Time-Table for the Development of the Silkworm, *Bombyx mori*

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It is important for the technical improvement in sericulture to know the mechanisms controlling growth and development of the silkworm, *Bombyx mori*. Since the pioneering achievements by Bounhiol (1937)[1] and Fukuda (1944),[2] extensive studies have been carried out from endocrinological standpoint [see Kobayashi (1978) for review[15]], but the control of development has not been as yet fully elucidated. In an approach to the analysis of the control mechanisms, we attempt to prepare a developmental time-table for the silkworm. The purpose of this trial is to make clear the mutual time-relations of various developmental events occurring during larval development and metamorphosis. In this review, the author will describe a basic framework of the time-table. Special emphasis is placed on how to determine the precise developmental stages of the insect.

Endocrine system of *Bombyx mori*

Silkworm larvae grow well on either mulberry leaves or artificial diets.[8,9] When a newly hatched larva feeds and reaches a critical size, the epidermis secretes enzymes which digest the old cuticle. The epidermis also secretes a new larger cuticular exoskelton, and finally the insect moults, namely sheds the partially digested old cuticle. Such a larval-larval moult occurs four times before the larva reaches the final fifth instar. During the 5th-instar, the larva follows a somewhat different course of development; for example, the silkglands rapidly grow and produce a large amount of silk proteins (fibroin and sericin). Epidermis switches from a commitment (i.e., secretory capacity) to make larval cuticle to that for pupal cuticle. Larva undergoes external metamorphosis into pupa at the next moult. All developmental events mentioned above are under hormonal control.

The endocrine regulation of silkworm development is principally depends on the three following hormones; prothracicotropic hormone (PTTH), moulting hormone (ecdysone, 20-hydroxyecdysone) and juvenile hormone (JH) (many recent reviews on general insect endocrinology are available).[4,10,15,16] Initial endocrine event for moult induction is the secretion of PTTH in response to an integration of external and internal signals. PTTH is a polypeptidic neurohormone which is synthesized in the neurosecretory cells of the brain and released into the haemolymph through the retrocerebral complex of corpora cardiaca and corpora allata. It activates the prothoracic glands to synthesize and secrete ecdysone, a kind of steroid hormone. In many peripheral tissues ecdysone is rapidly converted to 20-hydroxyecdysone (ecdysterone) which is the active hormone form rather than ecdysone itself. During larval life corpora allata secrete, also in response to neurohormonal stimulation by the brain, the juvenile hormone (JH). This hormone is a unique acyclic sesquiterpene that controls larval development by modulating the action of 20-hydroxyecdysone; when the moulting hormone attains its critical titre in the presense of a high concentration of JH, a larval-larval moult is induced. Whereas, when it reaches the critical level at a low titre or in the absence of JH, larval-pupal or pupal-adult development is initiated. Accordingly, information on these hormones is fundamentally important for the preparation of time-table.

