INTRODUCTION

Ambrosia beetles (Platypodinae and Scolytinae) exploit host trees primarily as a “fungal garden” to complete their life-cycles (e.g., Beaver, 1989; Kajimura, 2003). Adults transport an ectosymbiotic fungus in highly specialized saclike organs termed mycangia (Batra, 1963, 1966) and inoculate fungal spores taken from the mycangia into the walls of galleries that they excavate in the sapwood of host trees. Larvae that hatch from eggs laid in these galleries feed on the fungi that grew before the eggs hatched. The beetles are wood-boring pests, but at the same time, they are also natural recycling agents that promote turnover of the woody vegetation in both coniferous and broadleaved forests (e.g., Lindgren, 1990).

Reproduction and gallery construction by some species of scolytine ambrosia beetles in host trees have been studied in detail by splitting and examining infested wood (e.g., Kinuura and Hijii, 1991; Kajimura and Hijii, 1994). However, the results obtained from this field data vary widely because of non-homogeneous natural conditions. To provide more consistent data, various methods for artificial rearing have been developed. For example, adults of various species in the genus Xyleborus have been reared on semi-artificial diet composed of sawdust, potato starch, dried yeast, sugar, and distilled water in glass containers (Kawanami et al., 1976), but other researchers have also added antibiotics to the diet to prevent contamination by fungi other than those inoculated into the diet by the adult beetles (e.g., Takemori et al., 1973; Batra, 1985; Norris and Chu, 1985). Recently, we developed a new diet system that involved a three-layer structure, with different diets placed in a lower layer (all ingredients), a middle layer (with lower starch content than in the lower layer), and an upper layer that impedes the growth of contaminat-

Effects of ingredients and structure of semi-artificial diet on the reproduction of an ambrosia beetle, Xyleborus pfeili (Ratzeburg) (Coleoptera: Curculionidae: Scolytinae)

Takahiko MIZUNO*† and Hisashi KAJIMURA

Laboratory of Forest Protection, Graduate School of Bio-agricultural Sciences, Nagoya University; Nagoya 464–8601, Japan

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Abstract

To develop a better system for rearing ambrosia beetles, we tested semi-artificial diets with different ingredients and structures. For the rearing of Xyleborus pfeili (Ratzeburg), we prepared diets composed of Douglas-fir sawdust, potato starch, dried yeast, sugar, and distilled water. The addition of antibiotics (streptomycin and penicillin) to a diet with a single-layer structure did not inhibit fungal contamination, and resulted in fewer X. pfeili offspring. Diets containing neither the starch nor the yeast effectively prevented fungal contamination, but did not produce offspring. A diet with a two-layer structure, in which different diets were placed in the lower layer (all ingredients) and the upper layer (sawdust, sugar, and water) in a glass tube, greatly increased the reproductive success of X. pfeili. The length of the gallery system, the number of offspring, their sex ratio, and the timing of gallery boring and oviposition by mother beetles on the two-layer structure were not significantly different from those on a three-layer structure used in previous research that also contained a thin layer of Douglas-fir resin. Thus, the diet with a two-layer structure appears to be a useful and simpler method for rearing ambrosia beetles.

Key words: Contamination control; gallery construction; rearing method; wood-boring insect; Xyleborini
ing fungi (sawdust, sugar, and water) in a glass tube, with Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) resin added between the middle and upper layers (Mizuno et al., 1997; Mizuno and Kajimura, 2002). The controlled conditions permitted by these rearing methods have contributed to ecological and applied studies of sex ratios, mating systems, and reproductive strategies in the beetles (Roeper et al., 1980; Norris, 1992; Mizuno et al., 1999; Mizuno and Kajimura, 2002).

However, it is clearly desirable to avoid including antibiotics in these diets because they increase the cost and complexity of the experimental setup, and increase the risk of causing antibiotic resistance. In addition, some *Xyleborus* species are attracted more strongly to ethanol than to terpenoids (Flechtmann et al., 1999), which are chemical components of the resin. Therefore, the addition of antibiotics and resin to the diets may not be necessary for rearing of the beetles.

