mother's milk gland, a highly modified accessory gland. At the end of the third instar the larva is deposited (larviposition, parturition) and, after a brief (1–2 h) free-living existence (the wandering period), it pupariates. No feeding occurs after the larva has been deposited, and during this wandering period the larva burrows into the soil. The wandering period can be prolonged or shortened by environmental stimuli (Žďárek and Denlinger, 1991) but, once pupariation behavior begins, the larva progresses without interruption through a stereotypic sequence of activities that includes retraction of the anterior segments, longitudinal contraction and smoothing of the body as a result of cuticular shrinkage.

Like other fly larvae (e.g. Fraenkel, 1934), the ganglia of tsetse are fused into a ventral nerve mass located directly posterior to the brain. Thus, the CNS and associated glands are localized in the anterior part of the body and can be easily isolated by ligation and other severance techniques.

Ligation of aborted larvae

Larviposition in tsetse is a gated response (Denlinger and Ma, 1974; Denlinger and Žďárek, 1991), and larvae with black polypneustic lobes that are mechanically expressed from the female after the daily peak of larviposition would not normally be deposited until the following day. In this experiment, larvae with black polypneustic lobes were aborted 4 h after the mean larviposition time, and if they did not pupariate within 4 h of abortion they were classified as stage III (Denlinger and Žďárek, 1991) and ligated posterior to the CNS. Cuticular changes were observed for 2 days after ligation (Figs 1A, 2A). All larvae tanned in front of the ligation but none did so in the hind part. However, tanning in the anterior part was not completely normal: the cuticle was not intensely tanned and remained rather soft, possibly due to the thinness of the endocuticle that was deposited by the time of abortion. This observation of tanning in the anterior half but not in the posterior half is thus consistent with the classic scheme: the region containing the CNS and associated glands can proceed with development while the hind part, devoid of the CNS, fails to do so.

Ligation of normally deposited larvae

The response was different in larvae ligated at later stages of development. Larvae
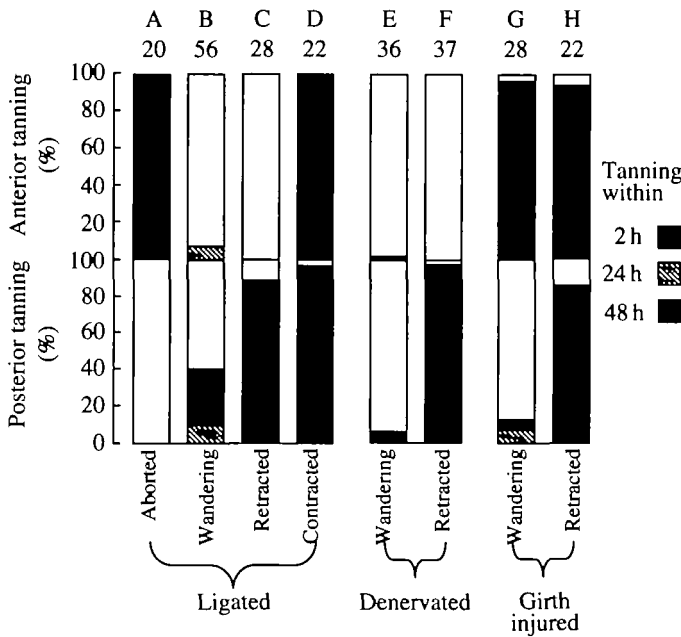
Fig. 1. Percentage of tsetse larvae tanning in the anterior or posterior portion of the body after being ligated, denervated or subjected to a complete band of epidermal cells injury around the girth of the larva. All manipulations were performed at mid-body (behind the CNS) using mechanically aborted third (final) instar larva, wandering larvae that had been deposited normally, larvae that had stopped wandering and had begun to retract their mouthparts or larvae that were already contracting to form a puparium. Cuticular tanning was scored 2, 24 and 48 h after the manipulation. Sample size is indicated above each column.

