Silkworms: diets for pre-dyed silks encourage environmental saving

Research Questions: Which colors of modified diet enable silkworms to directly produce colored cocoons and do they affect health and growth of the silkworms?

Members:
1. Thaksaporn Sirichanyaphong (May)
2. Narissara Pracharktam (Nik)

Introduction:
Modern clothes manufacturing tend to interfere silkworm’s life cycle the most since silkworms are the primary producer of silk.[1] Besides being the main producers, their silks are naturally shiny, have light weight –however are stronger than a filament of steel, and have poor heat conduction; therefore, they are cool in summer while warm in winter. Nevertheless, due to silkworm’s physical color, they can only produce cocoons in the range of white and yellow to gray. With this limitation, silk industries prefer to artificially dye the cocoons to produce varieties of colored silk. Meanwhile, tremendous amount of water is being used throughout the dyeing and bleaching processes, causing water pollution as toxins produced in water are untreatable.[2]

Since certain silkworms’ diets do have effects on their body’s color and their silks, to determine which type of colors in the modified diets are and the impacts on their health, silkworms will be fed with synthetic dye-blended mulberry leaves.[4] Since life of silkworms can be divided into four stages. In their fifth stage, which their bodies elongate and they molt, silkworms will be fed on synthetic dye-blended mulberry leaves in the last week of larva stage. The silkworms will be divided into four groups due to their diets. One group will be the control group, which feeds on the blended mulberry leaves without dye. The other three groups feed on three different dye-blended mulberry leaves: Rhodamine B, Methylene Blue and Green Fabric Dye.[6] Then, the silkworm’s body color will be primarily observed whether there is a change and is recorded. After 2-3 days, when the larvae fully develop, they start to spin their cocoons to protect their developing pupa. During the pupa stage, their color of cocoons will be recorded to see if the cocoons’ color corresponds to their body’s color. Throughout this experiment, physical features such as size and length will also be observed to determine whether the diets affect the silkworm’s health, and factors affecting their growth needed to be controlled such as temperature, sunlight, and humidity.

Objectives:
To test whether Rhodamine, Methylene Blue, and Green Fabric Dye in the modified diets will affect silkworms’ physical color, as well as their cocoons, regardless transgenic methods. Furthermore, to observe the effects of the modified diet toward silkworms’ health and growth.
Significance and Implications of the Study:

Since original silk produced by silkworms are not purely white and fabric industries demand varieties of silk colors, they bleach and dye the silks with synthetic color. Through the coloring processes, when huge amount of fresh water is being used to bleach, the water reservoirs get contaminated with chemicals. It is extremely hard and takes time to recover the contaminated water; therefore, this manufacturing process eventually results in water pollution and impacts other organisms, especially humans as we rely on water the most to sustain our life. Regarding the drawbacks of post-dyed silks, this experiment, by feeding dye-dipped mulberry leaves to silkworm which is pre-dyed silks, is conducted to create more alternatives for fabric industries to not only satisfy with various colors but also to not harm the surrounding environment.

Materials:

- 280 fifth-instar white silkworms (4 group with 70 per group)
- Distilled water
- 7 kg of fresh Mulberry leaves
- 140 mL of 3.5 M DYLON Green Fabric Dye
- 140 mL of 0.01M Rhodamine B solution
- 140 mL of 0.01M Methylene blue solution
- Newspaper
- 4 32*23-cm-paper boxes
- Blender
- Oven lab
- Closed glass jar
- Desiccant
- Balance
- Spatula
- Stirring rod
- Heater
- Beaker
- Thermometer
- 4 38*32-cm-plastic tray
- Gloves
- Sifted flour
- Plastic grille
- Robe
- Permanent maker
- Metal bowl
- Ruler
- Cutter
Procedure\[5\]:

**Making the dry-mulberry leaves powder**

1. Collect 7 kg of fresh mulberry leaves (the leaves should not be too wither).

![Figure 1: Fresh mulberry leaves](image1)

2. Dry all of the leaves in an oven lab overnight, adjust the temperature to 70 degree Celsius.

![Figure 2: Dry mulberry leaves](image2)

3. Blend all dry leaves using a blender for 1 minute using level two-switch, makes sure that the leaves are fine enough then use a sifted flour to filter the blended powder (the powder left on the sifted flour should be blended again and use the sifted flour to filter again).

