# CORRELATED EFFECTS OF ECDYSONE AND NEUROSECRETION IN PUPARIUM FORMATION (PUPARIATION) OF FLIES*

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*Abstract.*—A neurohormone accelerates the formation and tanning of the fly puparium in the presence of ecdysone. When larvae of *Phormia regina* are divided by ligation into two parts before the critical period, the hind part remains untanned, unless injected with blood from a pupariating larva. This confirms earlier observations and is in contrast to recent data with another genus. When *Sarcophaga bullata* larvae are ligated after the critical period, the anterior part starts to tan about two hours before the posterior part. Injecting neurosecretory material from the brain, the pars intercerebralis, or the corpora cardiaca of *S. bullata*, or from the brain or c. cardiaca of *Periplaneta* or *Pyrrhocoris* into the posterior part induces it to tan before the anterior part. Injection of the same material into normal larvae after the critical period similarly accelerates puparium formation and onset of tanning. Injection of ecdysone or ecdysterone does not have these effects. Thus, the neurosecretory material does not act by causing the release of ecdysone from the ring gland and its function is different from that of the classical brain ("activating") hormone. When injected before the critical period together with ecdysone it potentiates the effect of ecdysone. It is suggested that its biological significance is to coordinate puparial contraction and tanning.

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Pupariation in flies proceeds in two steps: The larva first contracts to form the barrel shape of the puparium; then the cuticle hardens and darkens by phenolic tanning. That this latter process is induced by a hormone (now known as ecdysone) was demonstrated by one of us 35 years ago[1] in the following manner: Larvae of *Calliphora erythrocephala* were divided into two parts by ligating before a critical period. The hind parts, which under these conditions remain untanned, were induced to tan by injecting hemolymph from pupariating larvae. This experiment subsequently became the assay method by which ecdysone was isolated.[2] It is still used today with little modification as the standard test (the "Calliphora test") for demonstrating and assaying ecdysone (e.g., refs. 3 and 4). The validity of the original experiment was questioned by Ohtaki *et al.*,[5] who failed to confirm it with a member of a different family, *Sarcophaga peregrina*. They also used a different technique. They were apparently unaware of two publications—one giving experiments with *C. erythrocephala*[6] and the other with *Didacus ciliatus*[7]—which had fully confirmed the old data and included the proper negative controls that the early papers had omitted.

In the present paper we shall demonstrate that the original experiment can be readily reproduced with *Phormia regina* and describe a concurrent effect of a neurohormone in pupariation which has to be considered when assessing the action of ecdysone in this process.

*Materials and Methods.*—The experiments on the interaction of ecdysone and neurosecretion were performed with *Sarcophaga bullata* Parker and *Phormia regina* L. The former species, like other species of this genus, shows clear symptoms of tanning previous to pupariation. Three to four hours before this event the cuticle between and around the hind spiracles begins to tan. The spiracles are thus surrounded by a gradually enlarging and darkening brown spot which is easily visible in the crawling larvae 2 hr before pupariation. Thus, incipient pupariation is recognizable up to 4 hr before the event, well past the critical period after which the hind part always pupariates despite ligaturing.

The larvae were immobilized for ligating or injecting by being placed on ice. Injections were made with finely drawn glass pipettes with a diameter of about 40 $\mu$ at the tip, manufactured with the aid of a Micropipette Puller MI (Industrial Science Associates, Inc., Ridgewood, N.Y.). Pressure was applied by mouth via a piece of rubber tubing fixed to the pipette. Amounts injected were estimated by comparison with a calibrated length of the same tubing. Extracts were prepared by grinding tissues in a Potter-Elvehjem homogenizer in Insect Ringer solution and centrifuging them at low speed (less than 2000 *g*) for 5 min. The supernatant was injected. The blood of larvae and puparia was transferred directly from the donors to the test hind parts. The blood of adult flies was collected by the method described earlier.[8]