ligated during the wandering period (less than 15 min after deposition) seldom showed signs of pupariation in either half of the body within 2 h (Fig. 2B). Some tanning occurred in the posterior part 24–48 h later (Fig. 1B). As larvae aged, the effect of ligation changed. When ligated after immobilization and retraction of the anterior segments (Žďárek and Denlinger, 1991), the majority of larvae tanned only in the hind part (Figs 1C, 2C). Even if the ligature was placed very far back on the larva (last segment in front of the polypneustic lobes) most (86 %, $N=21$) failed to tan anterior to the ligature. When the ligature was applied still later (larvae were already barrelled but still soft enough to allow the ligature to be tightened), the larvae invariably pupariated in both parts of the body within 2 h (Figs 1D, 2D). These results imply that a developmental transition occurs after the larva is deposited, resulting in the establishment of an inhibition that prevents the onset of cuticular tanning in the region containing the CNS. The presence of tanning in both halves of larvae ligated during the barrelling process indicates that the inhibition has been lifted by that time.

Non-invasive severance of nerves

To determine whether the inhibition we observed with ligation was due to nervous or humoral conduits, we destroyed the neural connections but not the humoral channels
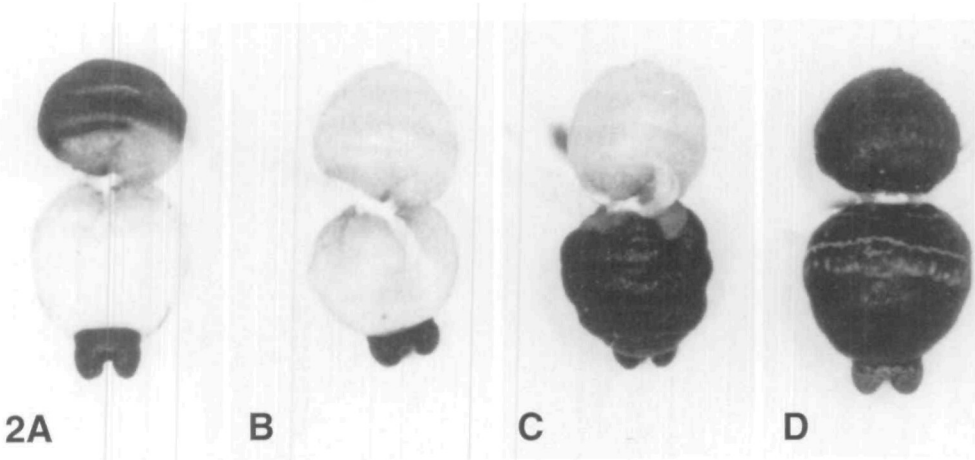
Fig. 2. Representative pupariation responses in tsetse larvae ligated at mid-body. Third-instar larvae dissected from the uterus of the female and ligated immediately usually pupariate only in the front half (A); most larvae ligated shortly after natural deposition, while still in the wandering stage, fail to pupariate in either half (B); older larvae that have stopped wandering and begun to retract their mouthparts pupariate only behind the ligature (C); larvae that are older still, those ligated when they have already begun to contract their body, pupariate on both sides of the ligature (D). Photographs were taken 24–48 h after ligation.

between the two parts of the body by severing the nerves (and other internal organs) with a blunt blade firmly applied to the cuticle. When this manipulation was performed at mid-body on wandering larvae of *G. m. morsitans*, the hind part was immediately paralyzed and practically no tanning was observed in either part of the body (Fig. 1E). In contrast, when the same operation was performed on larvae that had already retracted their anterior segments, most larvae tanned completely in the hind part, but no tanning was observed in the front part (Figs 1F, 3A). These experiments show, in both cases, that nerve severance elicited the same response as ligation (compare Fig. 1B and C with Fig. 1E and F), even though the humoral channels remained intact. This suggests that the inhibition of tanning was due to disruption of neural communication between the periphery and the CNS.

When nerve severance was performed on the last abdominal segment (immediately anterior to the black polypneustic lobes) of either wandering (*N*=24) or retracted (*N*=17) larvae, no tanning occurred anterior to the nerve severance. This suggests that intact innervation of the polypneustic lobes is essential for providing the sensory information needed to initiate tanning in the regions of the integument innervated by the CNS.

