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Silkworm larvae (*Bombyx mori*) can learn cues associated with finding food

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ABSTRACT: The present study investigated the ability of silkworm *Bombyx mori* (Lepidoptera: Bombycidae) larvae to learn. Silkworm larvae were trained to consume food that was placed on red paper; consequently they became attracted to red, rather than blue paper even in the absence of food. In contrast, untrained controls had no preference for either red or blue paper. These results suggested that silkworm larvae learned to associate red paper with food, and that they can discriminate colors.

Keywords: *Bombyx mori*; Color discrimination; Food; Larva; Learning; Silkworm.

1. INTRODUCTION

Learning has been investigated in insects for several decades [1], which has significantly contributed to the understanding of learning in animals [2, 3]. The fruit fly *Drosophila melanogaster* and the honeybee *Apis mellifera* have served as excellent model systems of learning: fruit flies are suitable for molecular genetic approaches [4, 5], and honeybees are highly adaptive and can continuously incorporate new experiences and even learn conceptual relationships [3, 6, 7]. The learning abilities of herbivorous insects, including lepidopterans, in host-selection and host-finding behavior have also been investigated [8-10]. Studies of the plant-related learning behaviors of herbivorous insects could be applied to pest control [11] and are contribute to understanding the coevolution of plants and insects [12].

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is a typical experimental lepidopteran insect that offers several advantages as an animal model [13]. For instance, it can be easily reared to produce large populations with a genetically uniform background and the body is a suitable size for surgical procedures. Moreover, silkworms are economically important as major components of the sericulture industry. Nevertheless, the learning abilities of silkworms have not been explored in detail. A recent study has shown that silkworm moths can learn in the oviposition behaviour [14]. However, as far as we can ascertain, the learning abilities of silkworm larvae have not been described in published papers except a conference proceeding, which reported the spatial learning ability of silkworm larvae [15]. Therefore, the present study examined whether silkworm larvae can learn to associate colored paper with food. We trained silkworm larvae to consume food that was placed on red paper, then assessed whether they would be attracted to red or blue paper without food.

2. MATERIALS AND METHODS

Silkworm eggs of the c10 (https://shigen.nig.ac.jp/silkwormbase/ViewStrainDetail.do?name=c10) and p50 (https://shigen.nig.ac.jp/silkwormbase/ViewStrainDetail.do?name=p50) strains provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Science, Sports and Culture of Japan (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp) were incubated at 25°C. Hatched larvae were fed *ad libitum* with the artificial diet Silkmate PS (Nihonnousan Kogyo Co. Ltd., Kanagawa, Japan), and reared at 25°C under a daily 12 h light-12 h dark cycle. A white fluorescent lamp (FL10EX-D-Z, Toshiba Corporation, Tokyo, Japan; color temperature 6700K; average of Rendering index (Ra) 88; spectral distribution https://www.akaricenter.com/chokkan/toshiba/img/3-fl20ssex-bunko-exdexn.jpg) was the source of illumination with 100–150 lux at the level of the silkworms, which were handled during the daytime. First and 2nd-instar larvae were housed in transparent plastic boxes (Foodpack Toku-2-Shin SE, Chuo Kagaku Co., Ltd., Saitama, Japan) measuring 25 × 15 × 5 cm (H × W × D) .

We trained the silkworms from day 1 of the 3rd until the end of the 4th instar in 26 × 20 × 5 cm opaque ivory-colored plastic containers (MS-120, ENTEC, Niigata, Japan), and 5th instar larvae were trained in those opaque ivory-colored containers (MS-109, ENTEC) measuring 32 × 23 × 6 cm. One half of the inside of these containers was covered with red (PI-N85R) and blue (PI-N83B) paper (Maruai Inc., Yamanashi, Japan), respectively (Fig. 1a and Fig. 2a). Larvae (n = 20) were fasted for 2 h, then placed on the borderline between the red and blue paper at the bottom of the containers, where food was placed in a 5 cm diameter circular area in the center of the red paper. The larvae were left in the containers until the following day. This was repeated daily for 11 days until day 1 of the 5th instar. Untrained control animals were housed in the same type of opaque ivory-colored containers without colored paper on the inside. Control animals were fed *ad libitum*.

