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Authors: Yasuda, Keiko, and Takahashi, Sohji

Source: Zoological Science, 14(3) : 435-442

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.435

ZOOLOGICAL SCIENCE **14**: 435–442 (1997)

Relationship between the Number of Annuli of Adult Antenna and the Length of Embryonic and Larval Period in *Samia cynthia ricini*

Keiko Yasuda* and Sohji Takahashi

Department of Biological Science, Faculty of Science, Nara Women's University, Nara 630, Japan

ABSTRACT—In the eri-silkworm, *Samia cynthia ricini*, the adult antennal flagellum is segmented into many annuli. Although the number of annuli is an important parameter in the morphogenesis of adult antenna, it is not clear when and how the number of annuli is determined. In the present study the fifth instar larva of the eri-silkworm was studied histologically to clarify when the antennal imaginal disk began morphogenesis and when the number of annuli of adult antennal flagellum was determined. In addition we studied whether the length of the embryonic and larval period might have any influence on the number of annuli in the eri-silkworm, and the influence of other factors such as body size and sex was also examined. Serial histological study of the imaginal disk during the larval period suggested that the number of annuli was determined by the second day after gut-purging, since the segmentation of the pupal antenna was almost finished by this time and the number of segments of pupal antenna was nearly equal to the number of annuli of adult antenna. The embryonic and larval period was closely related with the number of annuli. When the insects were reared at 25°C, the number of annuli was almost equal to the number of the days in which the insects passed from oviposition to gut-purging. In addition, the number of annuli tended to increase one by one from 27 to 34 as the embryonic and larval period was extended day by day from 28 to 35 days. When the insects were reared at 18°C, the larval period was doubled, whereas the number of annuli remained in the same range (28-34) as that reared at 25°C. The body size did not correlate with the number of annuli. Although the number of annuli was significantly larger in female than in male, this difference seemed to be due to the difference in the length of the embryonic and larval period in both sexes.

INTRODUCTION

The insect body generally consists of many segments. It is generally accepted that the number of segments of the insect body is genetically regulated and that the number does not vary in each insect. The antenna, one of head appendages, consists of three parts; scape, pedicel and flagellum. The adult antennal flagellum is further divided into many small annuli. The process of annulation is an important step in the morphogenesis of adult antenna. In Hemimatabola, Orthopteroid insects, the annuli of antennal flagellum increases in number as they grow from one instar to the next (Snodgrass, 1935). In hemimetabolous insects, the antenna grows progressively through ecdysis without a dramatic change. In contrast, in Holometabola, the anlage of adult antenna is maintained as undifferentiated cells (the imaginal disk) underneath the larval antenna in the larval stage. There are some reports on the growth and differentiation of the adult antenna in Lepidoptera (Essa, 1953; Nüesch, 1965; Sanes and Hildebrand, 1973; Takahashi and Oka, 1973). These studies have concentrated on the antennal development during metamorphosis. Takahashi and Oka (1973) reported that an excess of 20-hydroxyecdysone administered soon after pupation did not influence annulation of adult antennal flagellum, whereas it suppressed barb formation in eri-silkworm, *Samia cynthia ricini*. Therefore it was suggested that the number of annuli was determined before pupation. Little has been known about the earlier process of antenna formation in which the prospective annulation is programmed. In the present study the fifth instar larva of the eri-silkworm was studied histologically to clarify when the number of annuli was determined. In addition we studied whether the length of the embryonic and larval period might have any influence on the number of annuli in the eri-silkworm, and the influence of other factors such as body size and sex on the number of annuli was also examined.

MATERIALS AND METHODS

The eri-silkworms, *Samia cynthia ricini* (Saturniidae, Lepidoptera), were used in the experiments. They were reared on the fresh leaves of castor oil plant, *Ricinus communis*, under 14L10D photoperiod at 25°C, unless noted otherwise. The eggs were collected from several pairs of the insects that mated on the same day, and were kept at 25°C. It took 10 days from oviposition to hatching. Within 24 hr after

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* Corresponding author: Tel. +81-742-20-3411;
FAX. +81-742-20-3411.