When the nerves were severed only on one side of the body, in both wandering and retracting larvae, tanning in the front part of the corresponding side was inhibited, whereas the contralateral half tanned normally (*N*=25). As a result, the puparium remained untanned in one quadrant, and consistently the boundary between the tanned and untanned cuticle was well defined (Fig. 3B). If the blade used in this procedure inflicted injury across less than half of the body, or if the procedure was used on the ventral or dorsal half of the body, no inhibition was observed. The entire cuticle tanned normally. Thus, nerve severance to the midline of the body was essential to obtain
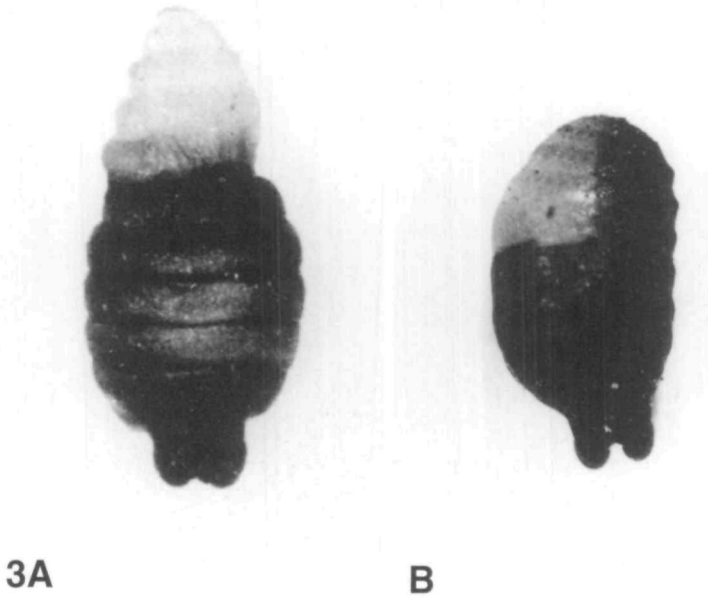
3A   B

Fig. 3. Pupariation in tsetse larvae in response to non-invasive nerve severance across the entire mid-section of the body (A) or in only the left side of the body (B). Nerves were severed shortly before the normal onset of pupariation, when the larvae were beginning to retract their mouthparts.

inhibition in the quadrant anterior to the point of injury, and complete nerve severance across the entire width of the body was required to inhibit tanning in the anterior half.

At the microscopic level, it is clear that the transition between tanned and untanned cells in the sagittal plane is abrupt. Fully tanned cells were adjacent to cells that were completely inhibited, and very few cells showed an intermediate response (Fig. 4).

Severance of the body

When the larval body was cut into two halves and the wound sealed with Parafilm®, the isolated hind part invariably pupariated if the operation was performed on retracted larvae ($N=19$). If the body was severed earlier, i.e. during the wandering stage, neither half pupariated ($N=15$). Whole-body severance thus produced the same effect as ligation or denervation.

Injury of the integument

To eliminate the possibility that our procedure for severing the nerves disrupted some form of surface communication operating across the epidermal cells, we also cauterized or, using a pair of fine forceps, mechanically destroyed a band of cells encircling the body at mid-region. When the operation was performed using a hot wire, only anterior parts tanned in wandering larvae (Figs 1G, 5), while both parts did so in retracting larvae (Fig. 1H). Tanning in cauterized wandering larvae was greatly accelerated in the anterior part (31.4 ± 1.2 min, $N=14$) in comparison to intact controls (99.3 ± 11.2 min, $N=14$).

Cauterized larvae never crawled, but the anterior segments performed vigorous retractory movements and finally became convulsively contracted, giving the body a 'pear-shape' appearance. Posterior to the cautery the body remained immobile.