In this study, we examined the effects of variations in the ingredients and layer structures of semi-artificial diets on the reproduction of an ambrosia beetle, *Xyleborus pfeili* (Ratzeburg), to develop a better system for rearing the beetles (Mizuno and Kajimura, 2002). We conducted three rearing experiments to determine the effects of different ingredients on the beetle’s reproduction in a diet with a one-layer structure (Experiment 1) and in a diet with a two-layer structure (Experiment 2), and we compared the results from an optimal diet with a two-layer structure with those from a comparable diet with a three-layer structure plus resin (Experiment 3).

**MATERIALS AND METHODS**

**Insects.** *Xyleborus pfeili* is a cosmopolitan species of ambrosia beetle (Wood and Bright, 1992; Bright and Skidmore, 1997, 2002). The tribe Xyleborini, which includes the genus *Xyleborus*, has an inbred polygynous mating system in which female adults mate with their male siblings in their galleries before dispersal flight (e.g., Kirkendall, 1983).

Dispersing female adults of *X. pfeili* were collected from Douglas-fir logs at Omaezaki-cho, Shizuoka prefecture, in central Japan (34°N, 138°E). The collected adults were pre-reared on the same diets described by Mizuno and Kajimura (2002) at 24°C in darkness for 8 weeks in a laboratory at Nagoya University. In this study, we used newly dispersed female adults (mother beetles) of the successive generations in our experiments. Before use, they were surface-sterilized by immersion in 70% ethanol for 10 s and then rinsed with sterilized water.

**Diet and containers used in the experiments.** We prepared eight different diets (A–H), which were composed of Douglas-fir sawdust (sieved through 0.84-mm mesh), potato starch, yeast extract, sugar (sucrose or granulated sugar), antibiotics (streptomycin sulfate and penicillin), and distilled water (Table 1) for use in Experiment 1. Diet H is the same one that was previously used for *X. perforans* by Kawanami et al. (1976). Diet C was used for several species of ambrosia beetle by Batra (1985). Diets D and E were made by omitting the antibiotics from diet C.

In Experiment 1, 20 g of a diet was placed in a small glass tube (10 cm in depth, 2 cm in diameter), whose open bottom end was plugged with a silicone stopper (Fig. 1a). After packing of the other end of the tube with a cotton plug, the tube and diet were autoclaved. Diets C and D were autoclaved for 15 min at 15 psi/2.27 kg/cm² according to the method of Batra (1985). The other diets were autoclaved at 120°C for 40 min. All the autoclaved diets were immediately compacted with a sterile wooden stick to fill in any cracks. In Experiment 2, we created a diet with a two-layer structure (diets B, F, G, and H, chosen based on the results of Experiment 1) in the tube (Table 1, Fig. 1b). In this experiment, we combined 17 g of diet F, G, or H (as the lower layer) with 3 g of diet B (as the upper layer) in the containers. In Experiment 3, we used large glass tubes (15 cm in depth, 3 cm in diameter) to create a two-layer structure (Fig. 1c) by combining diets B (5 g) and H (65 g) or the three-layer structure used in previous studies as a control (Fig. 1d); this structure combined 5 g of diet B, 10 g of diet F, and 55 g of diet H, with a thin layer of Douglas-fir resin (10 mg) between diets B and F (Mizuno and Kajimura, 2002). All the diets in Experiments 2 and 3 were autoclaved at 120°C for 40 min, and were then pressed down with the wooden stick as described above.

**Experimental design.** In Experiments 1 and 2, we introduced 50 mother beetles per diet type into glass tubes, one per tube, and reared them at 24°C.
### Table 1. Ingredients and autoclave conditions used in the test of diets with a one-layer structure for the rearing of *Xyleborus pfeili* ambrosia beetles

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>A</th>
<th>B(^a)</th>
<th>C(^b)</th>
<th>D</th>
<th>E</th>
<th>F(^a)</th>
<th>G</th>
<th>H(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Douglas-fir sawdust (g)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Potato starch (g)</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Yeast extract (Difco) (g)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Streptomycin sulfate (g)</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin (units)</td>
<td>0</td>
<td>0</td>
<td>1,250</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Distilled water (mL)</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
</tr>
</tbody>
</table>