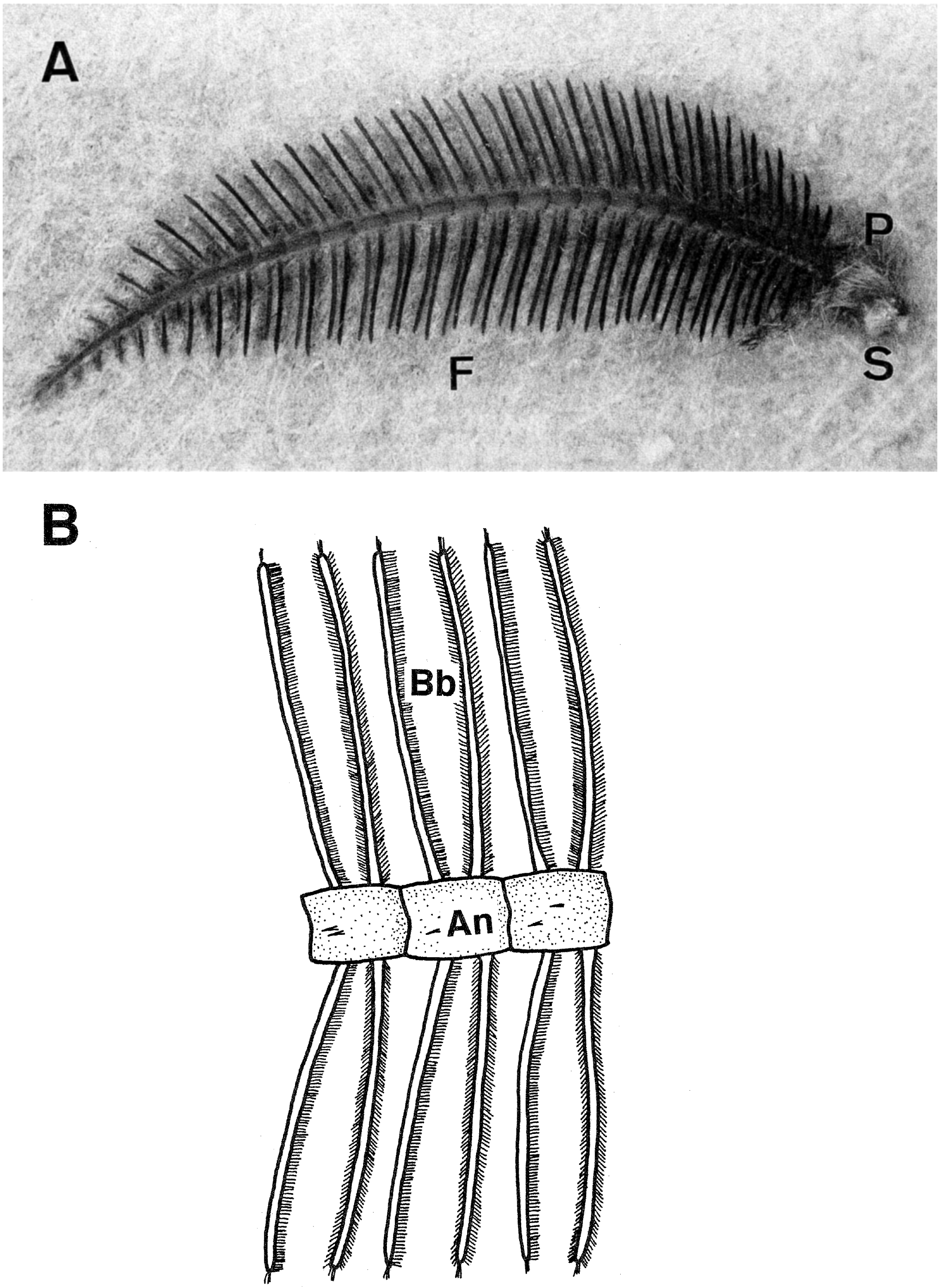
hatching, the insects were collected, and divided into two experimental groups. One group of insects were maintained at 25°C, the other at 18°C.

When the insects were reared at 25°C, they molted synchronously every three days until the fourth ecdysis. Some larvae stopped feeding five days after the fourth ecdysis and purged gut (gut-purging), whereas in others, gut-purging occurred 6 days or later after the fourth ecdysis, and spun cocoons. Shortly after gut-purging and before spinning, the body weight and the head width of the insects were measured.

When the insects were reared at 18°C, the ecdysis occurred every 6 days until the fourth ecdysis. The fifth instar larvae purged gut 10 days or later from the fourth ecdysis. Their larval periods were consequently prolonged two fold as long as those in larvae at 25°C.