*Results.*—(a) *Induction of tanning in the ligatured hind part by injection of hemolymph:* In view of the criticisms mentioned above, the experiment[1] was repeated, following the original method but using *Phormia regina* (in the absence of a culture of *C. erythrocephala*). The original results were fully confirmed. The number of tanned hind parts was $^2/_{16}$ (13%) with injection of 3 $\mu$l of hemolymph, $^7/_{24}$ (29%) with that of 5 $\mu$l hemolymph, and $^{11}/_{23}$ (48%) with that of 8–10 $\mu$l hemolymph. A similar experiment run with *Sarcophaga bullata* gave negative results, confirming those with *S. peregrina*[5] in which a different method was used. Details of these experiments will be published elsewhere.

(b) *Role of ecdysone and neurosecretion in tanning and pupariation:*

(1) *Timing of tanning in ligatured anterior and posterior sections:* When larvae of *Sarcophaga bullata* are ligated after the critical period, both halves ultimately tan. The anterior part invariably begins to tan before the hind part. Figure 1 shows the time difference in tanning of the two parts in larvae ligatured at different ages after the critical period, which had been found to be 10–12 hours in this species. Independent of the intervening period between ligaturing and tanning, the posterior part lags by about two hours. In this experiment, larvae from one rearing were ligatured at the same time and after most of them had passed the critical period. In another experiment, larvae at the beginning of

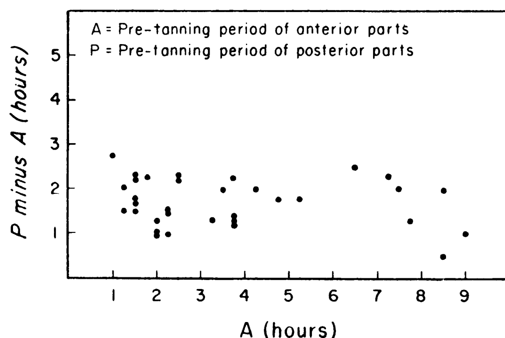


FIG. 1.—*Sarcophaga bullata* ligatured after the critical period. The length of time between ligaturing and onset of tanning in the anterior part (*abscissa*) is plotted against the difference in the length of time between beginning of tanning in the posterior and anterior parts (*ordinate*). Each dot represents a single larva.

the red-spiracle stage were selected from a culture at the same time and divided randomly into two lots, one of which was ligatured. The ligated anterior parts started to tan at about the same time as the nonligated controls.

The ligated hind part is lacking in the ring gland (which is the source of the hormones) and the central nervous system, which in fly larvae is fused into a single anterior mass. Disconnection from the central nervous system leads to tanning without a puparial contraction but is not the cause of the delay in tanning. When a ligature is tightly applied and immediately removed, both sections tan simultaneously (Table 1, expt. 1) although the shape of the puparium is highly abnormal. In this case the anterior part is contracted while the posterior part is paralyzed (because of the nervous separation from the central nervous system) and tans in the shape of the larva. Therefore, the delay in tanning behind the ligature is due to the absence of a blood-borne factor produced in the front part. Further experimentation led to the discovery of an effect of neurosecretion on the onset of pupariation. Many of the experiments to be described in the following sections are summarized in Table 1. We used the following assay method.

Larvae of *Sarcophaga bullata* were ligated three to four hours before they would normally have pupariated, and the posterior part was injected as described above. The time from ligating to the first signs of tanning in the anterior ($A$) and posterior ($P$) parts was recorded (pretanning period). Each experiment comprised 10 to 15 specimens. The effect which injection had on the onset of tanning was expressed in two different ways. $P/A$ represents the quotient of the mean pretanning period in the injected posterior and noninjected anterior parts. $P - A$ represents the difference in the pretanning period in the two sections. Since the noninjected front part pupariates at about the same time as the nonligated control, it also serves as a negative control to the injected hind part. As already mentioned, the noninjected hind part tans one to two hours after the front part. Thus, $P/A > 1$, and $P - A$ is a positive value.