Mechanical girth injury inflicted by pinching the epidermal cells with forceps produced essentially the same pupariation response as girth cautery: 86 % (N=14) of the wandering larvae pupariated only in the front region, and 92 % (N=12) of retracted larvae pupariated in both parts. If the circumferential injury to the epidermis was as far back as possible (i.e. close to the hind spiracles) tanning anterior to the injury was the predominant response in all larvae, regardless of age. These observations suggest several conclusions. First, the
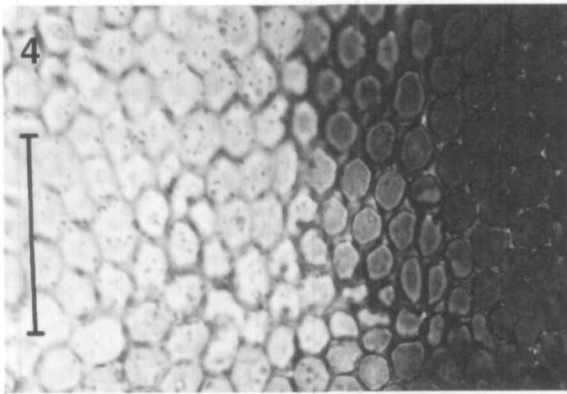

Fig. 4. Microscopic view of the epidermal cells showing the abrupt transition between cells on the right that show the pupariation response (tanning) and cells on the left that remain inhibited. Scale bar, 0.1 mm.
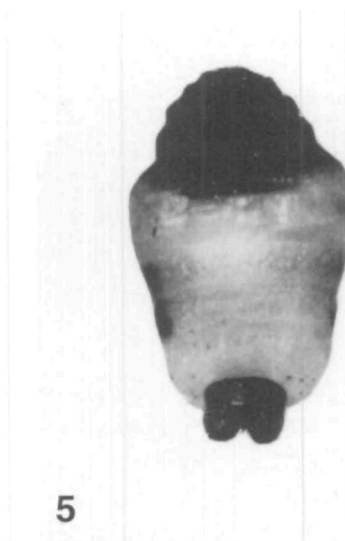

Fig. 5. Pupariation response in a tsetse larvae that had a band of epidermal cells mechanically injured by cautery during the wandering stage.

accelerated pupariation of the body anterior to the girth injury can be the result of a strong mechanical or thermal stimulus, a feature well recognized from our earlier study (Žďárek and Denlinger, 1991). Second, the intense neuromuscular activity that occurs in response to girth injury suggests that hyperactivity of the CNS is involved in the accelerated pupariation. And, third, failure of girth injury to prevent tanning anterior to the site of injury implies that the sensory information from the posterior part of the body that lifts the anterior inhibition (compare Figs 1C and 1H) is not transmitted across the epidermis by cell to cell communication.

Injury of the polypneustic lobes

To evaluate the role of the polypneustic lobes as a possible source of sensory information in the posterior region, we injured the lobes of wandering and contracting larvae by applying a drop of very hot olive oil with an electrically heated wire. This 'wet cautery' of the lobes inhibited tanning in larvae of all ages (84 % inhibition in wandering larvae, $N=31$; 85 % inhibition in retracted larvae, $N=27$). Wandering larvae responded by contracting convulsively, while the older larvae formed folded and contracted puparia that did not tan immediately, although some of them did so 24 h later. Control larvae that received a drop of cold olive oil pupariated normally ($N=10$). Damage to this area thus caused the same inhibitory effect as denervation or ligation of wandering or retracted larvae (compare with Figs 1B,C,E,F).

Effects of hormonal agents

To test the possibility that ecdysteroid levels were insufficient to stimulate tanning in the untanned regions, we injected 10 μg of 20-hydroxyecdysone into wandering larvae before ligation. Injection of this large dose of 20-hydroxyecdysone did not increase the incidence of pupariation in either part of the body (Table 1). Likewise, an injection of 20-

Table 1. *Failure of a 10 µg injection of 20-hydroxyecdysone to increase the incidence of tanning in ligated or denervated tsetse larvae*

Larval treatment	N	Percentage tanning in 48 h
Ligated wandering larvae		
Anterior half		
Untreated control	26	26.9
20-Hydroxyecdysone	27	22.2
Posterior half		
Untreated control	26	61.5
20-Hydroxyecdysone	27	55.6
Denervated retracting larvae		
Anterior half		
Untreated control	37	0
20-Hydroxyecdysone	21	4.8
Posterior half		
Untreated control	37	97.8
20-Hydroxyecdysone	21	90.5

hydroxyecdysone failed to promote pupariation in the front parts of retracted larvae whose posterior nerves had been severed.