After adult emergence, the annuli of right and left antennal flagella were counted under a stereoscopic microscope. Since we found no difference in the number of the annuli of right and left antennae, only the results from right antennae are shown.

For histological studies, the antennal imaginal disks were



dissected in saline and immediately fixed in Bouin's fixative. The tissues were dehydrated through a graded series of ethyl alcohol and embedded in paraffin. The serial sections of 5 µm were prepared and stained with Azan-Mallory triple staining.

RESULTS

The structure of the adult antenna

In the eri-silkworm, the adult antenna consisted of three parts; scape, pedicel and flagellum (Fig. 1A). The flagellum was made up of many annuli. Two barbs branched out at each side of every annulus except for the ones at the top of flagellum (Fig. 1B).

Morphogenesis of the antennal imaginal disk

In order to clarify when the antennal imaginal disk began morphogenesis and when the number of annuli was determined, histological studies were performed, in which the morphology of antennal imaginal disk during the whole process

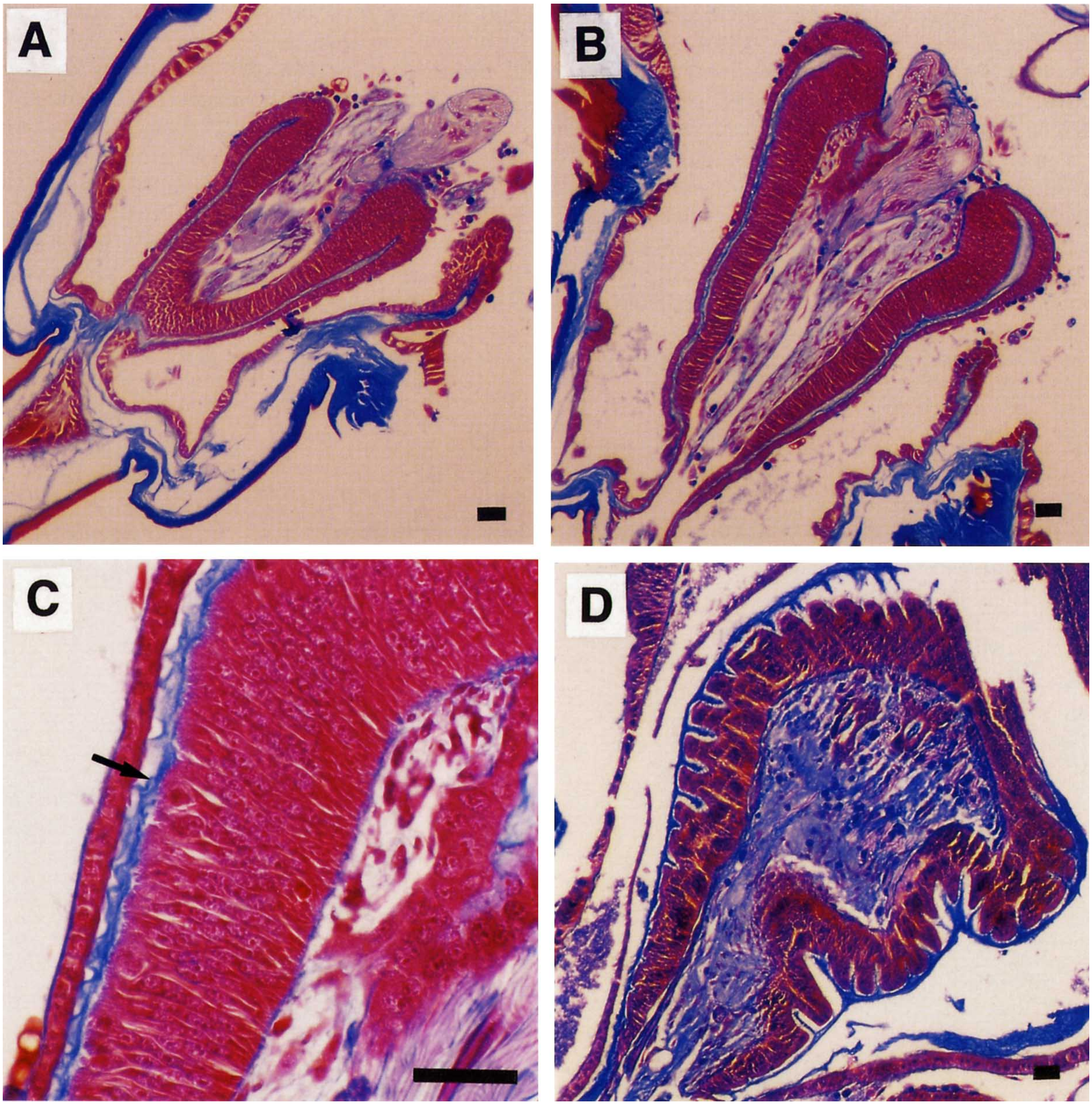


Fig. 2. Development of the antennal imaginal disk. (**A**) The imaginal disk on the second day in the fifth instar larva. (**B**) The imaginal disk at gut-purging stage. (**C**) Higher magnification of the imaginal disk at gut-purging stage. (**D**) The imaginal disk on the second day after gut-purging. Arrow shows the one segment formed in the imaginal antennal disk. Bar = 20 µm.

Fig. 1. Features of adult antenna in eri-silkworm, *Samia cynthia ricini.* (**A**) The whole shape of adult antenna. Adult antenna was about 10-mm long and consisted of three parts; scape (S), pedicel (P) and flagellum (F). Flagellum was made up of many annuli. (**B**) The shape of annulus (An). Two barbs (Bb) branched out from one annulus at each side.