Autoclave conditions: strong\(^d\), strong\(^d\), weak\(^e\), weak\(^e\), strong\(^d\), strong\(^d\), strong\(^d\), strong\(^d\)

\(^a\) Mizuno and Kajimura (2002).
\(^b\) Batra (1985).
\(^c\) Kawanami et al. (1976).
\(^d\) Autoclaved for 40 min at 120°C.
\(^e\) Autoclaved for 15 min at 15 psi/2.27 kg/cm² (Batra, 1985).

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**Fig. 1.** The rearing system used for *Xyleborus pfeili* ambrosia beetles. All diets were prepared in glass tubes [(a) and (b), small tubes; (c) and (d), large tubes] according to the descriptions in Table 1. (a) The diet with a one-layer structure used in Experiment 1. (b) The diet with a two-layer structure used in Experiment 2. The diets with (c) two-layer and (d) three-layer structure used in Experiment 3 (Mizuno and Kajimura, 2002).
in the dark. After 15 days, all the diets were carefully dissected to expose gallery walls and determine whether the inoculated beetles were alive or dead. From each gallery system (tube), we removed and counted the offspring (eggs, larvae, pupae, and new adults). We then measured the lengths of the galleries constructed by the beetles in each tube.

We used 110 mother beetles per diet type in Experiment 3. They were individually reared in separate glass tubes under the same conditions as in Experiments 1 and 2. Every day until the 30th day after inoculation, we randomly selected three tubes to record the number of offspring at each developmental stage and the gallery lengths. The mother beetle and her offspring (new female adults) were distinguished by the difference in their body colors. After 40 days, the remaining 20 tubes in each diet type were carefully dissected to count the mother beetles and the total offspring as in Experiments 1 and 2. In addition, we determined the sex ratio of the offspring in each tube, relying on clear differences in body size between females and males at the pupal and new adult stages.

**Data analysis.** Differences in the mean total length of the gallery system and total number of offspring per tube between the diet types were examined after 15 days in Experiments 1 and 2 using Fisher’s PLSD test. We tested for differences until the 30th day in Experiment 3 using the Mann-Whitney U-test because of the small sample size (n=3). After 40 days in Experiment 3, we compared the mean number of offspring and sex ratio per tube between the diet types using Student’s t-test and the Mann-Whitney U-test, respectively.

**RESULTS AND DISCUSSION**

**Effects of ingredients in diets with a one-layer structure on the reproduction of X. pfeili (Experiment 1)**

In all the tubes, mycelial growth of contaminating fungi, which invaded from the upper side of the diets and grew down into the galleries, was obvious by the 4th or 5th day after introduction of the beetles. The contamination tended to spread more rapidly at higher potato starch and dried yeast contents. Both eggs and larvae of X. pfeili were found in successful galleries.

Table 2 shows the results of rearing of X. pfeili on semi-artificial diets with a one-layer structure in small tubes. On diets A and B, where little contamination occurred, mother beetles began boring a straight gallery and extended it to average lengths of 13.9 and 11.5 mm, respectively. Of the 50 original beetles, 14 and 19, respectively, remained alive at the end of the gallery. However, they constructed no branch tunnels and laid no eggs. Thus, diets A and B effectively inhibited both fungal contamination and beetle reproduction. Diets C through E did not differ significantly from each other or from diets A and B in the mean total length of the gallery systems (Fisher’s PLSD test, p>0.05), although offspring were observed in two tubes of diet C and three of diet E. Diet E produced better survival of mother beetles than diets C and D. The antibiotics and autoclave conditions had little or no effect on the reproductive success of X. pfeili.

