Hormonal and Nervous Control of Tanning in the Fly

Darkening of the adult fly involves neurosecretion and the action of a hormone other than ecdyson.

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Abstract. In the newly emerged fly, darkening and hardening of the cuticle (tanning) starts after 15 to 20 minutes but can be delayed under certain conditions. Ligaturing off the head abolishes tanning. Tanning is initiated by a signal from neurosecretory cells in the brain, transmitted to the thorax via both the central and stomatogastric nervous system. Blood from a 15-minute-old fly induces tanning when injected into the body of a head-ligatured fly. The active factor in blood is probably neither a substrate nor an enzyme necessary for tanning. It could be a new hormone, because it is neither ecdyson, nor the juvenile hormone, nor a brain hormone. This factor is entirely unspecific, being present in newly molted cockroaches (*Periplaneta*) and newly emerged adults of the beetle *Tenebrio*. A mechanism that inhibits tanning also plays a role in this reaction.

When an adult fly emerges from the puparium, it is very much smaller than its ultimate size, is little pigmented, has a soft skin, and has not yet unfolded its wings. Twenty-six years ago one of us (*1*) described certain processes connected with the behavior and the morphological changes by which the fly *Calliphora erythrocephala* attains its ultimate size, color, and shape. It pumps air into the intestine through the proboscis, thereby blowing itself up like a balloon, and expanding the wings by forcing hemolymph into the wing veins. This process is completed about 15 to 20 minutes after emergence. During the following 1 to 2 hours the cuticle gradually darkens and hardens by a process usually designated as "tanning" and, in certain species, ultimately assumes a typical metallic sheen. For the sake of briefness we shall henceforth use the term "tanning" to refer to this darkening, although it is by no means certain that we are dealing here with a typical case of tanning as it occurs in the formation of the puparium. When a newly emerged fly is kept in a confined space, for example, sand, sawdust, or a narrow glass tube, it continues the normal digging movements of the ptilinum, and inflation and darkening may be delayed by many hours. The significance of this delay seems to have escaped the attention of the many investigators who have studied the hormonal processes involved in the metamorphosis and egg production of flies.

Tanning in insects after a molt represents one of the last steps in the molting process which is initiated and controlled by the hormone ecdyson. Ever since the tanning of the fly puparium was recognized as being brought about by what is now called ecdyson (*2*) it has probably been silently assumed that all tanning that occurs in various insects after a molt is due to an action of this same hormone. We must, however, not forget that tanning of the puparium is a somewhat aberrant process, and occurs in the absence of a molt. Curiously, the emergence of the fly from the puparium represents another aberrant case where tanning after a molt is separable from molting, since it can be delayed for many hours or altogether prevented. We would, therefore, expect this particular reaction to be an especially favorable system for a study of what controls tanning as distinct from what controls molting.

In the work which is reported below three very common species of flies were used: *Phormia regina, Calliphora erythrocephala*, and *Sarcophaga bullata*. No significant differences were found among the three species.

Phormia regina exhibits the delay of tanning to a remarkable degree. A simple way of demonstrating this phenomenon is to let a fly crawl head first into an empty puparium immediately upon emergence. In one such experiment 12 flies which had been thus confined for 8 hours had, when released, the normal appearance and activity of newly emerged flies. Five of these subsequently developed into normal flies, and four more became almost normal. Even flies which had been thus confined for 24 hours were apparently still in the original state, although much weakened and not moving normally. Five out of 12 became fully tanned and four partly tanned, although none of them became inflated.

Further analysis of these phenomena was attempted by placing ligatures between the head and thorax or between the thorax and the abdomen immediately after emergence of the flies. Flies with only the head ligatured off never tan on the head or the thorax-abdomen and serve as a convenient standard preparation in which to study the effects of injections or other manipulations on tanning. A ligature placed between the thorax and the abdomen did not have a predictable effect. In some cases tanning occurred in either the isolated anterior or posterior part, sometimes in both, but there seemed to be no correlation between tanning of one part or the other. The isolated anterior part seldom became inflated. If the ligature was placed 1 to 2 mm posterior to the base of the abdomen, the head-thorax section usually became inflated and fully tanned, while the abdomen tanned more rarely or to a lesser extent than did a whole abdomen isolated at the base. With two ligatures, one on the neck and the other behind the thorax, the thorax never tanned, while the isolated abdomen, as already

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reported, did so in a certain number of cases.

These results could be changed by delaying the application of the ligature to 5 to 10 minutes after emergence. After a 5-minute delay about half the flies which had been ligatured behind the head became tanned on the thorax and abdomen; after a 10-minute delay all became tanned. Similar results were obtained with the tanning of the abdomens of flies which had been ligatured between the thorax and abdomen 5 or 10 minutes after emergence. In this case the anterior parts which tanned usually also became inflated and the wings were expanded.

These observations suggested the following interpretation. All the prerequisites for tanning are present at the time of emergence, as witnessed by tanning in the isolated abdomen. Tanning, however, is normally inhibited by something located in the thorax. Since tanning never occurs when the head is ligatured off, it follows that in the intact fly tanning must be initiated by a signal from the head to the thorax. This does not happen while the fly is in a confined space and is in the process of digging itself out. Five to 10 minutes after the fly has dug itself out the signal has been given and the isolated posterior part consequently tans.