of fifth instar larva was serially examined (Fig. 2). The antennal imaginal disk surrounded by thin epithelium, peripodial membrane, existed underneath larval antenna in the fifth instar larva. A system of tracheae penetrated between the two cell layers of the imaginal disk. On the second day in the fifth instar, the segmentation was not observed in the antennal imaginal disk (Fig. 2A). On gut-purging, the antennal imaginal disk had grown up rapidly (Fig. 2B). Higher magnification revealed that the segmentation of the pupal antenna had already begun (Fig. 2C). On the second day after gut-purging, the segmentation of the pupal antenna was almost finished (Fig. 2D). On the third day after gut-purging, the antennal imaginal disk had evaginated beneath the larval cuticule. On the fifth day after gut-purging, the insect pupated. The number of segments of pupal antenna was nearly equaled to the number of annuli of adult antenna, although pupal and adult antennae were morphologically different (data not shown). Therefore, it was suggested that the number of annuli of adult antennal flagellum was finally determined by the second day after gut-purging.

Distributions of the number of the annuli of flagellum and the length of the embryonic and larval periods

In order to examine the influence of the embryonic and larval period on annulation, the number of annuli were counted in 377 adult antennal flagella (206 males and 171 females). It was revealed that the number of annuli varied from 27 to 34 (Fig. 3A). The number of annuli in female (31.1 ± 0.1; mean ± SE) was significantly larger than that in male (30.4 ± 0.1; mean ± SE) (Student's t test, $P < 0.05$).

The period through which the insects passed from oviposition to gut-purging (the embryonic and larval period) was examined in 598 specimens (310 males and 288 females) (Fig. 3B). The period varied from 27 to 35 days. The embryonic and larval period in female (30.3 ± 0.1; mean ± SE) was significantly longer than that in male (29.5 ± 0.1; mean ± SE) (Student's t test, $p < 0.05$). The distribution of the numbers of the embryonic and larval period (days) was similar to that of the numbers of the annuli in both males and females.

The relation between the number of annuli and the embryonic and larval period

Since the histogram of the number of annuli and that of the embryonic and larval period showed similar patterns, it was suggested that these two parameters might be closely related with each other. It was also suggested that the sexual difference in the number of annuli might be due to the