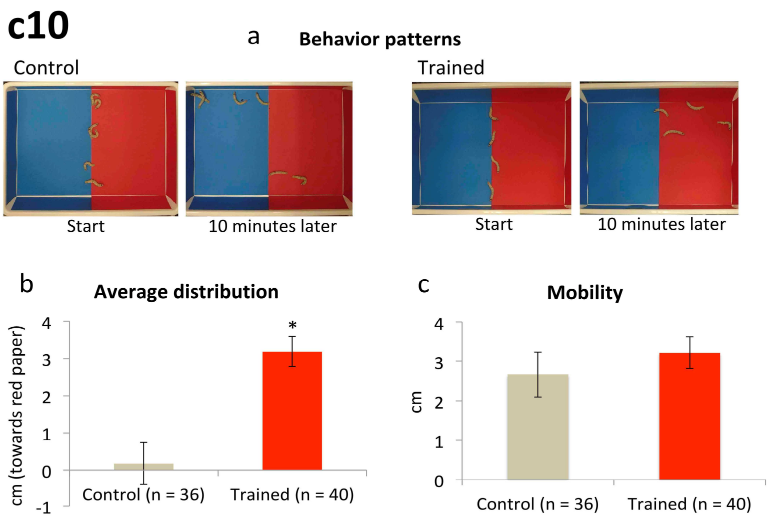

Figure 1. Effects of training silkworm larvae (c10 strain) to find food placed on red paper. a) Representative behaviors. b) Average distribution. c) Mobility. *P < 0.01, Student *t*-tests. Values are shown as means ± SEM.

We examined whether the trained larvae were attracted to red or blue paper in the absence of food on day 2 of the 5th instar. Groups of larvae (n = 4–10 each) were fasted for 2 h, then placed on the border between clean red and blue paper at the bottom of the same types of containers in which they were trained. The larvae were allowed to move freely for 10 min (Fig. 1a and Fig. 2a), and their behavior was video recorded. The average distribution of individuals was determined by measuring the distance from the border (starting line) to where the head of each larva faced the red paper (values for larvae distributed towards the blue paper were taken as negative). Mobility was calculated as the absolute value of the distance moved by each larva from the border regardless of direction. All data are expressed as means ± standard error of means (SEM). Data were statistically analyzed using Student *t*-tests, and $P < 0.05$ was considered to represent significance.

3. RESULTS AND DISCUSSION

The results of the c10 strain showed that trained larvae were attracted to red, rather than blue paper even in the absence of food. Significantly more trained larvae were distributed on red, than blue paper (Fig. 1a, b). In contrast, control animals did not indicate any preference as each individual moved randomly towards either color (Fig. 1a, b). However, mobility did not significantly differ between the trained and control larvae (Fig. 1c). These results suggest that silkworm larvae learned to associate the color red with food.

On the other hand, we could not demonstrate the learning ability in the p50 strain. In this strain, trained larvae did not indicate any preference for either red or blue paper (Fig. 2a, b). Both trained and control larvae stayed near the starting line and did not move much (Fig. 2a), and p50 larvae were less mobile than c10 larvae (Fig. 2c).

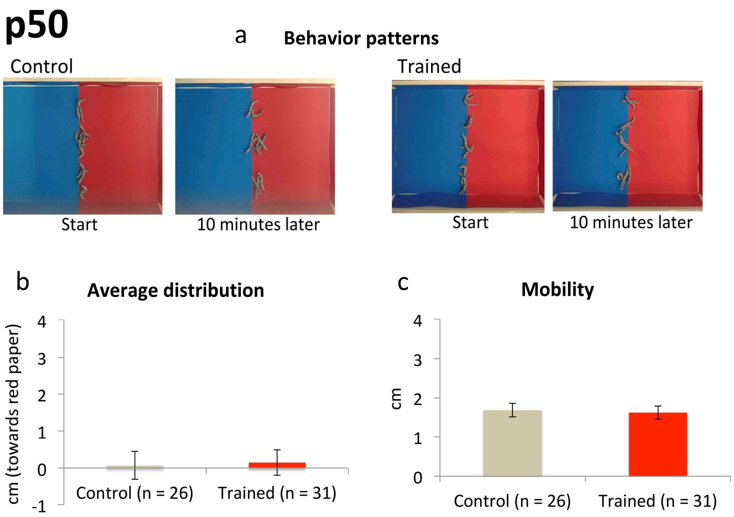

Figure 2. Effects of training silkworm larvae (p50 strain) to find food placed on red paper. a) Representative behaviors. b) Average distribution. c) Mobility. *$P < 0.01$, Student *t*-tests. Values are shown as means ± SEM.

Learning is well documented in wild lepidopteran larvae [16-19]. Silkworms feed only on mulberry leaves, and they have been domesticated for thousands of years. Nevertheless, they appear to have retained learning ability associated with finding food at the larval stage. Our results were positive with the highly mobile c10 strain of silkworms, but not with the p50 strain that has been the standard for silkworm studies, which is not particularly mobile. Our breeding experience of silkworm strains has found that c10 larvae tend to wander.

One advantage of silkworms is that they have pharmacokinetic parameters and metabolic pathways similar to mammals, and thus are useful animal models [13]. Therefore, our system of assessing silkworms could be applied to screening for drugs that modulate learning efficiency in mammals. We believe that our results have potential for future investigation and development.

The present study found that silkworms could distinguish between red and blue paper, indicating the ability to discriminate color. However, the possibility that they could also detect other factors such as brightness, smell or the texture of colored paper cannot be ruled out. Further detailed investigation is required for conclusive validation. Some behavioral studies have shown that adult lepidopterans can discriminate colors [20] and electrophysiological findings have found that the larval eyes (stemmata) of lepidopterans, including silkworms contain different types of color receptor cells [21]. However, behavioral studies have produced little evidence of color discrimination by lepidopteran larvae [22]. Our system using silkworms could be suitable for studying color discrimination in other lepidopteran larvae. Studies of color discrimination in lepidopteran larvae might prove useful for pest control, as these larvae can seriously damage crops.