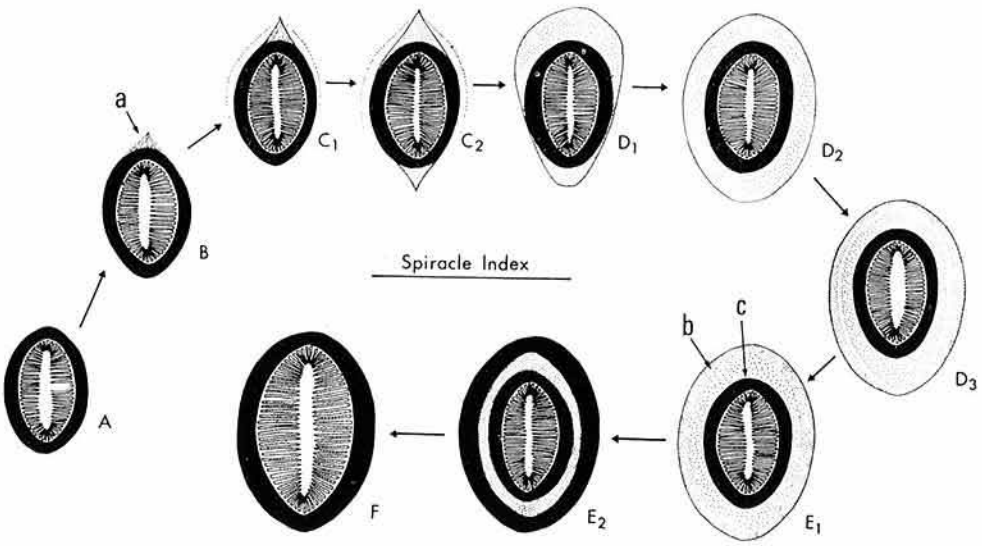

Fig. 1. Spiracle index as a basis for staging the larval moult
A: No distinct feature around the spiracle. B: Appearance of a dim spot (a) on the dorsal side of the spiracle. C_1–C_2: The spot becomes a triangular-shaped semi-transparent area with a clear border (C_1). The same feature appears on the ventral side of the spiracle (C_2). D_1–D_3: Both transparent areas fuse (D_1), then form an oval ring (D_2). The new cuticle of the peritreme is deposited as a white ring within the oval region (D_3). E_1–E_2: The new white peritreme begins to melanize (E_1). This stage is very clear. Then the new sieve-plate region gradually turns dark (E_2). F: New 5th-instar spiracle after ecdysis. a: first sign of initiation of the larval moult, b: new peritreme, c: old peritreme.

Time-table of the larval moult cycle[13)]

Precise staging of a larval moult cycle is crucial for such studies, because duration of larval development varies significantly depending on the genetic background, nutrition and environmental conditions. We found that the externally visible characteristics of the formation of new spiracle provides a particularly good criterion for the staging of moulting silkworm larvae. As shown in Fig. 1, spiracular apolysis is the first visible sign of the initiation of larval moulting. After the apolysis, formation of new spiracle can be seen clearly through the old cuticle when the larva is examined under a dissecting microscope. Visible changes permit distinction of 10 morphological larval stages (A–F) during the 4th moulting period, which are referred to as the spiracle index. Developmental stage of the larva is well defined by the combination of spiracle index and the time (hr) from the 3rd ecdysis.

In the next place, correlation of the spiracle index stages with morphological and endocrinological events during the moulting cycle was investigated. At certain spiracle index stages, we have determined the haemolymph level of ecdysteroids (i.e., the moulting hormone steroids) by radioimmunoassay procedure described by Horn et al. (1976)[6)] and Gilbert et al. (1977)[3)] (Fig. 2). We have also studied histological changes in the larval integument, and determined the critical periods for the secretion of juvenile hormone and ecdysone, using allatectomy and abdominal ligations. Fig. 3 presents a schematic developmental time-table so far prepared.

As shown in Fig. 3, the time of initiation of spiracular apolysis almost coincides with the end of the critical period for the corpora allata. Therefore, juvenile hormone, secreted from the corpora allata prior to spiracular apolysis, is responsible for the induction of larval moult. After spiracular apolysis this hormone is irrelevant to the character of moult, but can still affect the marking pigmentation in the newly

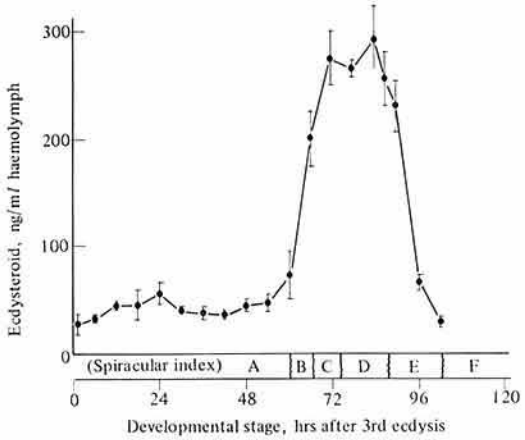

Fig. 2. The ecdysteroid levels in the haemolymph of *Bombyx mori* during the larval moulting period
Results are expressed as ng of ecdysone equivalent/m*l* of haemolymph. Each point represents the mean ±S.D. in 3 samples of 10 μl haemolymph. Experimental animals are the larvae of F_1 hybrid (J.124×C.124) reared at 25°C on mulbery leaves under continuous light conditions.

