Relative humidity and maintenance of p50 silkworms reared on artificial diet

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We found that artificial diet rearing of the silkworm strain p50 under high humidity was harmful to adult emergence. Specifically, median relative humidity (RH) greater than 74% during the 5th instar induced a high rate of occurrence of naked pupae, which then died without emerging as adults (Experiment 1). To determine the specific stage in which mortality induced, we divided the last instar period into three stages—early, middle, and late—and then subjected the larvae to low (L: 47% median RH) or high (H: 80% median RH) humid conditions. The occurrence of naked pupae decreased not according to the specific stage of the larvae but to the duration of exposure to the “L” condition (Experiment 2). We propose that to maintain p50 strains on an artificial diet, they should be reared in lower humidity more than 2/3 period in the last instar stage.

Key words: Bombyx mori, cocoon, high humidity, naked pupa, rearing environment

INTRODUCTION

Silk spinners (e.g. spiders, moths, mites, mayflies) and cocoon makers (e.g. spiders, moths, bees, flies) have repeatedly evolved in arthropods, and human beings have selected some of these moths for use in silk production. Environments sometimes harmfully affect cocoon quality as well as the spinners that inhabit them. For instance, the parasitoid bee, Cotesia glomerata, forms cocoon clusters to protect themselves from hyperparasitism (Tanaka and Ohsaki, 2006). However, high humidity causes individual cocoons to be thinner and the clusters to be loose (Tagawa and Satoh, 2008). The harmful effects of high humidity on the quality of cocoons and silk threads are well known in sericulture (Yamamoto, 1975; Kataoka, 1977). Recent experiments have shown that very high humidity negatively affects successful mounting and results in an increase of naked pupae (Watanabe et al., 2014). These are different from genetic naked-pupa (Nd) (Nakao, 1950) that cannot produce fibroin, which leads either to cocoons being constructed exclusively of sericin or to naked pupae. The frequency of adult eclosion has been found to be 88.7% in the naked pupae of Nd mutants (Nakao, 1950). Another naked pupae can be experimentally induced by blocking the spinneret opening, causing them to be naked and thus not become adults (Akao, 1942). These results suggest that physically induced naked pupae are defective and unable to become moths.

Two silkworm genome data were deposited into public databases, one originating in Japan (Kaikobase: http://sgp.dna.affrc.go.jp/KAIKObase/) and the other in China (SilkDB: http://silkworm.genomics.org.cn/). These open-source databases have been widely used by entomologists, especially lepidopteran molecular scientists (The International Silkworm Genome Consortium, 2008; Daimon et al., 2014; Xia et al., 2014). The strains, p50T (Daizo) and Dazao used for the respective genome projects, should be very close relatives. A p50T is a single-paired descendant of individuals of strain p50 (a derivative from Daizo kept in the national bioresource project (NBRP) Silkworm) (see http://shigen.nig.ac.jp/silkwormbase/top.jsp). Since the p50 individuals are available through the NBRP Silkworm project and are possible to rear not only with mulberry leaves but also with artificial diets (e.g. Silk Mate, NOSAN Corporation), these silkworms are one of the most important reference strains for lepidopteran studies (Fujiwara and Nishikawa, 2016). Artificial diet rearing has advantages in keeping the silkworms in an incubator throughout the year.

In sericulture adequate temperatures for rearing silkworms with mulberry leaves are considered at 27-29°C for 1st instar larvae. Then, breeders may decrease temperature at 1°C after each molting up to 22-24°C in the last instar larvae (The Japanese Society of Sericultural Science, 1979). Relative humidity (RH) is recommended at 85-90% in the beginning and to decrease at approximately 5% in every ecdisis until last instar stage (Kawaguchi and Yanagawa, 1992). The Japanese Society of Sericultural Science (1979) and, Kawaguchi and Yanagawa (1992) claimed that the last instar stage is the most important for silkworm rearing. Ueda et al. (1988) recognized the general importance of lower humidity for parental silkworms for hybridization reared on artificial diets. Their standard—70-80% RH during the 5th instar for the parental strain rearing—sounds similar to preferable RH for mulberry leaf rearing.

Kaneko (personal communication) has enough experience of artificial diet rearing for a practical hybrid (Kinshu × Showa) with the standard condition but has little occurrence of naked pupae. However, the same rearing proce-
dure made p50 silkworms naked pupae with very high mortality. Such that, here we investigated whether the p50 individuals reared in high humidity and fed an artificial diet have a high risk of becoming naked pupae.

