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An efficient production management of the silkworm variety "Daewhangjam" through low temperature in a pupa

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Abstract

The Daehwangjam has excellent marketability and quality. However, precise silkworm breeding data on the recent high-temperature environment is not enough collected, and it is necessary to check whether stable Daewhangjam silkworm egg production was possible in poor environments. In this study, the timing of metamorphosis of the Daewhangjam parents Jam323 and Jam324 silkworms, in an optimal condition and a general condition similar to breeding grounds, was investigated to confirm that appropriate mating was achieved. Surprisingly, a number of female moths were discarded due to the metamorphosis of Jam324 female moths much earlier than Jam323 male moths in either an optimal or a general condition. To improve this, during the Jam324 female pupation period, the mild low-temperature of 15℃ was treated for the pupation period to delay the metamorphosis into female moths. Depending on the treatment period, the metamorphosis of the female pupa was delayed by more than 4 days, allowing normal mating with the Jam323 male moth. The eye pigments in a pupa also slowly accumulated in these treatments. In order to discover the side effects of low-temperature treatments, the voltinism of the Daewhangjam eggs was investigated, but most of them remained univoltine. Taken together, the low-temperature treatment during the female pupation period made it possible to use Jam324 female moths that were should be discarded, which means that stable and efficient management of Daewhangjam egg production is possible.

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Introduction

Silk is believed to have been produced by breeding silkworms from about 4,600 years ago (Kurin, 2002), and the sericulture industry was considered to have a very important economic value until the modern era. As the silkworm industry in Korea has a long history, the industrial system is very well organized. According to the state-designated silkworm variety distribution and management system, Korea raises the parent silkworms of varieties for farmhouse distribution, and local institutions or special farms produce silkworm variety eggs and distribute them to farms that need them. The variety silkworm raised

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by farmers is the F1 hybrid silkworm obtained by mating two different silkworms. This is to produce high-quality silkworms using heterosis. For stable production, the Rural Development Administration (RDA) breeds the parent female moth and male moth of the variety silkworm and distributes them to variety silkworm egg producers. In order to produce F1 hybrid silkworm varieties, parent male moths and female moths of the original silkworm species for the variety must be metamorphosed at the similar time to proceed with breeding. At this time, the male moth should be metamorphosed first to prepare for mating. After that, the female silkworm breeding period is adjusted so that the female moth metamorphoses later. If there is no male moth to mate immediately when the female moth metamorphoses early, the female moth doesn't produce normal eggs and must dispose of most of them. This causes economic losses in the production of silkworm variety eggs and confusion in the plans for the distribution of varieties managed by the government. Therefore, in institutions and special farms that produce varieties for distribution to farms, it is necessary to metamorphose male moths first when producing silkworm variety eggs, and the technique for this will also be required.

Silkworm varieties are simply classified by the color of silk among the methods of classifying them. It is mainly divided into varieties that produce white silk or varieties that produce colored silk. Recent studies have shown that silk-colored varieties have the ability to transform or accumulate phytochemicals such as carotenoids derived from mulberry leaves in various ways (Tsuchida and Sakudoh, 2015). Therefore, color silk could have additional and positive effects to human, but these effects have been discovered to date. Currently, the representative silk-colored variety mainly distributed in Korea is the Golden-Silk (GS) variety with yellow silk. GS, which is a variety developed in Korea, is an F1 hybrid silkworm obtained by cross-breeding Jam311 and Jam312 parent silkworms, and is small in size compared to other varieties, but has strong, excellent cocoon quality, as well as high productivity (Kang *et al.*, 2007). GS has a different functionality from white silk-colored silkworms, and is attracting expectations from special patients and medical staffs (Nguyen *et al.*, 2020; Nguyen *et al.*, 2021). In particular, compared to other silkworm varieties, it has been found to improve the function of the brain nervous system, which seems to have the similar effect of other yellow silk-colored silkworms such as the Daewhangjam as well as the GS (Guo *et al.*, 2021). However, GS, which began to be distributed in 2006, has been developed as a main variety for a long time, and the original parent silkworms are deteriorating, so it may not produce the same stable quality as before. There is a growing demand for a replacement from farms to better new varieties. Accordingly, RDA developed a high-quality Daewhangjam with yellow silk like GS in 2018, paving the way to replace GS. However, it was difficult to collect precise silkworm breeding data on the actual various breeding environments, and there were doubts about whether Daewhangjam could actually be produced stably. This has been an obstacle for farmers to produce Daewhangjam instead of GS, and is the reason why Daewhangjam cannot be widely distributed to farmers despite the good quality.