secreted larval cuticle.[11,12] About 6 hr after the first sign of spiracular apolysis, epidermis detaches from the cuticle at the ventral midline, the last area in which the surface of the newly formed epicuticle becomes visible through the partly digested old cuticle. Thus, this may be regarded as the time of "general apolysis," namely, the beginning of pharate 5th instar.[5]

As indicated by the abdominal-ligation experiments, the critical period for ecdysone secretion by the prothoracic glands ends at the beginning of the C_1 stage. Apparently sufficient ecdysone has been released by the time when general apolysis is complete to initiate all the following events in new cuticle deposition. After general apolysis, the inner layer of the old cuticle is digested progressively, and the epidermis secretes epi- and then exo-cuticle. Each of these events can also be correlated with the readily visible spiracle index. Consequently, the spiracle index provides a time marker by which we can infer the proper developmental status during the larval moult.

As to the relationship between ecdysteroid level and developmental events, low but significant levels of ecdysteroid (30–50 ng/m*l*) are maintained during the first half of the 4th-instar. Midway in the feeding stage, spiracular apolysis is initiated when the hormone titre begins to rise to 60–70 ng/m*l*. As the ecdysteroid level increases, apolysis continues throughout the seg-

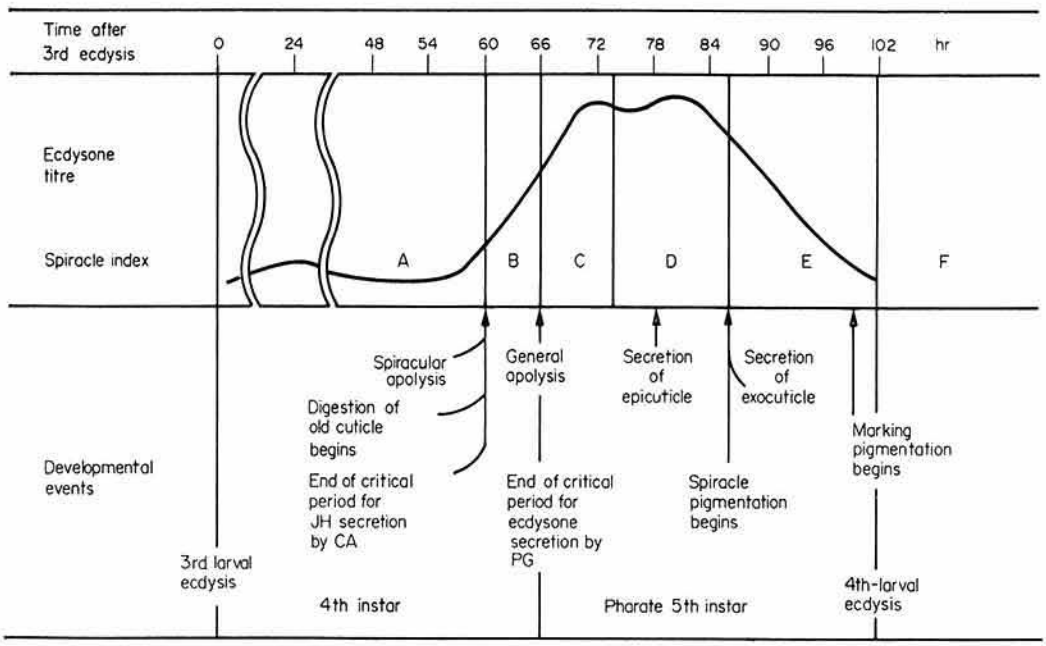

Fig. 3. Schematic developmental time-table for the larval moulting period in the silkworm, *Bombyx mori*