**MATERIALS AND METHODS**

**Silkworm rearing and artificial diet until 5th instar**

Two groups of approximately 500 p50 silkworm hatchinglings each were reared in polypropylene cases with lids (298 × 398 × 56 mm, W × D × H; Hi-PACK S-138, Entec Co. Ltd) under a 12L:12D condition. Three and four cases were used for the 3rd and 4th instar larvae, respectively, and the number of larvae was adjusted to approximately 150 and 90, respectively, in the boxes. The artificial diet Silk Mate PS (Nosan Corporation) was fed once per day. The amount of diet fed to the silkworms during the 1st, 2nd, 3rd, and 4th instars was 76.2 g, 105.4 g, 335.3 g, and 1335.3 g respectively, for Experiment 1, and 136.2 g, 256.2 g , 363.1 g, and 1089.7 g, respectively, for Experiment 2. Temperature and RH were measured with a digital thermo-hygrometer (HTC-2; Toloyo). Median temperature was the same for both experiments and was 25.2°C (25.1-25.4°C) while RH was 69% (62%-79%) and 76% (64%-84%), respectively, for Experiments 1 and 2.

**Experiment 1**

Fifth instar larvae were reared under three RH treatments—low (L), intermediate (I), and high (H). Three replicates of the treatments with 30 individuals each were subjected to the three RH conditions. The L treatments (43.5±2.1%, 44.0±3.3%, and 43.5±6.1%) were prepared by maintaining the lids half open. The I treatments (56.5±4.5%, 52.0±9.0%, and 58.5±5.3%) were obtained by placing the rearing cases into other plastic cases (319 × 449 × 155 mm, W × D × H; NV Box, Astage Co. Ltd). To create the H treatment conditions (74.0±10.9%, 80.0±8.8%, and 81.0±1.8%), we kept the lid closed and added an aeration hole. The temperature was maintained at 25.1-25.3°C throughout the experiment. We checked temperature and RH twice per day during the light period just before administering the artificial diet.

**Experiment 2**

The 5th instar was divided into three growth periods: the first two days, the middle two days, and the remaining days until mounting started (3-4.5 days, see Table 1). The L and H treatments were applied to each growth period for a total of eight different RH-growth period combinations. Average temperature ranged from 24.7 to 25.3°C. Average RH in the L and H treatments was 41.0-54.3% and 69.6-86.1%, respectively (Table 2). We checked temperature and RH three times per day during the light peri-

**Table 1. Combination of relative humidity in Experiment 2**

<table>
<thead>
<tr>
<th>combination</th>
<th>days for each period</th>
<th>total days of H condition</th>
</tr>
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<td>2 2 4 4</td>
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<tr>
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</tr>
<tr>
<td>LLL</td>
<td>2 2 4 0</td>
<td></td>
</tr>
</tbody>
</table>

H: high relative humidity (69.6%-86.1%)
L: low relative humidity (41.0%-54.3%)

od just before feeding the artificial diet.

**Pupae and adult**

All the spinning larvae were mounted at approximately 35% RH and we kept the condition up to adult emergence. Five to 6 days after mounting, we checked the pupal status and measured the weight of 10 each of naked and coocooned pupae of both sexes. Adults from Experiment 1 were mated to check the fertility. To compare the fertility and egg numbers we used p50 adults reared with mulberry leaves under routine condition.

**RESULTS**

**Experiment 1**

Naked pupae occurred in only 1.1% of the L treatment, increased to 8.0% in the I treatment, and jumped to 86.5% in the H treatment (Fig. 1, shown in degrees). The 5th instar periods lasted 9, 8 and 7 days in the L, I, and H treatments, respectively. All coocooned pupae except for one individual (99.4%) successfully achieved the adult stage irrespective of the treatment applied (Fig. 1). All naked pupae in H failed to eclose, while one pupa each in the L and I treatments became adults (Fig. 1). Adult fertility in H (86.9%, n=3) was statistically comparable to that of the other groups (95.9% in L and 95.3% in I, n=9 each) as well as to that of moths reared with mulberry leaves (96.5%, n=3). Scheffe’s post hoc comparison showed that the number of fertilized eggs in L (236.7, n=9) and H (194, n=3) was significantly smaller than that of those reared with mulberry leave (382.3, n=3). While the egg number in I (298.9, n=9) had no significant differences from those in others.