(2) *Effect of ecdysone on the onset of tanning in the hind part of larvae ligated after the critical period:* It was at first supposed that a delay in pupariation in the hind part was due to a lower titer of ecdysone which may be continuously produced in the ring gland in the anterior part. However, an injection of ecdysone which adds to what is already present at the time of ligation delays tanning rather than accelerates it. Both ecdysone and ecdysterone were used in these experiments, since it has recently been shown that ecdysterone, rather than ecdysone, is the natural hormone in insects,[9] including flies.[10] In preliminary experiments the value of the tanning unit of ecdysone for *S. bullata* was found to correspond roughly to the estimate by Ohtaki *et al.*[5] for *S. peregrina*, i.e., 0.035 $\mu$g of ecdysone per ligatured abdomen. In one experiment, injection of 1.75 $\mu$g of ecdysone or ecdysterone had a marked delaying effect on tanning (Table 1, expt. 24). In another, injections of 0.017 to 0.35 $\mu$g of ecdysone all had delaying effects (expt. 25). At no dosage was there an accelerating effect.

(3) *Effect of injected hemolymph on the onset of tanning:* The hemolymph from puparia one to two hours old greatly accelerated tanning in the hind part, while that from larvae more than four and less than 12 hours before pupariation (esti-

TABLE 1. *Effect of injections of homogenates of various tissues, hemolymph or ecdysone, into hind parts of larvae of Sarcophaga bullata.*

| Materials injected into hind parts of larvae | Volume of injected (μl) | Donor equivalent* | Specimens (no.) | Pretanning Period (min) Anterior | Pretanning Period (min) Posterior | P − A | P/A |
|---|---|---|---|---|---|---|---|
| *Expt. 1* | | | | | | | |
| Nothing injected, but ligature released immediately after application | ... | ... | 12 | 226 | 225 | −1 | 1.00 |
| Nonligated controls | ... | ... | 12 | 227 | | | |
| Ringer's solution | 5 | ... | 10 | 213 | 325 | 112 | 1.52 |
| *Expt. 24* | | | | | | | |
| Ecdysone (1.75 μg = 50 SU) | 5 | ... | 12 | 242 | 386 | 144 | 3.60 |
| Ecdysterone (1.75 μg) | 5 | ... | 12 | 245 | 398 | 153 | 1.62 |
| Distilled water | 5 | ... | 13 | 226 | 297 | 71 | 1.31 |
| *Expt. 5* | | | | | | | |
| CNS homogenates from red spiracles larvae | 5 | 1.5 | 13 | 234 | 114 | −120 | 0.49 |
| Ring gland homogenates from red spiracles larvae | 5 | 1.5 | 8 | 227 | 133 | −94 | 0.59 |
| Muscle homogenates from red spiracles larvae | 5 | ... | 12 | 236 | 302 | 66 | 1.28 |
| Ringer's solution | 5 | ... | 15 | 243 | 314 | 71 | 1.29 |
| *Expt. 6* | | | | | | | |
| Brain homogenates from red spiracles larvae | 5 | 1.5 | 10 | 172 | 99 | −73 | 0.58 |
| Ring gland homogenates from red spiracles larvae | 5 | 1.5 | 10 | 163 | 128 | −35 | 0.79 |
| Thoracal ganglion from red spiracles larvae | 5 | 1.5 | 12 | 196 | 140 | −56 | 0.71 |
| *Expt. 7* | | | | | | | |
| Pars intercerebralis of larval brains | 5 | 1.5 | 15 | 236 | 107 | −129 | 0.45 |
| Remaining parts of the brains | 5 | 1.5 | 14 | 250 | 336 | 86 | 1.34 |
| *Expt. 12* | | | | | | | |
| C. Cardiaca with part of ring gland | 5 | 4.0 | 10 | 142 | 72 | −70 | 0.51 |
| C. allatum with part of ring gland | 5 | 4.0 | 12 | 148 | 174 | 26 | 1.18 |
| Ringer's solution | 5 | ... | 9 | 152 | 208 | 56 | 1.37 |
| *Expt. 9* | | | | | | | |
| CNS homogenates from 3-day-old larvae | 5 | 1.5 | 14 | 180 | 93 | −87 | 0.52 |
| CNS homogenates from 4-day old larvaeä | 5 | 1.5 | 15 | 167 | 95 | −72 | 0.57 |
| *Expt. 8* | | | | | | | |
| CNS homogenates from 5-day-old larvae | 5 | 1.5 | 13 | 228 | 127 | −101 | 0.56 |
| *Expt. 10* | | | | | | | |
| Brain homogenates from adult flies | 5 | 1.2 | 11 | 186 | 111 | −75 | 0.60 |
| *Expt. 11* | | | | | | | |
| Pars intercerebralis of adult brains | 5 | 1.2 | 12 | 196 | 104 | −92 | 0.53 |
| Remaining parts of adult brains | 5 | 1.8 | 11 | 217 | 306 | 89 | 1.41 |
| *Expt. 13* | | | | | | | |
| C. cardiaca from *P. americana* | 5 | 0.7 | 17 | 157 | 86 | −71 | 0.55 |