In this study, the difficulties in producing a Daewhangjam variety were identified according to the environmental conditions. In addition, experiments were planned to find a way to stably produce Daewhangjam eggs and maximize their yield through temperature treatment in pupation stage of parent silkworms. In addition, the stability of the pupa in temperature treatment was confirmed by investigating the existence of side effects about the results.

Materials and Methods

Silkworm strains and breeding method

The Daewhangjam's parent silkworms Jam323 and Jam324 silkworms were provided by the RDA which maintains pure protection systems of Korean silkworm resources. Silkworms were bred based on the silkworm breeding standards from the RDA, excepting temperature conditions. Depending on the experimental conditions, silkworms were raised at variable temperature conditions of 25°C optimal conditions or 22 to 28°C general conditions, and both conditions maintained about 70% of humidity. The temperature according to each condition was maintained until metamorphosis into moths. The silkworm fed fresh mulberry leaves three times a day. The mulberry leaves were produced by the RDA.

Temperature treatment and investigation into the pupation period

On the 7th day after pupation from a silkworm, each female pupa was treated at a low temperature of 15°C for 2 or 4 days. After the low temperature treatment, the female pupa returned to the corresponding control treatment and investigated the entire period of pupation until metamorphosis to a female moth. The number of metamorphosed silkmoths was investigated according to the date.

Voltinism of Daewhangjam eggs

The Daewhangjam eggs produced by the mating of metamorphosis-delayed Jam324 female moths and Jam323 male moths which grew under optimal conditions, were investigated to determine the voltinism. The mating Jam324 female moths laid eggs, and the female moths were removed a day later. The Daewhangjam eggs laid on paper were treated at 25°C for two weeks. In the case of univoltinism, the color of the egg turns black within three days. If it is not univoltine, the egg color changes slowly and the silkworm hatches within two weeks. The number of black eggs was investigated after two weeks.

Statistical analysis

An Oneway Analysis of Variation (ANOVA) by SPSS Statistics 23 (IBM, USA) was used for statistical analysis, and the significance of each mean was determined by Duncan's multiple (P<0.05).

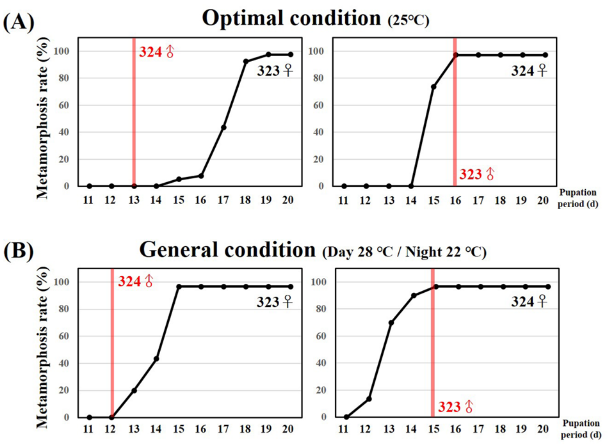

Fig. 1. Differences in the pupation periods between Jam323 and Jam324 pupae under optimal conditions or general conditions for the production of Daewhangjam eggs. Under an optimal condition, there are differences between the appearance timing of (A) the Jam323 female moth and the Jam324 male moth and between (B) the Jam324 female moth and the 323 male moth. Also, under a general condition, there are differences between the appearance timing of (C) the Jam323 female moth and the Jam324 male moth and between (D) the Jam324 female moth and the 323 male moth. The black line refers to the number of metamorphosed female moth according to the date, and the red line refers to the first metamorphosis day of each male moth group.