4. Pour the filtered powder into the glass jar, close the jar and add a bag of desiccant to avoid a humidity.
Preparing the silkworms’ batches
1. Label each paper box using a permanent marker with 4 different colors: Rhodamine B, Methylene Blue, Green Fabric Dye and control.
2. Lay a sheet of a newspaper into each box
3. Put 70 fifth-instar white silkworms into each box
4. Make sure that the temperature of the room should be at 25 degree celsius all the time

Making the modified artificial diet
Entering the fifth instar, the last growth stage which lasts about 22-28 days since birth, silkworms in each group needed to be fed by certain artificial diets. In this instar, the worms will start to spin their silks, forming cocoons.
1. Use a balance to weigh 100 g of dry-mulberry leaves powder and pour it into a 500-mL beaker
2. Add 140 mL of 3.5 M Green Fabric Dye solution into the beaker
3. Use a stirring rod to mix the solution with the dry-mulberry leaves powder until they form homogenous
4. Mold it into a cylinder shape within 6 cm length and 1.5 cm diameter
5. Feed the silkworms in the labeled box with the molded diet according to the color added in the artificial diet
6. Repeat step 1-5 again but adjust the new color in the diet: 140 mL of 0.01M Rhodamine B solution and again with 140 mL of 0.01M Methylene blue solution
7. Make sure to remove the silkworms’ feces and the newspaper in each box everyday
8. Also, remove the old molded diets and add the new ones every three days
9. After feeding them 1-2 days, observe the silkworms in each box and record their colors in Table 1
**Figure 4:** Silkworms are eating the Green Fabric Dye artificial diet in the first and second day (26-27\textsuperscript{th} February).

**Figure 5:** Silkworms are eating the Methylene Blue artificial diet in the first and second day (26-27\textsuperscript{th} February).

**Figure 6:** Silkworms are eating the artificial diet in control batch in the first and second day (26-27\textsuperscript{th} February).
Preparing the plastic net for the silkworms to spin their cocoons

After the silkworms enter their fifth instar for 5-7 days, the silkworms are going to spin their silks to form cocoons. During this period most of the worms will eat less than before and stay still to spin the silk so we support them by adding small pieces of fresh leaves, however we have not yet removed the artificial diet.

1. Prepare the plastic trays and label them according to the color added in the artificial diet
2. Cover the bottom of each tray with a sheet of newspaper
3. Add 4 fresh mulberry leaves in each tray
4. Fold the net back and forth until its height is the same as the height of the plastic tray, as shown in Figure
5. Estimate the size of the folded net to be the same as the plastic trays’ and cut it (the folded net should be fit into the basket).
6. Use a rope to tie the net with the plastic tray and make sure it does not spring from the tray. (this plastic net is for the worms to spin the cocoons with)
7. Remove the silkworms from each paper box to the labeled trays on the top of the plastic net
8. Record the number of color-changed silkworm and cocoon each batch and the observation in Table 2-6

Figure 7: Silkworms are eating the Rhodamine B artificial diet in the first and second day (26-27th February).

Figure 8: Silkworms in the control batch are going to spin the silk to form cocoon in the third day (28th February).
Figure 9: Silkworms in the Rhodamine B batch are going to spin the silk to form cocoon in the third day (28\textsuperscript{th} February).

**Drawing the silk from cocoons**

1. Pour 200 mL water into a metal bowl and place the bowl on a heater
2. Turn on the heater and adjust the temperature to 260 degree celsius.
3. While waiting for the water to boil, choose 6 cocoons from Rhodamine B batch (Note: choose the most intense pink cocoons which are not stained)
4. When the water boils, put all cocoons into the boiling water.
5. Stir and dip all cocoons into the water and wait for 2 minutes
6. Use forceps to pull the silk from each cocoon and gather each silk together
7. Use your hands to twist and draw the gathered silk to coil using a ruler (make sure to wear gloves)
8. Draw the silk until the end cut the silk from its pupa
9. Observe and record the color of the silk before and after boiling in Table 7
10. Repeat step 1-9 again with other batches

Figure 10: Boiling pink cocoons from the Rhodamine B batch and drawing the silk
Collecting the cocoon samples and terminating the pupas

All the pupas must be terminated in order to avoid the chemical contamination to the ecosystem so we only collect the cocoon but take the pupa in the cocoon out.