**Experiment 2**

Since rearing in high humidity through the 5th instar was harmful for adult emergence, we addressed to detect the specific period in which the damage occurred. Similar
to the results of Experiment 1, few naked pupae were produced when reared in all dry conditions (LLL) but increased (59.3%) when reared under high humidity (HHH) (Fig. 2). Some naked pupae also occurred when reared with double H plus single L combined conditions (30.8 and 23.3% in HHL and LHH) but were rare (0 to 8%) in single Hs as well as HLH (Fig. 2). Adult emergence rates were higher in conditions in which more cocooned pupation occurred. We could not detect the specific period that negatively affected spinning in the eight experimental conditions. Being subject to longer periods of high humidity seemed to be more harmful to adult emergence irrespective of the stage. The weights of naked pupae (female average: 88.6±5.7 cg; male average: 68.7±7.7 cg) were significantly heavier than those of cocooned pupae (female average: 71.6±5.6 cg; male average: 51.5±4.2 cg) in both sexes.

**DISCUSSION**

Development of an artificial diet for silkworms (Hamamura, 1959; Fukuda et al., 1960) and its improvement (Fukuda et al., 1962; Horie and Ito, 1963) have advantageously contributed to sericulture. This progress has enabled *B. mori* to be a more useful insect for entomological researches. The stock resource, p50 (Daizo), is one of the most important strains of *B. mori*. Our results clearly revealed that rearing the silkworms on an artificial diet in high relative humidity (RH) during the 5th instar caused the p50 larvae to become naked pupae (Fig. 1).
of the cocooned pupae (see results), which is similar to what occurs to operation-induced naked pupae (Akao, 1943). Akao (1943) speculated that all naked pupae induced by silk gland-removal are destined to die because of an amino acid overload syndrome called aminoacidemia. Spinneret removal also forced larvae to stop spinning, which made silk proteins overflow into the body cavity and cause 100% death (Kiyosawa and Kiuchi, 2001). Kiyosawa and Kiuchi (2001) also presumed that aminoacideemia caused this mortality. In genetic mutants, individuals with heavier pupal weights tended to have a higher risk of dying (Nakano, 1950; Doira, 1973). Very high mortality in heavier naked-pupae in the present study coincided with the results of these other studies.

Adults emerging from 3 RH conditions copulated normally and had similar fertility to those fed mulberry leaves (see results). This indicated that high humidity did not affect adult fecundity but only larval spinning. The combination of stress and high spinning speeds change soluble silk proteins to thread (Magoshi and Magoshi, 2007). High humidity causes sticky silk proteins to plug the spinneret during the spinning stage, which results in naked pupae. Hence, high-humidity induction of naked pupae in the spinning stage (Watanabe et al., 2014) may be explained by sticky soluble silk proteins. While, naked pupation observed in this study may not occur by the same process because we subjected the wandering larvae to dry conditions, and silkworms employ dry-type spinning for silk fiber drawings (Tanaka et al., 2003). High humidity disturbs and/or delays the drying process, which makes the silk protein sticky. Tanaka et al. (2003) found that 40% freezing water formed pores in the silk surface, which may be important for the process of quickly drying the silk. Our results indicated that high humidity for a period longer than 2/3 of the 5th instar increased the production of naked pupae irrespective of the stages (Fig. 2). If the freezing water were to be decreased by longer exposure to high humidity, silk proteins could become stuck in the spinneret of p50.

Ueda et al. (1988) developed standard procedure to rear practical silkworms with artificial diet. The rearing temperature and RH proposed for feeding stages were at 28-29°C with 80-90%, 27-28°C with 80-90%, 26-27°C with 75-80%, 25-26°C with 75-80%, and 24-25°C with 70-80% for respective 1st, 2nd, 3rd, 4th and 5th instar larvae. These conditions were similar to those of silkworm rearing with mulberry leaves (The Japanese Society of Sericultural Science, 1979; Kawaguchi and Yanagawa, 1992). However, their standard—70-80% RH during the 5th instar for practical strain rearing—would be too high for p50 strain individuals. Our results agreed with the general opinion that importance of lower humidity (Fig. 1) and suggesting that more precise humid control is important for 5th instar p50 individuals reared on an artificial diet. Hence, a humid control incubator is preferable for the p50 rearing with artificial diets. High humidity for 1/3 of the 5th instar did not severely affect p50 mortalities (Fig. 2), and artificial diets are rather easy to dry in the earlier stage of the 5th instar period. Hence, we propose practical p50 rearing with an artificial diet in an incubator without humid controller as follow: one might maintain high humidity (up to 80% RH) to keep the artificial diet edible for two days and then change to lower humidity (more than 45% RH). In this manner, we expect a sufficient number of p50 moths can be maintained on an artificial diet.

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