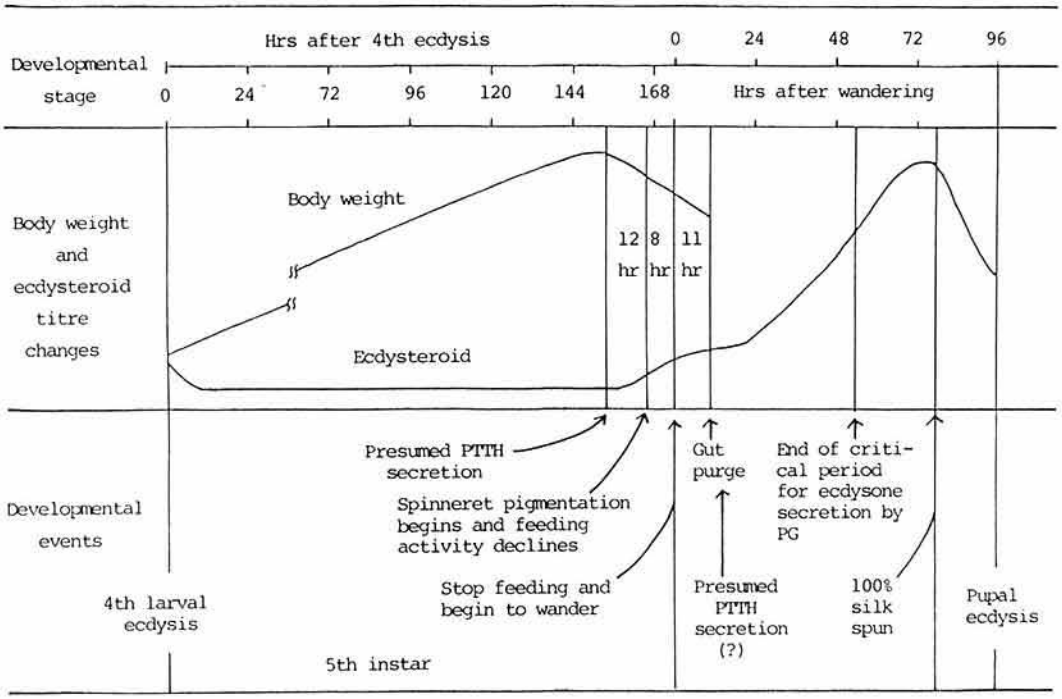

Fig. 4. Schematic developmental time-table for the larval-pupal moulting period in the silkworm, *Bombyx mori*

ment so that by 6 hr later whole epidermis is free from the overlying cuticle (C_1 stage). After this critical time, a high ecdysteroid titre is maintained for about 18 hr. During this time the new epi- and exo-cuticle are secreted. When the hormone level begins to decline, the newly formed spiracles begin to melanize—peritreme first, then the sieve-plate. Larval markings of the newly made cuticle begin to melanize when the ecdysteroid concentration has fallen to the initial low level (30 ng/m*l*). Thus, it is suggested that both the increase and the decrease of the haemolymph-ecdysteroid levels are physiologically significant for the production and the manifestation of various larval characters.

Time-table of the larval-pupal moult[14]

Fig. 4 is a schematic time-table so far prepared for the final 5th-instar and pupal moulting period. Due to reconstruction of the tracheal system, the spiracle index is not available for these stages. Consequently, we used a combination of several developmental events such as body weight changes, pigmentation of spinneret and various behaviour changes as criteria for the staging. From the table, we can understand some aspects of the mechanism controlling the larval-pupal transformation as follows.

Injections of partially purified PTTH confirmed that the first prothoracicotropic hormone (PTTH) release by the brain occurred when a 5th-instar larva reached a certain critical (maximum) body weight. The released PTTH stimulates the prothoracic glands to secrete ecdysone. Spinneret, which has been identified as the most sensitive organ to the moulting hormone, begins to melanize (Fig. 5). This is the first external sign of the initiation of metamorphosis in *Bombyx mori*.[7] It takes about 12 hr from the first PTTH secretion to the beginning of the spinneret pigmentation. The 5th-instar larva stops feeding and begins wandering about 8 hr after the onset of pigmentation. Then, it makes a scaffold on a suitable place for cocooning and subsequently purges the gut. The following spinning of the cocoon lasts about 60 hr. On the basis of some circumstantial evidences it is suggested that the 2nd PTTH release occurs around the time of the gut purge.