<table>
<thead>
<tr>
<th>Diet type</th>
<th>Number of mother beetles tested</th>
<th>Number of mother beetles alive</th>
<th>Number of mother beetles that oviposited</th>
<th>Total number of offspring per tube mean±SD</th>
<th>Total length of gallery system per tube (mm) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50</td>
<td>14</td>
<td>0</td>
<td>0 a</td>
<td>13.9±13.7 a</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>19</td>
<td>0</td>
<td>0 a</td>
<td>11.5±10.2 a</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>3</td>
<td>2</td>
<td>3.5±0.7 b</td>
<td>11.9±6.9 a</td>
</tr>
<tr>
<td>D</td>
<td>50</td>
<td>4</td>
<td>0</td>
<td>0 a</td>
<td>19.7±12.9 a</td>
</tr>
<tr>
<td>E</td>
<td>50</td>
<td>16</td>
<td>3</td>
<td>2.7±1.2 b</td>
<td>20.3±22.7 a</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>33</td>
<td>30</td>
<td>8.9±4.1 c</td>
<td>50.9±38.1 b</td>
</tr>
<tr>
<td>G</td>
<td>50</td>
<td>27</td>
<td>22</td>
<td>10.7±4.5 d</td>
<td>42.3±31.4 b</td>
</tr>
<tr>
<td>H</td>
<td>50</td>
<td>19</td>
<td>13</td>
<td>8.4±3.7 c</td>
<td>37.5±33.1 b</td>
</tr>
</tbody>
</table>

a Diet ingredients are described in Table 1 and their structures are illustrated in Fig. 1.

b Values in a column followed by the same letters did not differ significantly among the diet types (Fisher’s PLSD test, p>0.05).
However, the mean gallery lengths were significantly longer and the mean numbers of offspring were significantly larger in diets F, G, and H than in the other diets (Fisher’s PLSD test, \( p<0.05 \)). In particular, \( X. \) pfeili produced the most offspring on diet G (Fisher’s PLSD test, \( p<0.05 \)). The numbers of living beetles and ovipositing beetles increased from diet H to diet G to diet F.

These results suggest that two ingredients (potato starch and dried yeast) are indispensable for the reproduction of ambrosia beetles. Thus, only diets F, G, and H enable the beetle to produce many offspring. To develop a diet with a two-layer structure, diet B appears to be more useful than diet A as the upper layer (as a contamination barrier) because of the higher survival of the mother beetles. Diets F, G, and H should be used as the lower layer as a breeding base, because they produced the highest numbers of offspring. Thus, the combinations of diets B+F, B+G, and B+H appear to be suitable for rearing the beetle in a two-layer structure that does not include antibiotics.

**Effects of ingredients in diets with a two-layer structure on the reproduction of \( X. \) pfeili (Experiment 2)**

In Experiment 2, contamination occurred later than in Experiment 1 (by the 7th or 8th day after introduction of the beetles), indicating that the two-layer structure more effectively inhibited contaminant mycelial growth than the one-layer structure. Antibiotics produced by the fungus associated with the scolytine ambrosia beetle \( Euwallacea \) validus can suppress the growth of other microbes (Nakashima et al., 1982). Therefore, prior establishment of the ambrosia fungi in the lower layer of the structure would be a key to successful rearing of ambrosia beetles on the diets. Eggs and larvae of \( X. \) pfeili were observed on the 15th day after inoculation, as in Experiment 1.

Most of the mother beetles were able to reproduce on the three types of diet with a two-layer structure in small tubes, but two parameters differed dramatically among the diet types (Table 3): the mean number of offspring per tube was significantly greater on the B+H combination than on the other diets (Fisher’s PLSD test, \( p<0.05 \)), and the mean total length of the gallery system was significantly longer in the B+H combination than in the B+G combination, which was in turn significantly longer than in the B+F combination (Fisher’s PLSD test, \( p<0.05 \)). The only difference among diets F, G, and H is in the amount of potato starch: diet H has the highest starch content (Table 1). These results demonstrate clearly that a mother beetle is capable of manipulating her fecundity according to the expected quantity of the food resource (ambrosia fungus) by expanding the available space (i.e., the length of the gallery system), which is also shown in \( X. \) mutilatus (Kajimura and Hijii, 1994), in response to the quality of the diet (here, the starch content).