To investigate the possible involvement of pupariation factors (Žďárek, 1985) in controlling pupariation we injected 1 µl of active hemolymph from freshly formed (30–60 min old) 'orange puparia' of *Sarcophaga bullata* into wandering *Glossina* larvae that had been immobilized on ice for 30 min. The injected hemolymph contained a high titer of the pupariation accelerating factors that are known not to be species-specific (Žďárek, 1985). The injected hemolymph did not affect the speed of pupariation when the larvae were removed from ice. The mean time of tanning for larvae injected with the *Sarcophaga* hemolymph was 16.9±1.1 min (*N*=15), which did not differ from the tanning time of sham (1 µl of Ringer's solution) injected controls (17.1±6.9 min). Thus, the neurohormonal pupariation factors contained in *Sarcophaga* hemolymph were not effective in altering the pupariation rate of *Glossina* larvae.

Discussion

Critical period for molting hormone

A traditional approach to the study of hormonal control of fly pupariation is based on ligation of the third-instar larvae and subsequent evaluation of cuticular tanning. When a ligature is used to divide the body, the hind part (which lacks the CNS and ring gland) tans only if the larva has developed beyond the 'critical period' for molting hormone action (Fraenkel, 1934; Fraenkel and Žďárek, 1970). The molting hormone, ecdysone, is believed to be released from the endocrine centers (the ring gland) in the front part of the body (Possompes, 1953; Žďárek and Fraenkel, 1971). In most cyclorrhaphous genera (e.g. *Sarcophaga, Calliphora* or *Musca*) the critical period occurs 8–12 h before pupariation. But in a study with *Glossina* (Langley, 1967), larvae ligated after parturition invariably tanned in the posterior part only, and no indication of tanning was noted in the anterior part. Langley inferred from this observation that the larva had already passed the critical period for ecdysone by the time of parturition and that the CNS was capable of inhibiting the action of the hormone in the anterior half. This explanation was supported by Whitehead (1974), who observed anterior tanning in some ligated larvae that were expelled from the female prematurely.

Our study fully confirms Whitehead's observation that aborted and ligated third-instar larvae tan in the front part only. The tanning, however, occurred after a considerable delay and was less intense than in normally deposited larvae. The pupariation response we observed in larvae ligated after parturition was not as uniform as reported by Langley (1967). The response varied with larval age. Some larvae ligated during the wandering period did tan only in the posterior half, but usually with a considerable delay. Many wandering larvae did not tan at all. A more uniform response was observed in larvae that were ligated later, after they had already switched from wandering behavior to pupariation behavior (see Žďárek and Denlinger, 1991, for details of staging). Most larvae that were in the immobilization or retraction phase tanned only in their hind parts

and they did so within 2 h (Fig. 1C and F). Only those that had advanced to the stage of cuticular shrinkage by the time of ligation pupariated in both parts of the body (Fig. 1D).

These results suggest two conclusions. First, the critical period for molting hormone action must occur when the larva is still within the uterus, presumably several hours before parturition. This is supported by the fact that ligated hind parts of normally deposited larvae can tan (e.g. Fig. 1B,C,F) but those of premature larvae cannot (Fig. 1A). This is consistent with our previous results (Denlinger and ŽĎárek, 1991) showing that larvae expelled up to 8 h before the expected time of parturition can pupariate within 2 h of abortion, suggesting that the hormonal conditions for initiation of metamorphosis had been attained by that time. Second, the age-dependent variation in the effect of ligation on normally deposited larvae implies that still other controlling mechanisms, presumably those involving the CNS, are effective during the post-parturition period.

Why does the ligated anterior half not pupariate?

Failure of the front part of post-critically ligated larvae to pupariate has been observed in several flies, either occasionally *(Sarcophaga, Calliphora)* or on a regular basis *(Glossina, Lespesia)*. Ratnasiri and Fraenkel (1974a,b) and Chang (1972) suggest that this is caused by a shortage of oxygen preventing phenolic tanning. Whitehead (1974) rules out this explanation for *Glossina* by arguing that in tsetse larvae, which have no anterior spiracles, pupariation in the front part can indeed occur in premature larvae, and he favors Langley's (1967) suggestion that the CNS acts as an inhibitory center. Whitehead further suggests the existence of humoral factors 'produced in the abdomen' that can reverse the neural inhibition. Our experiments were designed to clarify this complicated situation in *Glossina*.