4. CONCLUSION

The present study demonstrated for the first time the ability of silkworm larvae to learn visual cues associated with food searching. It was also suggested that silkworm larvae discriminate colors. Our system using silkworms could be applied to screening for drugs that modulate learning efficiency, and might be suitable for studying color discrimination in lepidopteran larvae. We believe that our results have potential for future investigation and development.

Authors' Contributions: TT, TH and KS conceptualized and designed the study. TT and TH carried out material preparation, data collection and analysis. KS wrote, reviewed and revised the manuscript. YE managed the literature searches and edited the manuscript. KS supervised the study. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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REFERENCES

1. Thorpe WH, Jones FGW. Olfactory conditioning in a parasitic insect and its relation to the problem of host selection. Proc Royal Soc B. 1937; 124: 56-81.
2. Capaldi EA, Robinson GE, Fahrback SE. Neuroethology of spatial learning: the birds and the bees. Annu Rev Psychol. 1999; 50: 651-682.
3. Giurfa M. Learning and cognition in insects. Interdiscip Rev Cogn Sci. 2015; 6(4): 383-395.
4. Bust GU, Cervantes-Sandoval I, Davis RL. Olfactory learning in *Drosophila*. Physiology. 2010; 25(6): 338-346.
5. Davis RL. Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. Annu Rev Neurosci. 2005; 28: 275-302.
6. Avarguès-Weber A, Giurfa M. Conceptual learning by miniature brains. Proc Royal Soc B. 2013; 280: 19-21.

7. Capaldi EA, Smith AD, Osborne JL, Fahrbach SE, Farris SM, Reynolds DR, et al. Ontogeny of orientation flight in the honeybee revealed by harmonic radar. Nature. 2000; 403: 537-540.
8. Cunningham JP, West SA, Zalucki MP. Host selection in phytophagous insects: A new explanation for learning in adults. Oikos. 2001; 95: 537-543.
9. Du Y, Poppy GM, Powell W, Wadhams LJ. Chemically mediated associative learning in the host foraging behavior of the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae). J Insect Behav. 1997; 10: 509-522.
10. Dunlap AS, Nielsen ME, Dornhaus A, Papaj DR. Foraging bumble bees weigh the reliability of personal and social information. Curr Biol. 2016; 26: 1195-1199.
11. Little CM, Chapman TW, Hillier NK. Considerations for Insect Learning in Integrated Pest Management. J Insect Sci. 2019; 9(4): 1-14.
12. Jones PL, Agrawal AA. Learning in Insect Pollinators and Herbivores. Annu Rev Entomol. 2017; 62: 53-71.
13. Panthee S, Paudel A, Hamamoto H, Sekimizu K. Advantages of the Silkworm As an Animal Model for Developing Novel Antimicrobial Agents. Front Microbiol. 2017; 8: 373.
14. Gámez AM, León SP. The role of learning in the oviposition behavior of the silkworm moth (*Bombyx mori*). Behav Processes. 2018; 157: 286-290.
15. Tsunashima M, Takano Y, Awazu S. Spatial learning ability of silkworms [in Japanese]. In: Proceedings of the 78th Annual Convention of the Japanese Psychological Association. 2014; 3AM-1-087. https://www.jstage.jst.go.jp/article/pacjpa/78/0/78_3AM-1-087/_article/-char/ja
16. Blackiston DJ, Silva Casey E, Weiss MR. Retention of memory through metamorphosis: Can a moth remember what it learned as a caterpillar? PLoS One. 2008; 3(3): e1736.
17. Carlsson MA, Anderson P, Hartlieb E, Hansson BS. Experience-Dependent Modification of Orientational Response to Olfactory Cues in Larvae of *Spodoptera littoralis*. J Chem Ecol. 1999; 25: 2445-2454.
18. Deboer G. Diet-induced food preference by *Manduca sexta* larvae: acceptable non-host plants elicit a stronger induction than host plants. Entomol Exp Appl. 1992; 63: 3-12.
19. Pszczolkowski MA, Brown JJ. Single experience learning of host fruit selection by lepidopteran larvae. Physiol Behav. 2005; 86: 168-175.
20. van der Kooi CJ, Stavenga DG, Arikawa K, Belušič G, Kelber A. Evolution of Insect Color Vision: From Spectral Sensitivity to Visual Ecology. Annu Rev Entomol. 2021; 66: 435-461.
21. Ichikawa T, Tateda H. Distribution of color receptors in the larval eyes of four species of lepidoptera. J Comp Physiol A. 1982; 149: 317-324.
22. Süffert F, Götz B. Verhalten von Schmetterlingsraupen gegenüber farbigen Flächen [in German]. Naturwissenschaften. 1936; 24: 815.