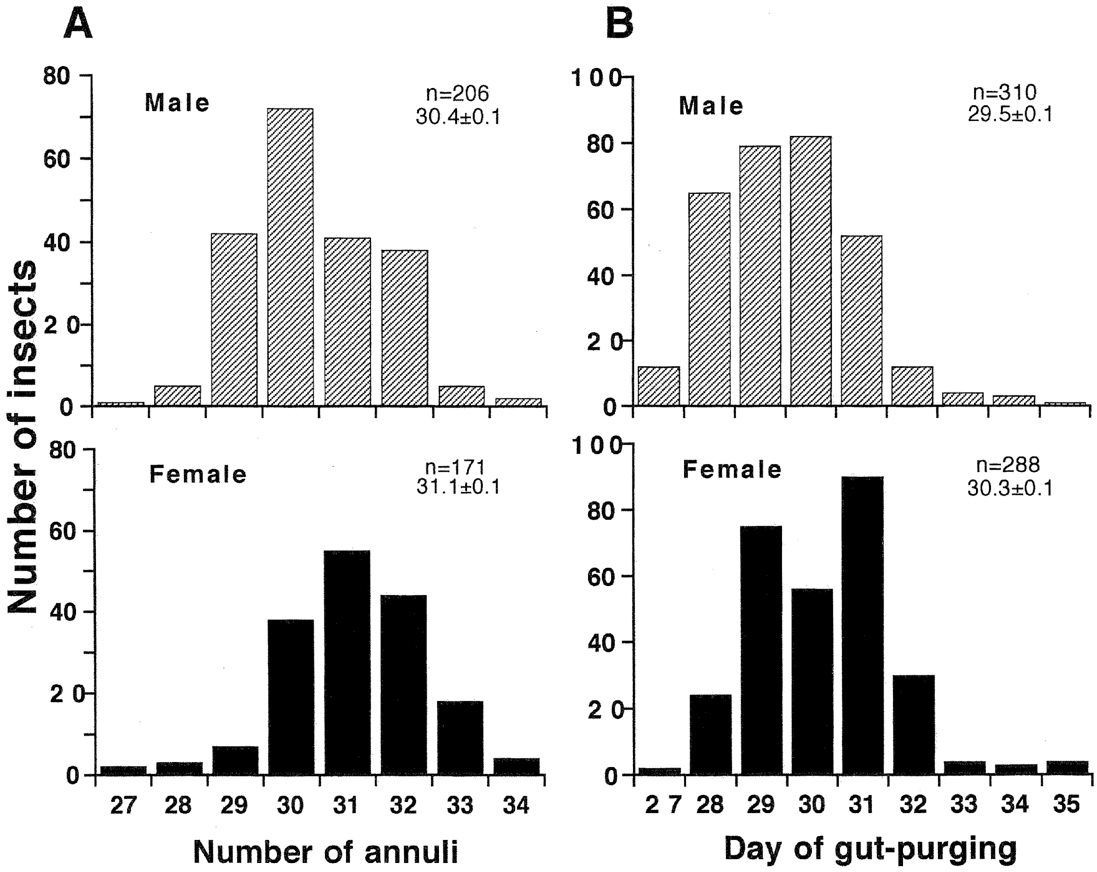


Fig. 3. Distributions of the number of annuli of adult antenna and the embryonic and larval period. (**A**) Distribution of the number of annuli of adult antenna. The annuli were counted in 206 males and 171 females. (**B**) Distribution of the embryonic and larval period. The days needed to pass from oviposition to gut-purging were examined in 310 males and 288 females. Since we found no difference in the number of annuli between right and left antennae, only the results from right antennae are shown.

difference in the embryonic and larval period. To confirm this relationship, prospective studies were performed, in which the period needed to pass from oviposition to gut-purging and the number of annuli formed were examined in the identical insects. The insects were grouped according to the length of the embryonic and larval period (days); the frequency distribution of the number of annuli in each group is shown in Fig. 4. When the insects were reared at 25°C, the number of annuli tended to increase one by one as the embryonic and larval period was extended day by day from 28 to 32 days (Fig. 4A). The number of annuli showing highest frequency in each group was larger than the number of days representing the embryonic and larval period by 2.

To further examine the influence of the embryonic and larval period on the annulation, we next reared the insects at 18°C, since it had been assumed that the larval period would