Table 1. (*continued*)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Expt. 23* | | | | | | | |
| Brain homogenates from *Pyrrhocoris apterus* | 5 | 3.0 | 7 | 176 | 144 | −32 | 0.82 |
| C. cardiaca homogenates from *Pyrrhocoris apterus* | 5 | 4.0 | 9 | 180 | 153 | −27 | 0.85 |
| *Expt. 15* | | | | | | | |
| Blood from 1- to 2-hr-old puparia | 10 | ... | 20 | 220 | 172 | −48 | 0.78 |
| Larval blood prior to red spiracles | 10 | ... | 21 | 186 | 265 | 79 | 1.42 |
| *Expt. 16* | | | | | | | |
| Blood from freshly emerged | 10 | ... | 29 | 198 | 316 | 118 | 1.60 |
| Blood from flies 1 hr old | 10 | ... | 21 | 197 | 268 | 71 | 1.36 |
| *Expt. 25* | | | | | | | |
| Ecdysone 0.35 μg + CNS homogenates | 5 | 1.5 | 10 | 142 | 81 | −61 | 0.57 |
| Ecdysone 0.035 μg + CNS homogenates | 5 | 1.5 | 9 | 155 | 95 | −60 | 0.61 |
| Ecdysone 0.017 μg + CNS homogenates | 5 | 1.5 | 9 | 142 | 95 | −47 | 0.67 |
| Ecdysone 0.35 μg in Ringer's solution | 5 | ... | 12 | 176 | 305 | 129 | 1.73 |
| Ecdysone 0.035 μg in Ringer's solution | 5 | ... | 12 | 165 | 306 | 141 | 1.85 |
| Ecdysone 0.017 μg in Ringer's solution | 5 | ... | 12 | 165 | 320 | 155 | 1.94 |

The larvae had been ligatured after the critical period at the beginning of the red-spiracle stage, 3 to 4 hr before they would have pupariated. The effect measured was the period between the injection and beginning of tanning in the anterior and posterior parts.

* Number of insects from which homogenates were made, per injection.

† Still feeding.

‡ End of feeding period.

§ 12–24 hr before pupariation.

mated by the state of the crop) had no effect (Table 1, expt. 15). The blood from adults taken less than one minute after emergence (bursicon absent) showed no activity, and neither did blood from two-hour-old donors (bursicon present) (Table 1, expt. 16). This eliminates the possibility that the active factor is bursicon (see *Discussion*).

(4) *Effect of neurosecretion on the onset of tanning behind the ligature after the critical period:* The following experiments proved unequivocally that the delay in tanning in separated hind parts can be annulled and tanning accelerated relative to the front part by the injection of suitable neurosecretory materials. In these experiments (in Table 1), the dosages of tissue extracts employed are expressed in terms of equivalents of number of insects from which the tissues for a single injection were derived.