Humoral versus neural control

We have shown that the innervated front part of the larval body fails to pupariate if it is separated from the hind part either by ligation (Fig. 1B,C) or by severance of the abdominal nerves (Fig. 1E,F) at any stage of larval life after parturition, except for a very brief period late in development when shrinkage of the cuticle has already started (Fig. 1D). This suggests the presence of communication channels between the periphery and the CNS carrying both stimulatory and inhibitory messages. Such messages could be either neural or humoral. By non-invasively severing the nerves leading from the CNS we were able to distinguish between these two possibilities. Wandering larvae with neural communication interrupted but humoral channels intact did not tan in either part of the body, but similar denervation performed on older, retracted larvae prevented tanning only in the front part (Fig. 3A). The possibility that cell to cell communication by the epidermal cells is the information channel was eliminated by our failure to disrupt the communication by destruction of a band of epidermal cells. We conclude that this inhibitory action of the CNS is achieved through intact nerves.

Experiments involving partial (monolateral) denervation provided further evidence for the involvement of nerves rather than humoral factors. Consistently, only the hind (denervated) portion of the operated side of the body tanned, whereas the cuticle in front

remained completely inhibited. The contralateral half of the body, with all nerves intact, tanned both at the front and at the back (Fig. 3B). The fact that the segregation between affected and unaffected cells in tsetse is so precise along the sagittal plane implies that the central control of this process is uncoupled between the left and right regions of the CNS. Each side apparently directs the response independently in one half of the body. When nerves are severed across less than half of the body, or if only the nerves in the ventral or dorsal half of the body are destroyed, pupariation can proceed. Thus, as long as some of the nerves on the left or right side of the body remain intact, the communication channel is operative.

The abrupt transition that is observed between tanned and untanned epidermal cells (Fig. 4) further eliminates the possibility that a blood-borne factor is involved in regulation of the cuticular changes. The process thus appears to be under direct neural control or possibly the nerves direct local release of a humoral factor from the nerve endings. Anatomical evidence has shown the extensive innervation of the epidermal cells and neurosecretory release sites are abundant in this area (Finlayson, 1972).

Temporal model

The posterior parts of ligated or denervated larvae readily tanned only when the severance was performed after the wandering period was over. This means that the abdominal integument must have been capacitated for puparial changes but that the actual event has been inhibited by the output of efferent nerves. Denervation of younger wandering larvae prevented pupariation in the whole body, suggesting that the signal activating the CNS to switch from larval wandering behavior to pupariation behavior and to activate the integument must originate from the posterior part of the body and must be neural. Because destruction of the last abdominal segment, including the spiracular lobes, inhibited pupariation in the rest of the body, we propose the existence of some sensory signal stimulation originating from this region. A variety of environmental stimuli can either terminate wandering behavior (e.g. pinching, lack of contact with the substratum, contact with ice) or prolong wandering (contact with water) (Finlayson, 1967; Žďárek and Denlinger, 1991), suggesting that this developmental switch is responsive to environmental conditions.

The neural mechanisms controlling pupariation in *Glossina* thus include (1) early sensory input to the central neurons from the posterior part of the body, a feature that is essential for termination of the wandering period, and (2) inhibitory effects of the CNS on the integument imposed on individual epidermal cells by different neurons. Our experimental results allow us to draw some conclusions about the timing of those regulatory signals.