1. Use a cutter to cut the end of the cocoons to take the pupa out
2. Separate the cut cocoons and the pupas in different trays
3. Keep the cocoon as samples but terminate all the pupas
4. Pour 200 mL water into a metal bowl and place the bowl on a heater
5. Turn on the heater and adjust the temperature to 200 degree celsius.
6. Wait for the water to boil then pour all pupas into the boiling water
7. Leave them for 5-10 minutes (make sure that they all die)
8. Discard died pupas in a bin

*Figure 11*: Cut the end of the cocoon using a cutter

*Figure 12*: Separate the cocoons from the pupas
Result:

**Table 1:** Observation of changed silkworms’ color in each batch in Day 1-2 after the silkworms start their fifth instar

<table>
<thead>
<tr>
<th>Batch</th>
<th>Change in silkworms’ color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine B</td>
<td>Pink</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td>(the color is too fade to observe)</td>
</tr>
<tr>
<td>Green Fabric Dye</td>
<td>No change</td>
</tr>
<tr>
<td>Control</td>
<td>No change</td>
</tr>
</tbody>
</table>

*Figure 13:* After feeding the silkworms with Rhodamine B artificial diet for 1 day, the silkworms changed their into pink.

*Figure 14:* After feeding the silkworms with Methylene Blue artificial diet for 1 day, the color is not obvious enough to observe.
Figure 15: After feeding the silkworms with Green Fabric Dye artificial diet for 1 day, the silkworms did not change their color.

Figure 16: After feeding the silkworms in the control with the artificial diet batch for 1 day, the silkworms did not change their color.

Table 2: Number of color-changing silkworms and cocoons in the first day

<table>
<thead>
<tr>
<th></th>
<th>Rhodamine B</th>
<th>Methylene Blue</th>
<th>Green Fabric Dye</th>
<th>Control</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pink</td>
<td>White</td>
<td>Blue</td>
<td>White</td>
<td></td>
</tr>
<tr>
<td>Silkworms</td>
<td>18</td>
<td>10</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Cocoons</td>
<td>23</td>
<td>19</td>
<td>0</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>29</td>
<td>0</td>
<td>70</td>
<td>0</td>
</tr>
</tbody>
</table>

Day 3 (28th Feb)
Figure 17: Silkworms in the Rhodamine B batch are going to spin the silk to form cocoon with the plastic net in the third day (28th February).

Figure 18: Silkworms in the Green Fabric Dye batch are going to spin the silk to form cocoon with the plastic net in the third day (28th February).
Figure 19: Silkworms in the Methylene Blue batch are going to spin the silk to form cocoon with the plastic net in the third day (28th February).

Figure 20: Silkworms in the control batch are going to spin the silk to form cocoon with the plastic net in the third day (28th February).

Table 3: Number of color-changing silkworms and cocoons in the second day

<table>
<thead>
<tr>
<th></th>
<th>Rhodamine B</th>
<th>Methylene Blue</th>
<th>Green Fabric Dye</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pink</td>
<td>White</td>
<td>Blue</td>
<td>White</td>
</tr>
<tr>
<td>Silkworms</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Cocoons</td>
<td>29</td>
<td>27</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>29</td>
<td>0</td>
<td>70</td>
</tr>
</tbody>
</table>

*Observation: Pink = bright pink + light pink*
Figure 21: Silkworms in the Rhodamine B batch are going to spin the silk to form cocoon with the plastic net in the fourth day (29th February).

Figure 21: Silkworms in the Methylene Blue batch are going to spin the silk to form cocoon with the plastic net in the fourth day (29th February).

Figure 21: Silkworms in the control batch are going to spin the silk to form cocoon with the plastic net in the fourth day (29th February).
Figure 21: Silkworms in the Green Fabric Dye batch are going to spin the silk to form cocoon with the plastic net in the fourth day (29th February).

Table 4: Number of color-changing silkworms and cocoons in the third day

<table>
<thead>
<tr>
<th></th>
<th>Rhodamine B</th>
<th>Methylene Blue</th>
<th>Green Fabric Dye</th>
<th>Control</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pink</td>
<td>White</td>
<td>Blue</td>
<td>White</td>
<td>Green</td>
</tr>
<tr>
<td>Silkworms</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Cocoons</td>
<td>29</td>
<td>27</td>
<td>0</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>29</td>
<td>0</td>
<td>70</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure 22:** Silkworms in the Rhodamine B batch are going to spin the silk to form cocoon with the plastic net in the fifth day (1st March).