The signal from the head almost certainly has its origin in the median and lateral neurosecretory cells of the anterior rim of the brain, the same cells which also control the development of the ovary in the adult fly (*3*). Removal of either the median or lateral groups alone of a newly emerged fly abolished tanning in about one-half of the cases, while removal of both groups completely abolished tanning. These neurosecretory cells are located on the surface of the brain below the membrane which encloses the brain. They can be removed with a fine pin or forceps without any obvious injury to the main body of the brain. Extensive damage to other parts of the brain affects the tanning reaction only slightly, if at all, which suggests that the neurosecretory cells in fact initiate the tanning reaction.

Attempts to delineate the pathway by which the tanning signal is communicated from the head to the rest of the body did not give unequivocal results. Interruption of the recurrent nerve connecting the brain with the ring gland complex of endocrine organs, or direct interference with these glands by removing the corpus allatum or the whole of the ring gland abolished tanning in from 50 to 70 percent of the cases. Similarly, cutting of the main ventral connectives in the neck abolished tanning to about the same extent. Only interruption of both the stomatogastric and central nervous systems abolished tanning completely. This, of course, simulates the condition where tanning is abolished by either ligaturing or cutting off the head. This would suggest that the tanning signal travels via both the central and sympathetic nervous systems, and not via the blood stream.

A perhaps more clear-cut indication of a neural transmission of the initial tanning impulse from the head was given in an experiment in which the neck of a newly emerged fly was firmly tied with a hair, and the ligature removed 15 minutes later. Tanning never occurred in such flies, although they remained alive for well over 24 hours. Since there were no external lesions and the heart pumped continuously, an effective humoral connection between head and body must have been maintained. Later examination of such flies revealed that both central and stomatogastric nervous systems were interrupted. This may have abolished tanning in the posterior part. (An alternative explanation is that the neurosecretory cells in the brain do not produce the impulse for tanning in the absence of suitable stimuli from the thorax.)

It is, however, easy to show that tanning can be successfully accomplished by humoral effects carried by the blood stream. The most clear-cut demonstration of such an effect is accomplished by injecting fly blood into the thorax of another fly that had the head ligatured off immediately after emergence. No tanning took place if blood from a newly emerged donor was injected, but the receiving thorax-abdomen invariably tanned if the blood was taken from a fly 10 to 20 minutes after emergence. Similar effects were produced under somewhat different conditions. Flies were ligatured between head and thorax, and between thorax and abdomen, and specimens were selected where the abdomen alone had tanned. If the ligature between thorax and abdomen was then removed, the thorax also tanned. In other experiments where a single ligature was placed somewhat posterior to the base of the abdomen, and where only the part anterior to the ligature had tanned, removal of the ligature led invariably to tanning of the posterior part. All these observations demonstrate that tanning can be induced in the absence of nervous connections by humoral effects alone.

At this point one is tempted to speculate that the signal for tanning, which originates in the neurosecretory cells of the brain, first travels to the thorax via the stomatogastric *and* the central nervous system, causing there the release of some hormonal agent which completes the chain of reactions. One additional fact in favor of this explanation is the observation that the isolated head never tans. (This may, however, be owing to the absence of spiracles, and hence an effective respiratory system, in the isolated head.) A serious objection to this hypothesis is the fact that removal of the whole ring gland system of endocrine organs never completely stops tanning as long as the central nervous system is intact. If neurosecretion in the brain stimulates the release of a hormone elsewhere, presumably in the thorax, from which tissue then would the hormone originate?

Ecdyson

The literature is curiously silent in regard to mechanisms which control tanning as it occurs after a molt. Tanning is usually considered as part and parcel of the molting process which is initiated and controlled by ecdyson, the secretion of the prothoracic gland; its production is under the control of a secretion from the neurosecretory cells of the brain. In flies the organ homologous to the prothoracic glands of other insects is located in the lateral arms of the ring gland. This organ persists through the pupa and is still present in the newly emerged adult, but subsequently disappears. Its disappearance in the adult has been recorded by several authors (*4*) though the exact timing of this event, or the morphological changes involved therein, never seem to have been investigated in detail. This would suggest that this organ may at this stage secrete ecdyson and thereby initiate tanning, and that it subsequently degenerates. This attractive hypothesis has, however, been disproved by the following experiments. (i) Blood from a fly larva which is about to pupate fails to bring about tanning when injected into the thorax of a newly emerged fly with the head ligatured off. It is well known that larval blood at this stage is loaded with ecdyson. (ii) In a converse experiment, blood from a 10- to 20-minute-old fly, injected into the ligatured hind part of fly larva where the anterior part alone had pupated within 24 hours after ligaturing, fails to cause tanning. This preparation is commonly used in the "*Calliphora* test" for ecdyson (*2*) where tanning of the hind part demonstrates the presence of ecdyson. This experiment rules out the possibility that the larva does not contain sufficient ecdyson for adult tanning, since in that event adult blood would have caused tanning in the larva. (iii) A highly concentrated preparation of ecdyson (obtained from P. Karlson-Munich) containing 100 *Calliphora* units per milligram was injected in doses of 5 and 15 *Calliphora* units per fly and proved entirely inactive. These experiments, therefore, definitely rule out a decisive role for ecdyson in the tanning of the adult.