The overall sequence of events involved in regulation of pupariation in *Glossina* is shown diagrammatically in Fig. 6. As a result of ecdysteroid action potentiated by the intake of food, the various larval tissues become committed to metamorphosis towards the end of the last larval instar, while the larva is still within the mother's uterus (Denlinger and Žďárek, 1991). After reaching this stage of ecdysteroid-induced competence for metamorphosis (sometimes called the 'critical period'), the larva waits for the next daily parturition gate to open, an event that occurs late in the photophase
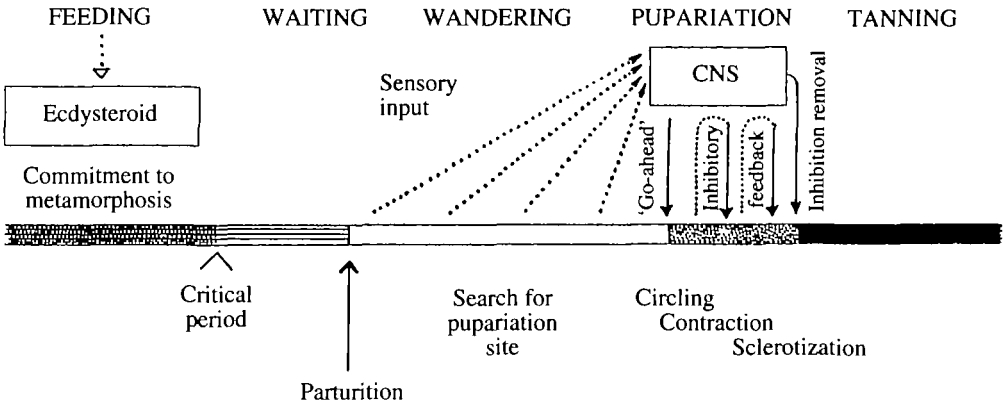
Fig. 6. Schematic representation of the sequence of events involved in regulation of pupariation and tanning in *Glossina morsitans*. The time line begins with the third-instar larva's commitment to metamorphosis and progresses through a period of waiting for the parturition gate, parturition, the wandering phase during which the larva seeks a pupariation site, pupariation, and culminates in tanning of the puparium. The commitment to metamorphosis is achieved in response to ecdysteroid and this, in turn, is dependent upon a period of feeding during the third instar. Sensory input directed to the CNS culminates in the release of a 'go-ahead' signal that initiates pupariation behavior, but the final events of sclerotization and tanning cannot proceed until CNS inhibition is removed. (See text for further details.)

(Phelps and Jackson, 1971; Denlinger and Ma, 1974; Ždárek *et al.* 1992). Following parturition, the newly deposited larva enters the wandering period and searches for a suitable pupariation site. During this period, the CNS of the larva is sensitive to posterior sensory stimulation that can affect the duration of this period of mobility. Certain not-yet-identified stimuli (or lack thereof) induce the CNS to release a 'go-ahead' signal and, in response, the larva switches from wandering behavior to pupariation behavior (Ždárek and Denlinger, 1991). A neural inhibition that prevents sclerotization and tanning persists but, meanwhile, the larva rotates in a circle, contracts and undergoes the complex morphological changes associated with the transformation of a soft larval cuticle into a smooth, ovoid puparium. Only then is the neural inhibition lifted so that the sclerotization process can begin. During pupariation, the CNS presumably receives feedback sensory information about the progress of puparial morphogenesis, and on this basis determines when to permit sclerotization. Only during a relatively short interval (i.e. between the 'go-ahead' signal and the lifting of inhibition) will the larval cuticle pupariate without having intact connections with the CNS. This mechanism ensures the proper timing of sclerotization and tanning with respect to the events of puparial morphogenesis.

Hormonal effects

The ineffectiveness of 20-hydroxyecdysone to increase pupariation in both ligated wandering larvae and in the front parts of denervated retracted larvae supports our previous conclusion that the larva has already passed the critical period for ecdysone action at the time of parturition, and that the failure to pupariate is not caused by molting hormone deficiency. The blood-borne pupariation factors that are active in the

hemolymph of *Sarcophaga* (Žďárek, 1985) were ineffective in *Glossina*. It is this hormonal input that appears to be missing in tsetse and is replaced by a system of neural control. How widespread this reliance on the nervous system may be is unknown, but the scattered reports, especially in flies, of anterior inhibition in ligated larvae (Price, 1970; Chang, 1972; Ratnasiri and Fraenkel, 1974*a*,*b*) suggest that a similar mechanism may be operating in other species. Our observations with tsetse suggest caution in assuming that the integumental transformation at metamorphosis is directed solely by blood-borne hormonal factors.