**Figure 23:** Silkworms in the Methylene Blue batch are going to spin the silk to form cocoon with the plastic net in the fifth day (1st March).
Figure 24: Silkworms in the Control batch are going to spin the silk to form cocoon with the plastic net in the fifth day (1st March).

Figure 25: Silkworms in the Green Fabric Dye batch are going to spin the silk to form cocoon with the plastic net in the fifth day (1st March).

Table 5: Observation of silk colors before and after boiling

<table>
<thead>
<tr>
<th>Batch</th>
<th>Color of silk before boiling</th>
<th>Color of silk after boiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine B</td>
<td>Pink</td>
<td>Light Pink (the color partly dissolves into the water )</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>No change (the color is too fade to observe)</td>
<td>No change</td>
</tr>
<tr>
<td>Green Fabric Dye</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Control</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

**Figure 26:** Boil cocoon from Rhodamine batch

**Figure 27:** Remains after boiling
Discussion:

Silkworm Life Cycle

The life cycle of silkworms can be divided into four different main stages of lives: egg, larva, pupa, and adult in which each stage shows distinctive changes in its physical body. Begin with a caterpillar hatches out of its egg, the little caterpillar in larva stage grows
bigger and bigger as we can divide it into five instars, ordering from the first instar to the fifth instar. After 20-30 days, the larva will then turn into a pupa stage as the silkworm spins the silk to form a cocoon around their body for protection. For 7-10 days later, the pupa changes into moth and moth comes out of the cocoon. Based on the experiment, the fifth instar silkworms are selected to examine our modified artificial diets since it is the last stage of silkworms before spinning silk. The silkworms tested in the experiment is called Bombyx mori. This species of silkworms’ bodies are all white and they produce white silk so it is easier to observe their changes than the other species.

Making the modified artificial diet

Since the silkworms chosen were the fifth instar and they were raised with fresh mulberry leaves since they were born, we first aimed to dye fresh leaves. However, when we dyed the leaves with a solution of dye powder, it was hardly possible to identify whether the color is absorbed into the leaves or only attached to their surface even though the leaves are tore apart to be observed, shown in Figure 30.

Figure 30: Testing if methylene blue is absorbed by the leaves after an overnight soaking.

Thus, this method was not conducted. After searching for silkworm artificial diet and observing how it is made, the new method is used. The artificial diet is mainly made up of very fine mulberry powder. It is mixed with water and vitamin and molded into a shape. To make very fine powder, mulberry leaves are dehydrated at 70 degree celsius; the result is shown in Figure 2. The dried leaves later are blended, resulting in fine mulberry powder. Preparing diet for each batch of silkworm, the powder is mixed with the solution of dye powder. Finally, the paste-like mixture is molded into a shape and introduced to a group of silkworm. By this way, it ensures that the chemical color is fully absorbed into the diet.

Color change in silkworms

According to our observation, after the fifth instar silkworms were fed with each color of modified artificial diets, in the half first day the silkworms in each batch refused to
consume the modified diets since these silkworms were previously fed with normal fresh leaves so they were unfamiliar with the shape and texture of our modified food. However, in the half day later, the silkworms adapted themselves to consume the diets but the observation was not obvious enough to record. Then the silkworms were left in the setting room with 25 degree celsius overnight. In the second day, we are able to observe that only the silkworms in Rhodamine B(Pink) batch changed their body colors from white to pink as shown in Figure 13. There are no change in Green Fabric Dye (Green) and control batches. Also, in Methylene Blue (Blue) batch, the color of the silkworms’ bodies are too fade to observe.

In Day 3 as recorded in Table 2, 41 silkworms from the total 70 silkworms in Rhodamine B batch changed their color into pink. Out of that number, there are 23 of them were spinning the cocoon around themselves. As shown in Figure 17, the color of the silk produced in Rhodamine B batch remains pink since Rhodamine B was normally used in textile industry and bacteriologic stain so it was able to dye the protein of silk. Therefore, the pink color of Rhodamine B was transferred from the diets through the silkworms’ body and also to the silk that was produced by the worms[8]. However, in Green Fabric Dye and Control batches, there was still no change in the silkworms color, although they were spinning the cocoon, their cocoons were white as shown in Figure 18 and Figure 20. Since DYLON Green Fabric Dye commonly could be used to dye wool and silk, nevertheless, the color might not be able to transfer through the protein of the silk or the solution of the dye might be too dilute so the color did not appear to observe[9]. For Methylene Blue batch, though the third day, the color of the silkworms didn’t get stronger. Also, the blue color of the silk produced was still too fade to observe so we recorded the result as no color change. We think that the reason of why the blue color of Methylene blue didn’t show an obvious result was because Methylene blue was the chemical used to dye animals’ cell to make the nuclei more visible[10]. Therefore, the color was stained deeply in the cell and was harder to observe by eyes. By comparing the number of cocoon forming in each batch, we found that Methylene Blue got the highest number which was 56 cocoons out of 70. Green Fabric Dye and Rhodamine B got lower than Methylene Blue while the control batch got the lowest number of cocoon forming.