\( Xyleborus \) pfeili reproduced better on the diets with a two-layer structure (B+F, B+G, and B+H; Table 3) than on diets with a one-layer structure (F, G, and H; Table 2). This finding confirms that the two-layer structure is more efficacious in producing offspring of ambrosia beetles on artificial diets than the one-layer structure. Among the diets with a two-layer structure, the B+H combination appears to be most suitable for reproduction.

**Comparison between a diet with a two-layer structure and a diet with a three-layer structure plus resin (Experiment 3)**

In the third experiment, we tested the optimal diet with a two-layer structure (B+H), which was selected based on the results of experiments 1 and

<table>
<thead>
<tr>
<th>Diet type (^a)</th>
<th>Number of mother beetles tested</th>
<th>Number of mother beetles alive</th>
<th>Number of mother beetles that oviposited</th>
<th>Total number of offspring per tube (^b) mean±SD</th>
<th>Total length of gallery system per tube (mm) (^b) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B+F</td>
<td>50</td>
<td>39</td>
<td>37</td>
<td>11.2±6.1 a</td>
<td>58.4±26.8 a</td>
</tr>
<tr>
<td>B+G</td>
<td>50</td>
<td>45</td>
<td>45</td>
<td>8.4±6.3 a</td>
<td>71.0±19.8 b</td>
</tr>
<tr>
<td>B+H</td>
<td>50</td>
<td>46</td>
<td>45</td>
<td>15.0±6.3 b</td>
<td>105.7±40.3 c</td>
</tr>
</tbody>
</table>

\(^a\) Diet ingredients are described in Table 1 and their structures are illustrated in Fig. 1.

\(^b\) Values in a column followed by the same letters did not differ significantly among the diet types (Fisher’s PLSD test, \( p>0.05 \)).
2, in comparison with a diet with a three-layer structure (B+F+H with Douglas-fir resin) that had been developed in a previous study (Mizuno and Kajimura, 2002).

Figure 2 shows the population growth of *Xyleborus pfeili* offspring on the two diet types until 30 days after inoculation with the mother beetles. Oviposition appeared to continue until day 29 or 30 on the B+H and B+F+H diets. The number of offspring per tube increased gradually after inoculation in both combinations, differing significantly on days 5 and 6 (Mann-Whitney *U*-test, *p*<0.05). Thereafter, the numbers fluctuated widely, but the mean value on B+H was significantly greater than that on B+F+H on days 15 and 28 (Mann-Whitney *U*-test, *p*<0.05). Larvae were first observed on days 11 and 10, respectively, pupae on day 18, and new adults on days 24 and 22. After adult eclosion, all developmental stages were present in the gallery systems of both diet combinations. Thus, the population growth pattern was basically similar between the two combinations.

Figure 3 illustrates the progress of the length of the gallery systems of *Xyleborus pfeili* on the two diet structures until 30 days after inoculation. Irrespective of the presence of Douglas-fir resin, mother
beetles bored galleries beginning on the 1st day after the inoculation. The total length of the gallery system also increased gradually over time on both diets, although there were significant differences in mean lengths on days 4, 6, 14, 15, 17, 18, and 28 (Mann-Whitney U-test, p<0.05). Thus, the gallery construction pattern was essentially similar between the two structures.

Table 4 shows the numbers and sex ratios of the *X. pfeili* offspring reared on the two diet structures for 40 days after inoculation. There were no significant differences in the mean number of offspring (Student's *t*-test, *p*>0.05) or their sex ratio (Mann-Whitney *U*-test, *p*>0.05) between the two structures.

Consequently, the middle layer (diet F) and the presence of Douglas-fir resin had little effect on the reproductive success of *X. pfeili*, which suggests that using the two-layer structure can save time and labor without reducing reproductive success.

**CONCLUSION**

Our results strongly suggest that a diet with a two-layer structure (diets B+H), but without either resin or antibiotics, is a practical tool for rearing ambrosia beetles, and offers the advantage of simplicity compared with the three-layer structure that has been previously used for these beetles. This new diet can thus be used easily as a standard tool in studies of the life history and behavioral ecology of these beetles. Future tests should confirm whether these results also apply to ambrosia beetles in the Platypodinae.

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