The active factor in blood proved entirely unspecific. Active blood from one fly caused tanning in the two other species, and vice versa, in all combinations, while the same did not happen with inactive blood. Blood from freshly molted nymphs or adults of the cockroach, *Periplaneta americana*, but not from fully darkened ones, induced tanning in the fly. Similarly, blood from newly emerged adults of the beetle, *Tenebrio molitor*, was active, but not blood from fully tanned larvae or adults.

Since ecdyson has been excluded as the active factor in adult fly blood which induces tanning in head-ligatured flies, what are the remaining possibilities? It would theoretically seem that there are three alternatives, namely, a hormone, or an enzyme (for example, a polyphenol oxidase), or a substrate necessary for tanning (for example, a particular polyphenol). No extensive isolation studies have yet been performed with this material, but some insight into the nature of the active material was obtained in the following way.

Active blood from about 30 flies was pooled under mineral oil. The activity was the same before and after this material was centrifuged. A drop of such blood, placed on filter paper, darkened within about 10 minutes. Darkening was somewhat more intense and faster upon addition of *l*-dihydroxyphenylalanine solution. When active blood was heated at 80° or 100°C for 5 minutes and the ensuing precipitate was removed by filtration, the supernatant still retained its activity, but no longer darkened on filter paper, not even upon addition of *l*-dihydroxyphenylalanine. Active blood could be diluted 20 to 30 times without losing its activity. The experiments suggest that the active factor is not a polyphenol oxidase, and that it is not likely to be a simple substrate. The chances therefore are that we are dealing with a hormone. Since this can be neither ecdyson, nor the prothoracotrope hormone of the brain, nor a corpus allatum hormone (since tanning can occur in the absence of the corpus allatum), we may be dealing with a new type of insect hormone.

All the work so far discussed was based on the premise that tanning in the fly is the result of some *activating* mechanism. There still remains the possibility that a delay or failure in the tanning of the newly emerged fly is not so much due to a lack of activation as to the presence of an *inhibitory* mechanism. The following observations indeed suggest such a mechanism. When ligatures are placed between head and thorax, and thorax and abdomen, of a newly emerged fly, the isolated thorax never tans, while tanning occurs in the isolated abdomen in an appreciable number of cases. An injury to an isolated untanned abdomen, even as small as the prick of a pin, frequently causes it to tan, while no such effect can be produced in the isolated thorax. This would suggest that an inhibitory mechanism located in the thorax prevents immediate tanning in the newly emerged fly, and that normal tanning is initiated by removal of this inhibition. If this were true, it should be possible to initiate tanning in the head-ligatured fly by removing the source of this inhibition. All attempts to localize such a tissue have so far failed (*5*).

References and Notes

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Discrimination and Generalization on a Dimension of Stimulus Difference

Abstract. Four pigeons were trained to peck at one key when two identical stimuli were displayed, and at another key when the stimuli were displayed with a wavelength difference of 40 $m\mu$. With stimulus combinations ranging in difference from 0 to 70 $m\mu$ in a generalization test, key preference was a function of the degree of difference between the stimuli.

The study of stimulus generalization has usually been confined to specific physical dimensions, such as intensity, size, wavelength, auditory frequency, or position of a tactile or visual stimulus. Generalization along derived dimensions defined by the relationship between stimuli has not been studied in animals. One such relationship is the degree of difference between stimuli. It is clear that animals can discriminate on the basis of identity versus difference of stimuli in solving the oddity problem (*1*) and matching to sample (*2*); the transfer of such a discrimination has been reported for chimpanzees by Robinson (*3*). But a quantitive analysis of this relationship as a stimulus dimension has not been undertaken, even though Ekman (*4*) and others have developed methods for psychological similarity scaling with human subjects.

In our study, monochromatic stimuli illuminating two response keys in an operant behavior apparatus were used to establish the difference dimension (*5*). Four pigeons were trained with the following problem: When the wavelength difference was zero, one key was correct (the "identity key"); when the difference was 40 $m\mu$, the other key was correct (the "difference key"). Sixteen training combinations were used with eight stimulus values ranging from 500 to 570 $m\mu$ in 10-$m\mu$ steps. For the eight "identity" pairs, one of these eight wavelengths was identically displayed on the two keys. For the eight "difference" pairs, appropriate combinations were used; thus, 500 and 540 $m\mu$ were presented, respectively, on the left and right keys, or the reverse; 510 and 550 $m\mu$ were presented together, and so forth. For two subjects, the left key was the identity key and the right key was the difference key; this system was reversed for the other two subjects. The stimulus values were produced by projecting collimated white light through Bausch & Lomb interference filters.

The sixteen training conditions were presented in randomized order three or