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Original Communication

Reconsideration of the abnormal larval behaviors used to characterize a strain of the silkworm *Bombyx mori* as a model of Parkinson's disease

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ABSTRACT

A mutant strain of the silkworm *Bombyx mori* (L.) (Lepidoptera: Bombycidae) was proposed as a model for Parkinson's disease. Homozygous *op* mutants exhibiting translucent larval integument are reported to exhibit occasional unique actions such as vibration. This study investigates the validity of a previous report on this abnormal behavior and movement of mutant and its suitability as a model for Parkinson's disease. A previously reported study movie documenting occasional unique actions of *op*/*op* larvae was considered; it revealed that these actions were simply reactions to the touch of other larvae. Moreover, xanthine oxidase injection did not rescue the phenotype of *op* mutant larvae, bringing into question the proposed role of the uric acid synthesis-modulating pathway in this mutant.

KEYWORDS: silkworm, Parkinson's disease, *Bombyx mori*, xanthine oxidase, abnormal behavior

INTRODUCTION

The silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae), is a lepidopteran model insect [1] that has been completely domesticated and is no longer found in the wild. However, a presumed ancestral species, *B. mandarina*, can still be found in mulberry fields. The silkworm has been used in genetic studies since the birth of genetics in the early 1900s, primarily, in Japan. *B. mori* is a convenient experimental model organism because a large variety of mutants are available in the egg, larval, pupal, and adult stages [2].

A draft sequence of the genome of the p50 (standard) strain of *B. mori* was constructed by 3-fold whole-genome shotgun (WGS) sequencing in Japan [3]. Moreover, a draft sequence of the p50 strain was constructed by 5.9-fold WGS sequencing in China [4]; ultimately, full-scale genome sequencing was performed [5].

Several genes considered to be involved in human diseases, including Parkinson's disease, were identified in the genome of *B. mori*. Parkinson's disease is a progressive disorder of the central nervous system (CNS), characterized by a reduced concentration of the neurotransmitter dopamine in the brain [6]. This dopamine deficit is caused by the premature death of dopamine-containing neurons in a region of the midbrain, leading to debilitating problems with tremor, muscular rigidity, and slowness of movement [7]. In insects, dopamine is involved in cuticlar sclerotization and melanization in addition to its role as a neurotransmitter [8]. Precursors for sclerotization are derived from the amino acid tyrosine in three enzymatic steps. First, the tyrosine is hydroxylated to 3,4-dihydroxy-phenylalanine (L-Dopa) by tyrosine hydroxylase (TH). The L-Dopa is then decarboxylated to dopamine by dopa decarboxylase, followed by the acylation of the dopamine amino groups with

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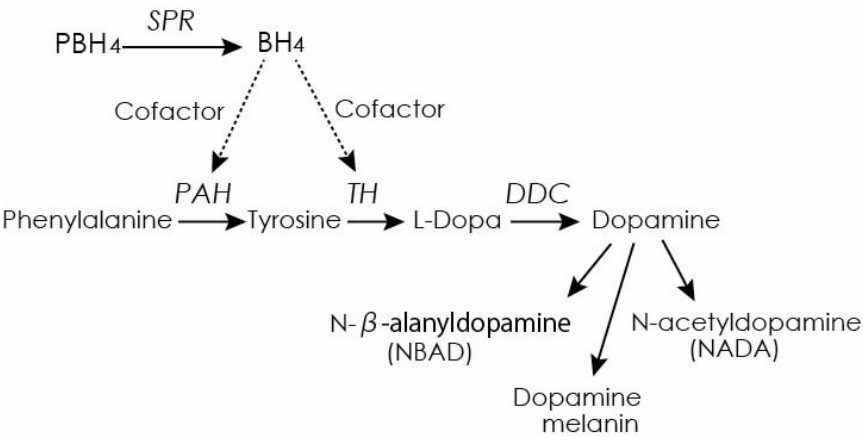

Figure 1. Pathways for dopamine biosynthesis along with its related products from phenylalanine. PBH_4, 6-pyruvoyl-tetrahydropterin; BH_4, tetrahydrobiopterin; L-Dopa, 3,4-dihydroxyphenylalanine; SPR, sepiapterin reductase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; DDC, Dopa decarboxylase.

either acetate or β-alanine to form the cuticle tanning precursors catecholamines N-acetyldopamine (NADA) or N-β-alanyldopamine (NBAD), respectively (Figure 1). Dopamine, the central molecule for both sclerotization and melanization, can also be channeled into the pathway for melanin production [8].