An accelerating principle is contained in extracts of the total central nervous system (including the ring gland) of fly larvae at the time of pupariation (Table 1, expt. 5) or one or two days before pupariation. It has also been found in homogenates of separated parts of the central nervous system of pupariating larvae, brain, fused thoracic-abdominal ganglion, and ring gland (expts. 5 and 6). Brains from adult flies were also active (expt. 10). An analysis of the brain, based on detailed dissection and bioassays of homogenates of its components,

revealed that all the activity of both larval and adult brains resides in the pars intercerebralis. The brain minus pars intercerebralis is inactive (expts. 7 and 11).

When ring glands are separated into front sections (containing the corpus allatum) and hind sections (containing the corpus cardiacum), with both retaining about equal amounts of side-arm (prothoracic) tissue, the activity is in the c. cardiacum section (expt. 12). Activity is also found in the corpora cardiaca of both males and females of the cockroach, *Periplaneta americana* (expt. 13), and in the c. cardiaca and brain of the bug *Pyrrhocoris apterus* (expt. 23). Brains of both sexes of *P. americana* have very low activity (expt. 13).

From the above experiments we conclude that the active principle causing accelerated tanning in the ligatured hind part is not ecdysone but a neurohormone deriving from the neurosecretory cells in the pars intercerebralis and thoracic-abdominal ganglion and stored by and presumably released from the corpora cardiaca at the time of pupariation. It is unspecific, showing activity in our test with preparations from species belonging to three different insect orders.

(5) *Experiments with normal (nonligated) post-critical-period larvae:* Larvae about $1^1/_2$ to 3 hours before pupariation were injected with extracts from the central nervous system taken from larvae of the same age. They pupariated much sooner than controls injected with Ringer or noninjected controls (Table 2). These precociously produced puparia were normal, showing the contraction into the barrel shape before tanning.

TABLE 2. *Effects of neurohormones and ecdysones on duration of period between injection and completion of white puparium (prepupariation period) and between completion of white puparium and onset of tanning (white puparium period).*

| | Materials injected | Specimens (ml) | Prepupariation Period (min) | Prepupariation Period (% control) | White Puparium Period (min) | White Puparium Period (% control) |
|---|---|---|---|---|---|---|
| 18 | 5 μl of Ringer's solution | 11 | 114 | 100 | 54 | 100 |
| | 1 central nervous system in 5 μl Ringer's solution | 17 | 56 | 49 | 29 | 54 |
| 24 | 10 μl of distilled water | 13 | 188 | 100 | 45 | 100 |
| | 2.0 μg of ecdysone in 10 μl of water | 13 | 158 | 84 | 126 | 280 |
| | 2.0 μg of ecdysterone in 10 μl of water | 12 | 207 | 110 | 93 | 206 |
| 27 | 5 μl of distilled water | 15 | 95 | 100 | 31 | 100 |
| | 1.6 central nervous system in 5 μl of distilled water | 15 | 53 | 56 | 18 | 58 |
| | 1.5 μl of ecdysone in 5 μl of water | 15 | 98 | 103 | 52 | 168 |
| | 1.5 μg of ecdysterone in 5 μl of water | 15 | 150 | 158 | 71 | 228 |

Normal larvae were injected $1^1/_2$ to 3 hr before they would have pupariated.

Ecdysone or ecdysterone injected into nonligated larvae in fairly large amounts (approx. 50 SU) delayed tanning but not the contraction into the puparium (Table 2, expts. 24 and 27), except in one experiment with ecdysterone.

These experiments prove that a neurohormone also accelerates the contraction into the barrel shape which precedes tanning and that this neurohormone is one

of the limiting factors in the normal animal in the timing of pupariation shortly before this event (another being ecdysone, since pupariation does not occur in its absence).