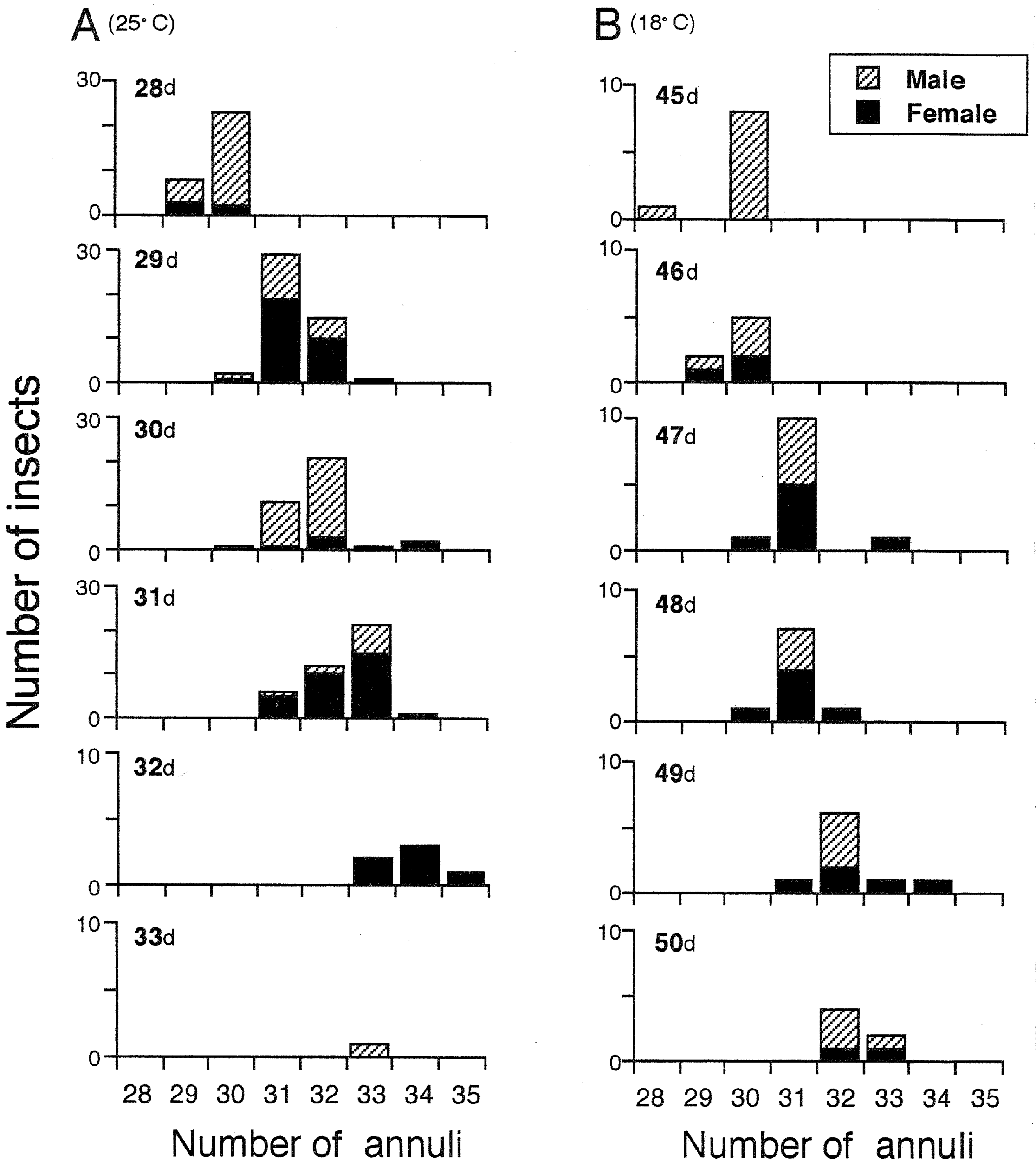


Fig. 4. Relationship between the number of annuli and the embryonic and larval period. The insects were grouped according to the length of the embryonic and larval period. The numerals depicted at top left of each graph shows the days needed to pass from oviposition to gut-purging. The experiments were repeated three times, in which similar results were obtained. (**A**) The insects were reared at 25°C. The embryonic and larval period varied from 28 to 33 days. The number of annuli differed significantly among the six groups (Kruskal-Wallis test; $P < 0.05$). (**B**) The insects were reared at 18°C. The embryonic and larval period varied from 45 to 50 days. There was no significant difference in the number of annuli between 45 and 46 days, between 47 and 48 days, and between 49 and 50 days, respectively. However, the number of annuli differed significantly among the three groups; 45 ~ 46 days, 47 ~ 48 days, and 49 ~ 50 days (Kruskal-Wallis test; $P < 0.05$).

be prolonged at lower temperature. At 18°C, the larval period (from hatching to gut-purging) was twice as long as that at 25°C (data not shown); consequently, the embryonic and larval period was prolonged to as long as 45-50 days (Fig. 4B). Contrary to our expectation, the number of annuli formed at 18°C did not double but remained in the same range (28-34) as that formed at 25°C. On the other hands, the insects seemed to obtain one annulus at every two-day period when reared at 18°C.