Results and Discussion

Differences in metamorphosis timing between the Jam324 female pupa and Jam323 male pupa for mating

A Daehwangjam is an F1 hybrid silkworm based on Jam323 and Jam324 silkworms raised and managed by the RDA in Korea. For the stable production of Deawhangjam, the mating of the parent moths must be made definitively. However, since the breeding period and pupation period vary depending on the silkworm resources which were preserved in Korea, an experiment was conducted to confirm the period, and the effect according to the breeding environment was also investigated to know the difference in the actual farmhouse. During the silkworm larva period, it is very complicated to control the period because various factors, such as environmental factors, diseases and the quality of mulberry leaves, affect it. Therefore, this study was focused on adjusting the pupation period of a pupa which is less related to diseases or feeding and can clarify environmental factors. First, the period of a pupa was investigated to find out the timing of moth metamorphosis of the Daewhangjam parent Jam323 and Jam324 at a constant temperature of 25 °C which is an optimal temperature for silkworm breeding (Fig. 1A). In the case of the Jam323 female moth, there is no problem with the production of Daewhangjama eggs because it metamorphosed later than the Jam324 male moth. The Jam324 female moth, meanwhile, metamorphosed about two days earlier than the Jam323 male moth, so most of the Jam324 female moth had to be discarded. This means that about half of the total Jam324 moths cannot be used to mate for producing the Daewhangjam eggs, and more silkworms should be raised to meet the Daewhangjam eggs distribution plan. As a result, more labor and space are needed and more mulberry leaves are also demanded, resulting in various economic losses. In a laboratory, silkworm breeding is carried out by precisely controlling the circumstances, while most mass breeding farms and local institutions may have difficulty controlling the conditions. In particular, high temperatures may occur during the spring season due to global warming and abnormal weathers. This environmental factor makes silkworm eggs to be produced at a wide range of environmental fluctuations, including high temperatures, at a time when breeding is concentrated in spring in Korea. Breed farmers and local institutions need silkworm production manuals for these environmental conditions. Therefore, to similarly implement the temperature condition based on general farms, silkworms were raised in a general condition with temperature fluctuations of 22 to 28℃ in this study, and the pupation period of the parent silkworm moth was compared with these conditions (Fig. 1B). As a result, the pupation periods of the parent moth were promoted by 1 to 3 days earlier under general conditions in which high and low temperatures were alternately treated than under optimal conditions in which a uniform temperature of 25°C was applied. It is suggested that the growth of silkworms was accelerated by high temperatures, leading to rapid moth metamorphosis. The Jam323 female pupae metamorphosed into moths from the 13th day of the pupa, and all pupae metamorphosed into moths on the 15th day. The Jam324 male moth occurred from the 12th day and was able to prepare for mating with the Jam323 female moth, and as a result, there was no problem in the production of Daewhangjam silkworm eggs. However, it was confirmed that mating between the Jam324 female moth and the Jam323 male moth is impossible in the general condition, as well as in the optimal condition. The Jam324 female moth metamorphosed very early from the 12th day in the general condition, and all Jam324 female moths metamorphosed on the 15th day. The rate of Jam324 female moths that metamorphosed at 15th day was 6.7% of all Jam324 female moths (Fig. 1B). Because the Jam323 male moth began to metamorphose on the 15th day, almost Jam324 female moths were unable to mate and had to be disposed of. It means that 93.3% of Jam324 female moths at the same time could not be used for mating, and that means labor and mulberry leaves were consumed in excess. In a word, when Jam324 female moths and Jam323 male moths were raised at the same time under either optimal or general conditions, the mating for production of Daewhangjam eggs was impossible due to the difference in metamorphosis timing between the two moths.