References

BUTENANDT, A. AND KARLSON, P. (1954). Über die Isolierung eines Metamorphose-Hormons der Insekten in Kristallisierten Form. *Z. Naturf.* 9b, 389–391.

CHANG, F. (1972). The effect of ligation on tanning in the larva of a tachinid parasite, *Lespesia archippivora. J. Insect Physiol.* 18, 729–735.

DENLINGER, D. L. (1972). Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). *Biol. Bull. mar. biol. Lab., Woods Hole* 142, 11–24.

DENLINGER, D. L. AND MA, W.-C. (1974). Dynamics of the pregnancy cycle in the tsetse *Glossina morsitans. J. Insect Physiol.* 20, 1015–1026.

DENLINGER, D. L. AND ŽĎÁREK, J. (1991). Commitment to metamorphosis in tsetse: temporal, nutritional and hormonal aspects of the decision. *J. Insect Physiol.* 37, 333–338.

FINLAYSON, L. H. (1967). Behaviour and regulation of puparium formation in the larva of the tsetse fly *Glossina morsitans orientalis* Vanderplank in relation to humidity, light and mechanical stimuli. *Bull. ent. Res* 57, 301–313.

FINLAYSON, L. H. (1972). Chemoreceptors, cuticular mechanoreceptors and peripheral multiterminal neurones in the larva of the tsetse fly (*Glossina*). *J. Insect Physiol.* 18, 2265–2276.

FRAENKEL, G. (1934). Pupation of flies initiated by a hormone. *Nature* 133, 334.

FRAENKEL, G. AND ŽĎÁREK, J. (1970). The evaluation of the 'Calliphora test' as an assay for ecdysone. *Biol. Bull. mar. biol. Lab., Woods Hole* 139, 138–150.

LANGLEY, P. A. (1967). Effect of ligaturing on puparium formation in the larva of the tsetse fly *Glossina morsitans* Westwood. *Nature* 214, 389–390.

PHELPS, R. J. AND JACKSON, P. J. (1971). Factors influencing the moment of larviposition and eclosion in *Glossina morsitans orientalis* Vanderplank. *J. ent. Soc. Sth Afr.* 34, 145–157.

POSSOMPES, B. (1953). Recherches experimentales sur le determinisme de la metamophose de *Calliphora erythrocephala* Meig. *Archs Zool. exp. gen.* 89, 203–364.

PRICE, G. (1970). Pupation inhibiting factor in the larva of the blowfly *Calliphora erythrocephala. Nature* 228, 876–877.

RATNASIRI, N. P. AND FRAENKEL, G. (1974*a*). Anterior inhibition of pupariation in ligated larvae of *Sarcophaga bullata* and other fly species: incidence and expression. *Ann. ent. Soc. Am.* 67, 195–203.

RATNASIRI, N. P. AND FRAENKEL, G. (1974*b*). The physiological basis of anterior inhibition of puparium formation in ligated fly larvae. *J. Insect Physiol.* 20, 105–119.

TOBE, S. S. AND LANGLEY, P. A. (1978). Reproductive physiology of *Glossina. A. Rev. Ent.* 23, 283–307.

WHITEHEAD, D. L. (1974). The retardation of puparium formation in Diptera: Could factors other than ecdysone control cuticle stabilization in *Glossina* and *Sarcophaga* species? *Bull. ent. Res.* 64, 223–240.

ŽĎÁREK, J. (1985). Regulation of pupariation in flies. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8 (ed. G. A. Kerkut and L. I. Gilbert), pp. 301–303. Oxford: Pergamon Press.

ŽĎÁREK, J. AND DENLINGER, D. L. (1991). Wandering behavior and pupariation in tsetse larvae. *Physiol. Ent.* **16**, 523–529.

ŽĎÁREK, J., DENLINGER, D. L. AND OTIENO, L. H. (1992). Does the tsetse parturition rhythm have a circadian basis? *Physiol. Ent.* **17**, 305–307.

ŽĎÁREK, J. AND FRAENKEL, G. (1971). Neurosecretory control of ecdysone release during puparium formation in flies. *Gen. comp. Endocr.* **17**, 483–489.