In Day 4 (Table 3), there was no more white silkworms in Rhodamine B batch turned into pink color. Also, in other batches there was no more change in color too. However, more silkworms including the pink silkworms were spinning the cocoons. Similar as Day 4 but in Day 5, all of the silkworms in Control batches were forming cocoons. More silkworms in Green Fabric Dye and Methylene blue batches were turning into cocoons while there is no change in the number of cocoons in Rhodamine B batch.
The Silkworms Disease

After feeding the silkworms with the modified artificial diets for five days, some of the silkworms in each batch seemed to appear a sign of Flacherie disease, especially in Rhodamine B batch\textsuperscript{11}. Since there was a stain of green or brown fluid appeared inside of their cocoons as shown in Figure 31. Moreover, some of the silkworms became flaccid and secrete juice\textsuperscript{11}. Their faces also became wet and soft (Figure 32). We thought that the disease might cause from the starvation of silkworms during the first day of adjusting the modified artificial diet and the accumulated humidity from uneaten artificial diets. However, there were around 8 silkworms died from the disease and 6 of them changed into pupa without spinning the cocoon. The rest survived and were able to form their cocoons normally.

**Drawing silk from the cocoons**
Figure 33: Cocoon from Rhodamine B batch

Figure 34: Drawing silk from the pink cocoons from Rhodamine B batch

Figure 35: After drawing the cocoon from Rhodamine B batch
The color observed from the Rhodamine B cocoon batch is bright pink. However, after boiling and drawing it, its silk color became light pink (Figure 35), and the boiling water turned into pink since Rhodamine B is soluble in water. Meanwhile, the cocoons from Methylene Blue batch are very pale blue. Thus, they can be concluded that their color before drawing, as well as after boiling, is unchanged compared to the control batch. Similar to Methylene Blue, Green Fabric Dye has no effect on cocoons’ color. They are white like the cocoons from control batch. Boiling also does not affect the color of cocoons from Green Fabric Dye batch and control batch (Figure 36-38).
**Color of the moths after hatching**

The color of the moths follows color of their cocoon. Moths from Rhodamine B batch appears to be pink as shown in Figure 39 while moths from other batches remain white color. As soon as they hatch and fully become moths, they mate and lay eggs. Eggs laid by moths feeding on Rhodamine B are obviously pink as shown in Figure 40. Yellow eggs were laid by moths from the control batch. Despite silkworms, their cocoons, and moths from Methylene Blue and Green Fabric Dye batches physically resemble those from control batch which their bodies’ color is white, green and blue eggs were hypothesized to be their eggs. Since we did not separate the moths according to their batch while they were mating, we could only propose the possibility that green eggs were the effect of Green Fabric Dye while blue eggs were the effect of Methylene Blue. However, though we tried to control all the unexpectation in the experiment, there were still some experimental errors occurred from the method of measuring chemical powder and counting the silkworms. As we used only a tiny scale of Rhodamine B and Methylene Blue, the balance was not accurate enough to measure. Also, the vibration from wind and weight of surrounding air affect the scale of the balance so resulted in an error on weighing. Besides, counting high movable animals liked silkworms was difficult. Therefore, this might cause a slight mistake on the result. Nevertheless, further experiment can be conducted to test if synthesized dye color consumed by silkworms is genetically passed to the next generation.

![Figure 39: Pink Moths, the effect of Rhodamine B](image-url)
Conclusion:

Despite a sign of Flacherie disease observed from the silkworms that feed on Rhodamine B, Rhodamine B is the most effective color chemical used in modified diet for silkworms since their bodies, cocoons, eggs appear to be pink. Green fabric dye and Methylene Blue do not have any effects on the silkworms’ physical characteristics; the silkworms’ color resemble those which feed on fresh mulberry leaves in the control batch.
References


http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3561333/