(6) *Effect of neurosecretion and ecdysones on tanning in hind parts of larvae ligatured well before the critical period:* When fly larvae are ligatured before the critical period when the crop is empty, the anterior part may pupariate after a shorter or longer period, but the posterior part never tans unless it is injected with ecdysone, ecdysterone, or certain ecdysone analogues (e.g., refs. 3 and 4). It was of interest to know whether injection of neurosecretion in addition to ecdysone potentiates the action of ecdysone or accelerates tanning. In an experiment with *Phormia regina* the hind parts were injected with ecdysone or ecdysterone in doses of 0.16, 0.033, 0.016, and 0.003 μg, and simultaneously with a homogenate from the central nervous system of *Sarcophaga bullata* from which the ring gland had been removed (to exclude the ecdysone contained in the ring gland) (Table 3). A higher number of specimens tanned when the CNS homogenate was added to doses of between 0.16 and 0.016 μg of the hormones. The onset of tanning was also accelerated.

TABLE 3. *Effects of ecdysone, ecdysterone, and neurosecretion on the tanning of hind parts of larvae of Sacrophaga bullata and Phormia regina ligated before the critical period.*

| Ecdysone (μg) | Central nervous system homogenate* | Tanned Andomens (no.) | Tanned Andomens % | Ecdysone (μg) | Tanned Abdomnns (no.) | Tanned Abdomnns % |
|---|---|---|---|---|---|---|
| 0.16 | 1.0 | 11/13 | 85 | 0.16 | 10/16 | 62 |
| 0.033 | 1.0 | 6/13 | 46 | 0.033 | 4/15 | 27 |
| 0.016 | 1.0 | 3/12 | 25 | 0.016 | 0/14 | 0 |
| 0.003 | 1.0 | 0/11 | 0 | 0.003 | 0/13 | 0 |
| Ecdysterone | | | | Ecdysterone | | |
| 0.16 | 1.0 | 16/17 | 94 | 0.16 | 11/18 | 61 |
| 0.033 | 1.0 | 7/18 | 39 | 0.033 | 3/15 | 20 |
| 0.016 | 1.0 | 2/18 | 11 | 0.016 | 1/18 | 6 |
| 0.003 | 1.0 | 0/17 | 0 | 0.003 | 1/17 | 6 |
| none | 1.0 | 0/15 | 0 | | | |

* Number of insects from which homogenates were made, per injection.

*Conclusions.*—According to Ohtaki *et al.*,[5] the blood of *Sarcophaga peregrina* contains only about 15 per cent of the titer of ecdysone necessary to induce tanning when injected into a ligatured hind part of this species. Hence, these authors considered earlier experiments showing tanning to be induced by this procedure in *Calliphora erythrocephala*[1] as "false positives." In substantiating their theory, however, they used an entirely different method and a different family of flies. It now appears that this controversy is, in part, a matter of a species difference. Not only had the original results been fully confirmed on two former occasions,[6,7] but we have also now reproduced them in a related species, *Phormia regina*. We have, however, failed to obtain a positive result in *Sarcophaga bullata*.

It would be premature to speculate on the identity of our new hormone with some of the numerous endocrine factors which have been described from brain, ganglia, and corpora cardiaca of insects and are commonly regarded to be polypeptides or proteins.[12, 13] To be sure, our factor is different from bursicon, another tanning hormone of the nature of a protein.[8] Blood from pupariating fly larvae contains our factor but has no detectable bursicon activity;[8] conversely, blood from young adult flies with high bursicon activity is largely ineffective during pupariation.

Our neurohormone not only accelerates tanning in the whole organism as well as in the isolated hind part, but in the whole larva it also accelerates the contraction into the puparium which precedes tanning. It shares this double function with ecdysone itself which, aside from tanning, also induces the puparial contraction.[14]

The function of the new hormone is different from that of the much-discussed brain ("activation") hormone which activates the prothoracic gland to release ecdysone.[15] In our test preparation, ecdysone is already present in the hemolymph in a titer high enough to induce tanning in the posterior part. The relation of our new neurohormone to other neurohormones of similar origin can only be decided after its isolation and chemical characterization.

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