The delayed metamorphosis of Jam324 female pupae through low temperature treatments

Since the metamorphosis timing between the Jam324 female moth and the Jam323 male moth was not appropriate, the mating was impossible. Previous studies have shown that treating low temperatures during the pupation period of silkworms, as well as other insects, delays the metamorphosis into moth (Bale, 2002; Liang, 1985; Mellanby, 1939; Nijhout and Williams, 1974). Based on this, low temperature was treated in the Jam324 female pupa to confirm whether there is a tendency to delay the metamorphosis of the Jam324 pupa which is one of the parent silkworms of Daewhangjam. In this study, for effective and stable production of Daewhangjam silkworm eggs, an experiment was conducted to delay the metamorphosis timing of the Jam324 female moth to make it similar to or later than that of the Jam323 male moth, especially focusing on the Jam324 female pupa. Since previous papers showed that the low-temperature treatment of 5°C strongly causes changes in physiology and gene-expression of insects, experiments were conducted by applying mild low-temperature treatment to stably maintain the quality of the silkworm variety (Kihara *et al.*, 2009; Moribe *et al.*, 2001). In the experiment, seven-day female pupae

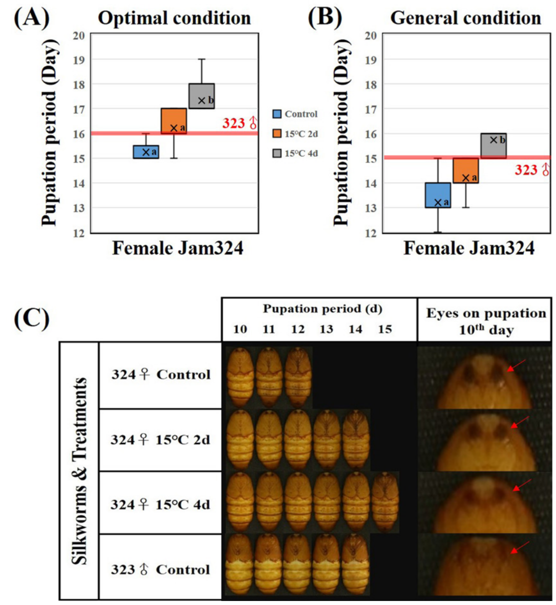

Fig. 2. Changed metamorphosis timing and pigmentation of pupa eyes in Jam324 female pupa due to low temperature treatments. The changes in the pupation period of the female pupa by low temperature treatments are shown in the quartile table. Changes in the metamorphosis timing of the Jam324 female pupa by low temperature treatments (A) under optimal conditions or (B) under general conditions. The red line indicates the day of first metamorphosis of the Jam323 male pupa for mating. The X in the box suggests the average pupation period for each group. Each group consists of more than 30 pupae. The different letters next to X mean significance with control group ($P<0.05$). (C) According to temperature treatments, pupae showed different eye colors, and low temperature treatments affect to delaying of pigment accumulation on Jam324 female pupa. The red arrow implies the location of a silkworm pupa eye.

after the cocoon began to be built were treated at 15°C for two or four days, and the pupae were transferred back to the control group treatment to compare the whole pupation period (Fig. 2). Under optimal conditions, Jam324 female pupae metamorphosed into moths on the 15th to 16th day, but when they were treated in the low temperature, the metamorphosis was delayed depending on the treatment period (Fig. 2A). When a pupa was treated for the two days of 15°C, the moth metamorphosis was slightly delayed by about a day under optimal conditions, and when treated for the four days, the metamorphosis was delayed by one to four days. Since a Jam323 male moth metamorphosed on 16th day, there is little loss of the Jam324 female moth by treating a low temperature to a Jam324 female pupa. Conclusively, it means that it is possible to produce Daewhangjam eggs stably by low temperature treatments. In general conditions where the temperature fluctuation between day and night occurred, the overall pupation period tended to be advanced due to the high temperature during the day (Fig. 2B). In the control group of the general conditions, the Jam324 female pupa had a wide range of pupation periods ranging from 12 to 15 days. Through low temperature treatments, the Jam324 female pupae of the general condition showed pupation periods of 13 to 15 days in two-day treatment at 15°C and 15 to 16 days in four-day treatment. Specifically, under general conditions, the low temperature treatment not only delayed the pupation period of the Jam324 female pupa, but also tended to narrow the range of the period. Under general conditions with the fluctuated temperature, the Jam323 male pupa started metamorphosis on the 15th day, so when the Jam324 female pupa was treated for four days at 15°C, the Daewhangjam egg was able to be produced. The degree of metamorphosis of a silkworm pupa can be also confirmed by comparing the changes in the eye color of the pupa. In the early stage of a pupa, there is a little pigment in the eyes, and as the moth metamorphosis approaches, the pigment in the eyes changes dark and dark. As shown in Figure 2C, under general conditions, the Jam324 female pupa had already been heavily pigmented on the 10th day of the pupa, but the Jam323 male pupa was still found to have no eye pigment at that time. However, when the Jam324 female pupa was treated at 15°C for two days, it was shown that the eye pigment was mildly black color compared to the control group, and when treated for 4 days at 15°C, it was confirmed that the eye pigment was lighter than that of 2 days at 15°C. Taken these results together, it suggests that moth metamorphosis was delayed by low temperature treatments at 15°C in a Jam324 female pupa and the pupal eye pigmentation indirectly implied the metamorphosis timing of a silkworm moth.