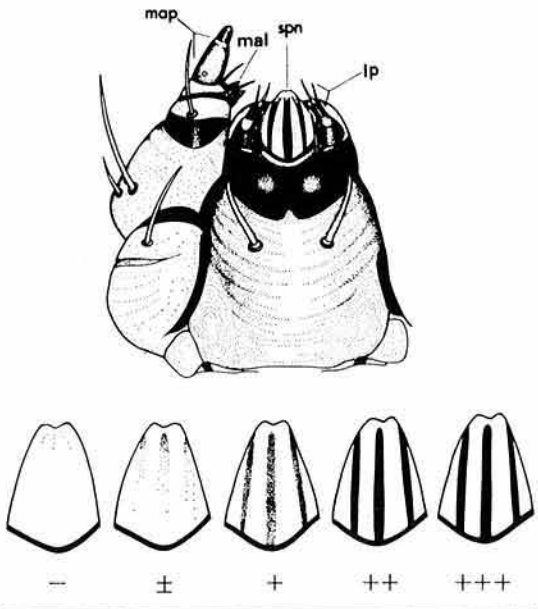

Fig. 5. A diagram showing the process of spinneret pigmentation
spn, spinneret; mal, maxillary lobe; map, maxillary palpus; 1p, labial palpus. Degree of spinneret pigmentation: (−) transparent, (±) yellowish, (+) light brown, (++) brown, (+++) dark brown.

Haemolymph-ecdysteroid levels during the 5th-instar are significantly different from those established during the larval moulting period. No ecdysteroid is detectable until 5th-instar larva reaches the critical body weight (Fig. 6). Very low level of ecdysteroid (10 ng/m*l*) first appears in the haemolymph at the beginning of spinneret pigmentation. After this time the level increases slightly through the wandering stage up to the time of gut purge (15–25 ng/m*l*), and then rises rapidly, reaching 700 ng/m*l* at the critical period for the ecdysone secretion by the prothoracic glands. The maximum level is observed just before the larva finishes cocoon-spinning (1500 ng/m*l*). From these relationships, it is likely that the spinneret pigmentation, wandering, gut purge, and cocoon-spinning behaviours are all closely connected with the titre changes of the moulting hormone.

Problems remaining

In this paper only basic frameworks of the developmental time-table are presented. Information on the titre changes of JH and PTTH,

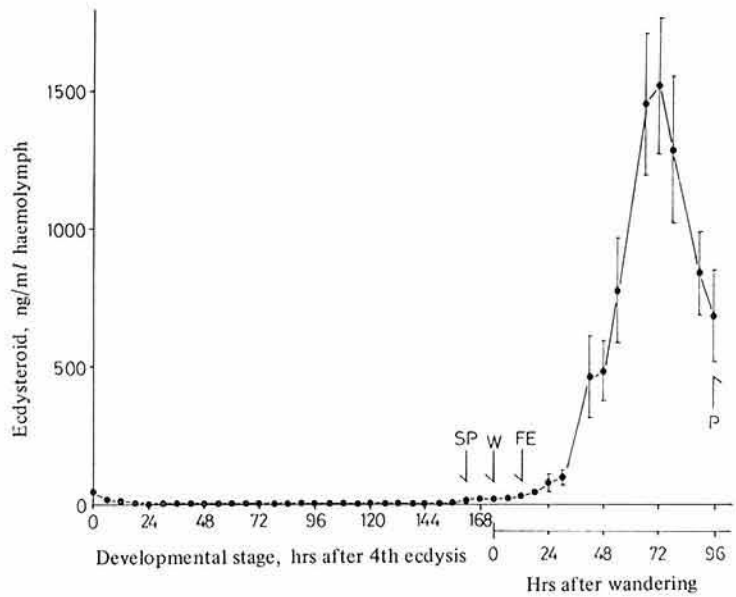

Fig. 6. The ecdysteroid levels in the haemolymph of *Bombyx mori* during the larval-pupal moulting period
For detail, see Fig.2 legend.

as well as additional physiological, biochemical and histological data, are needed before growth and development of *Bombyx mori* is fully understood. It is also expected that, by preparing the time-tables under various experimental conditions, we will be able to clarify the mutual relations among the endocrinological events, genetical, nutritional, and environmental factors. Such studies are now under way.

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