After the insects were grouped according to the embryonic and larval period, no difference in the results was observed between male and female, as shown in Fig. 4. Therefore it was revealed that the difference in the number of annuli observed between male and female (Fig. 3A) was due to the difference in the embryonic and larval period.

The relation between the number of annuli and the body size

We next studied whether the insect's body size might have any influence on the number of annuli. For this purpose, the body weight and the head width were measured soon after gut-purging; the relation between these parameters and the number of annuli was assessed. Figure 5 shows the results in the insects reared at 25°C. The body weight was significantly (Student's t test, $P < 0.05$) different between males and females, the females being heavier than the males, whereas the head width was not different between both sexes. Neither the body weight nor the head width was correlated with the number of annuli in either sex.

When the insects were reared at 18°C, the body weights of both males (3.55 ± 0.05 g; mean ± SE, n = 82) and females (4.24 ± 0.06 g, n = 83) were significantly (Student's t test, $P < 0.05$) heavier than those reared at 25°C, whereas the head widths of males (4.18 ± 0.01 mm; mean ± SE, n = 66) and females (4.29 ± 0.01 mm, n = 66) did not significantly differ from those reared at 25°C. Similar to the results at 25°C, no significant correlation was observed between either the body weight or the head width and the number of annuli at 18°C (data not shown).

DISCUSSION

The present study demonstrated the intimate correlation between the number of annuli and the length of the embryonic and larval period. There was the significant difference in the number of annuli between males and females, but this difference seemed to be due to that of the length of the embryonic and larval period in males and females. No correlation was found between the number of annuli and the body size in spite of the difference in body weight between the sex. Therefore, it is the embryonic and larval period that influences the number of annuli of adult antennal flagellum.

Although the larval period was doubled under low temperature (18°C), the number of annuli remained in the same