Univoltinism of Daewhangjam eggs from Jam324 female moths treated at low temperatures during pupation periods

The Korean government plans the silkworm industry. Under detailed planning, silkworm eggs of the varieties produced in spring, are distributed to farmers in the spring of the next year. Therefore, the silkworm eggs produced for distribution must be uniformly diapaused, and silkworm varieties that are bivoltine or polyvoltine will be difficult to proceed with the distribution according to the national plan. However, the low temperature treatments of a female pupa or a female moth adversely affects the eggs inside it, and becomes a factor that causes the change in the voltinism of fertilized eggs (Tsurumaki *et al.*, 1999; Zambrano-González *et al.*, 2023). In this experiment, the diapaused egg ratio of the fertilized Daewhangjam eggs was checked to determine whether the eggs of the low-temperature-treated Jam324 female pupa remained univoltine (Fig. 3). Since diapaused silkworm eggs are able not to hatch naturally in a month, the Daewhangjam eggs, which produced by the low temperature-treated Jam324 female moth, were at 25°C to investigate the presence of naturally hatched eggs in a month. As shown in Figure 3A, as a result, about 92 percent of the eggs from control group were determined to be diapaused. Nearly 95 percent of the eggs of the moth treated on the two days of 15°C were identified as diapaused eggs, which seemed slightly

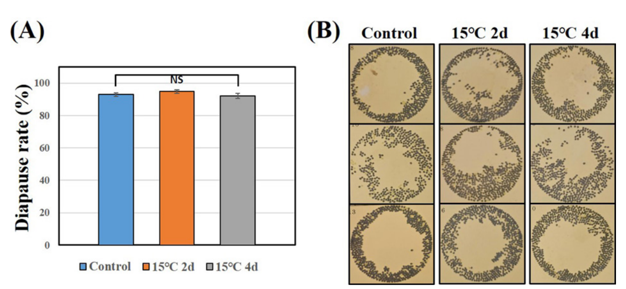

Fig. 3. The Voltinism of Daewhangjam eggs produced by Jam324 female moths treated with low temperatures. (A) Ratio of diapaused eggs produced by mating Jam324 female moths and general Jam323 male moths according to treatments. (B) Changes in egg colors after two weeks incubated at 25°C according to treatments. Black eggs mean diapaused. Each group was conducted with more than 7000 eggs. Ns means that there is no statistical significance.

higher than the control, but it was not significant. The eggs of the moth treated on the four days of 15°C were identified as approximately 92% diapaused eggs, showing no significant difference from the control. As shown in the control group of Figure 3B, the diapaused egg color changes black in two or three days when the eggs were incubated at 25°C. On the other hand, non-diapaused eggs slowly change color, and silkworms hatch from the eggs in two to three weeks. The Daewhangjam silkworm eggs of both 15°C two-day treatment and 15°C four-day treatment groups changed egg color quickly like those of the control group, indicating that most fertilized eggs remained univoltine even if Jam324 female pupae were treated at low temperatures of 15°C for 2 or 4 days. Therefore, it is believed that the low temperature treatment of the Jam324 pupa does not change the voltinism of the Daewhangjam eggs, and in conclusion, delays only the time of female moth metamorphosis without side-effects