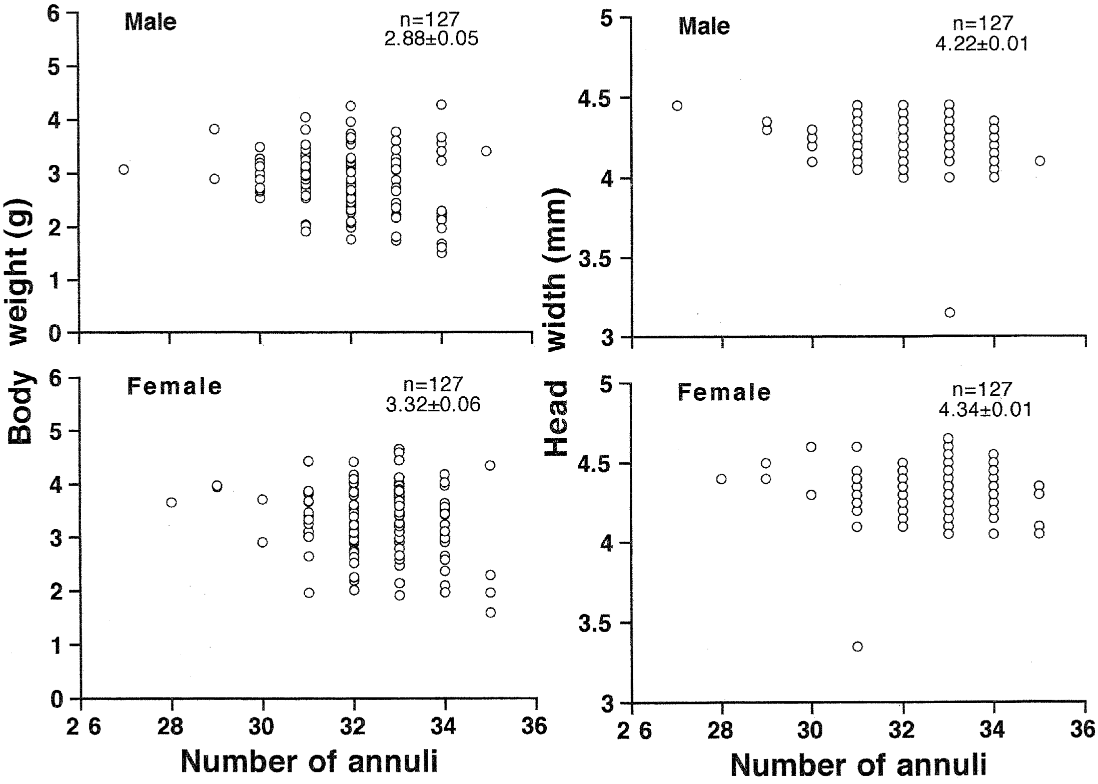


Fig. 5. Correlations between the number of annuli and the body size as assessed by the head width and the body weight. The insects were reared at 25°C. The numerals depicted at top right of each graph shows the number of insects and mean ± SE.

range as that of the insect reared at 25°C. Therefore it seemed that the number of annuli might vary in the fixed range.

Little has been known about the determination of morphology of adult antenna in Lepidoptera insects. Takahashi and Oka (1973) reported that the excess of 20-hydroxyecdysone administered soon after pupation did not influence the annulation of the flagellum, whereas it suppressed the barb formation. Their results suggested that events occurring in morphogenesis of adult antenna might be determined at their own stage respectively. It was also suggested that the number of annuli of flagellum might be earlier determined than other event in morphogenesis of adult antenna. Our histological observations clarified the stage of determination of the number of annuli. The segmentation of antennal imaginal disk was first observed at gut-purging, and almost completed at second day after gut-purging. The number of annuli of the adult antenna was finally determined by second day after gut-purging. In *Manduca sexta*, the rapid growth of the antennal imaginal disk was also observed during the last few days of the final larval instar (Sanes and Hildebrand, 1976). Morphogenesis of the antennal imaginal disk seemed to start in the last larval instar.

As compared with the antennal imaginal disk, more information has been obtained with regarded to the wing disk development. The actual rapid growth and differentiation were also observed in the wing disk after the second half of the last instar larvae (Nijhout, 1994). In *Bombyx mori*, Nagata (1961) found that there were two phases of cell divisions in the wing disk; the first cell division phase on the first to the second day in the fourth instar and the second phase in the last larval instar. Kurushima and Ohtaki (1975) suggested that the first phase of the cell division might be concerned with the acquisition of 'competence' of the wing disk. Their results suggested that the plan for the adult development might be determined long before the beginning of the morphogenesis. On the other hand, in our results the number of annuli of the adult antennal flagellum varied from 27 to 34 as the embryonic and larval period extended. This difference in the number of annuli seemed to be due to that in the length of last instar larval period (days), since the insects molted synchronously until the fourth ecdysis. Therefore, the number of annuli of the adult antennal flagellum was not strictly determined by the day of gut-purging.

The number of annuli of the adult antennal flagellum increased one by one as the larval period extended day by day in the eri-silkworm. The number of annuli was tended to coincide with days from oviposition to the second day after gut-purging. We speculated about the possibility that the annulus of the adult antennal flagellum might be prospectively determined one by one in the course of the larval period. The development of the imaginal nervous systems have been studied more actively. White and Kankel (1978) investigated the formation of the imaginal nervous system in *Drosophila melanogaster*. According to their report, the formation of the imaginal nervous system in *Drosophila* started in the first larval instar and continued throughout the larval period. The neuroblast in the central nervous system divided unequally, giving rise to a neuroblast and a ganglion mother cell. The latter cell arrested its development soon after it was born, and then matured into a functional adult neuron through metamorphosis. Similar observation was reported in the monarch butterfly, *Danaus plexippus plexippus* (Nordlander and Edwards, 1969a, b). Poulson (1950) reported the existence of the cell populations that would give rise to the adult optic lobe in *Drosophila* embryo. Therefore, it was suggested that the imaginal nervous systems developed continuously from the embryo to the pupa and then matured all at once into the functional adult neurons through metamorphosis. Although it has been generally believed that the imaginal disk remains undifferentiated and grows slowly during larval life, we think that plans of adult antennal development have been made in the course of larval period, and are carried out during metamorphosis. However, little has been known about the mechanism of determination of the annulation of the adult antennal flagellum. We next try to study how an endocrinologically modulated larval period influences the number of annuli of the adult flagellum, and also examine the morphogenesis of the adult antennal imaginal disk in the early larval stage.

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(Received June 18, 1996 / Accepted February 7, 1997)