An efficient production of Daewhangjam silkworm eggs through low temperature treatment in a Jam324 female pupa

The Daehwangjam is a promising silkworm variety that will replace GS which is a Korean main colored-silkworm variety, and is a potential industrial material and an important genetic resource that will continue the silkworm industry in Korea. Nevertheless, there is a reason why the Daewhangjam, known to have superior performances to the GS in terms of size and weight, is not yet preferred by farmers in Korea. It is the instability of a Daewhangjam production from the difference in the metamorphosis timing between female and male moths. Jam323 female moths metamorphose later than Jam324 male moths (Fig. 4A). The belatedly metamorphosed Jam323 female moth produces healthy Daewhangjam eggs through normal mating with a Jam324 male moth. However, the situation has a trouble between Jam324 female moths and Jam323 male moths. The Jam324 female pupa begins moth metamorphosis considerably earlier than the Jam323 male pupa (Fig. 4B). Jam324 female moths occurred on the 15th day of pupa in optimal conditions and on the 12th day of pupa in general conditions. At this time, Jam323 male moths began to metamorphose on the 16th and 15th, respectively. Since there is no male moth to mate with the Jam324 female moths, they cannot be used for the production of Daewhangjam eggs and should be discarded. For this reason, the plan to distribute eggs of the Daehwangjam variety in the following year may be disrupted, which also causes economic losses to the entire sericulture industry in Korea. In this paper, the metamorphosis of a female moth was delayed through low-temperature treatments in the Jam324 female pupa (Fig. 4C). The delayed Jam324 female moth had normal mating with a Jam323 male moth, and the produced fertilized eggs were confirmed to be univoltine. In conclusion, the loss of the Jam324 female moth decreased from 93.3% to 0% sharply through the low-temperature treatments of the 324 female pupa, and most of the moths could be used for the production of Daewhangjam eggs. These results suggests that the low temperature treatments could be applied not only to a Jam324 silkworm, a Daewhangjam source, but also to a number of other silkworm resources for other variety egg productions, and are expected to promote the development of the sericulture inustry in Korea.

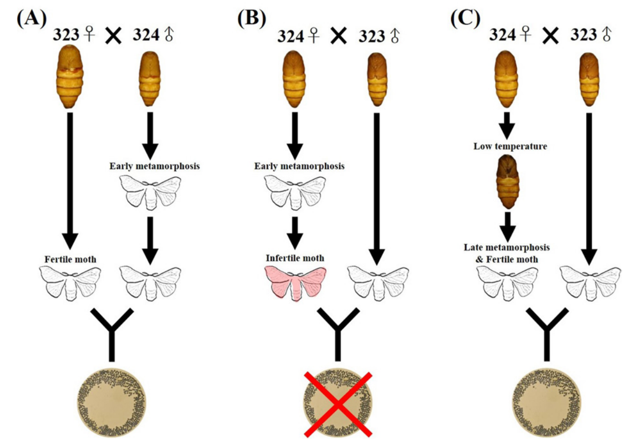

Fig. 4. An efficient production model of Daewhangjam silkworm eggs by low temperature treatments to the Jam324 female pupa. (A) In respect of mating between the Jam324 male moths and Jam323 female moths, Jam324 male pupa metamorphoses into a moth early on, allowing breeders to produce Daewhangjam eggs normally. (B) In the case of producing Daewhangjam eggs using Jam324 female moths and Jam323 male moths, the Jam324 female moths metamorphose at a fairly early stage, and most female moths are discarded due to absence of male moths. This makes it impossible to produce Daewhangjam eggs. (C) To overcome this, the female moth metamorphosis is delayed by treating the Jam324 female pupa at low temperatures. As a result, the late metamorphosed Jam324 female moth can be timed to mate with the Jam323 male moth, enabling efficient and stable production of Daewhangjam eggs.

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