A strain of *B. mori* considered to be a model for Parkinson's disease is the *op* mutant, which has translucent larval integument [9] (http://www.shigen.nig.ac.jp/silkwormbase/). In wild-type *B. mori*, uric acid, one of the end products of nitrogen metabolism, is synthesized in the larval fat body and transported to the epidermis. The uric acid accumulates as urate granules in epidermal cells and renders the larval skin opaque [10]. To date, more than 30 translucent mutants have been reported [11] with the responsible genes identified for the *oq* [12], *og* [13], *od* [14], and og^z [15] mutants. Silkworm larvae with translucent skin are conventionally called "oily" in Japan because the translucent skin looks like a paper blurred with oil. Therefore, the first letter "*o*" in the gene symbols stands for "oily". Through maicroarray analysis, Tabunoki *et al*. [9] identified a novel uric acid synthesis-modulating pathway, from DJ-1 to xanthine dehydrogenase (XDH), that is repressed in the *op* mutant. Human DJ-1 is considered to be the causative gene in PARK7-linked familial Parkinson's disease [16]. Xanthine oxidase (XO) catalyzes xanthine to uric acid [17]. Moreover, Tabunoki *et al*. [9] show that levels of DJ-1 and TH mRNA are lower in the brain of *op* mutant and propose that the *op* mutant is a potential *B. mori* model for Parkinson's disease. To support this proposal, the authors report that the *B. mori op* mutant exhibits spontaneous and pronounced translucency during the larval stage and demonstrates occasional unique actions such as vibration. A movie on comparing these actions between *op* and wild-type larvae is included in their report.

In this report, the unique actions of the mutant that was reported as a *B. mori* model for Parkinson's disease were considered. The actions of normal strains were filmed for comparison with those of *op* larvae, and the movie of *op* individuals provided by Tabunoki *et al*. [9] was studied. Whether the translucent phenotype of the *op* mutant is caused by the inability to synthesize uric acid, resulting from repressed expression of XDH (an equivalent enzyme of XO), was also examined. In addition, the head-wagging behavior of *Arcte coerula* larvae was studied as the best example of head-wagging behavior shown by lepidopteran larva. The results indicate that caution is warranted when relating the actions or behaviors of silkworms to those characteristics of human diseases.

MATERIALS AND METHODS

Silkworms

This study used the *op* (o751), and og^t (o35) strains maintained at the Institute of Genetic

Resources, Faculty of Agriculture, Kyusyu University (NBRP silkworm database, http://www.shigen.nig.ac.jp/silkwormbase/). The o751 strain includes *op*/*op*, *op*/+, and +/+ individuals because this strain results from crossing an *op*/+ female moth with an *op*/+ male moth. Homozygotes of *op* exhibit translucent larval skin, but *op*/+ and +/+ individuals exhibit normal opaque larval skin (Figure 2).

To observe the reactions of normal silkworm larvae for several stimulations, the og^t (o35), p50, and Kuroshima (black-striped larvae) strains were used. The p50 and Kuroshima strains are maintained at Tokyo University of Agriculture and Technology.

Study of the "occasional unique action" shown by *op* larvae

To reconsider the reported "occasional unique action" shown by *op* larvae, the movie depicting *op* individuals provided by Tabunoki *et al*. [9] was studied in detail.

Arcte coerula larva

The larvae of *Arcte coerula* were selected to provide the best example of lepidopteran larval head-shaking behaviors. The larvae of *A. coerula* were found on the campus of Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan. The *A. coerula* larvae grasp the food grass (*Boehmeria nivea* var. *nipononivea*) that grows in the field. To stimulate head-shaking (wagging) behavior for the video, the larvae were gently touched.

Video recordings

Videos of the actions and behaviors of the silkworms and *Arcte coerula* larvae were made using a Sony Handycam HDR-PJ590V digital HD video camera recorder (Sony Corp., Tokyo, Japan). Furthermore, to show the actions of larvae, blurring figures from the videos were obtained.

Injection of XO into silkworm larvae

Injection of XO changes the translucent skin of *og*/*og* larva to white and opaque [18]. Lyophilized XO (EC 1. 17. 3. 2) (0.32 U/mg powder) from buttermilk was purchased (Oriental Yeast Co. Ltd.). The solution of XO (0.15 mg/μL) was prepared by dissolving the enzyme in Grace's Insect Medium (1x) (Gibco/Life Technologies, Carlsbad, CA, USA). Silkworm larvae were anesthetized with diethyl ether on the first day of the 5th instar. The solution (20 μL) was injected at the base of the 3rd abdominal leg into the hemocoel. Injected larvae were fed on the mulberry leaves. The larvae were observed and photographed until 100 h after injection. As a positive control, the og^z/og^z larvae were used. The og^z mutant is known for its translucent character, and the og^z gene is an allele of og^t [15].

RESULTS

Analysis of the "occasional unique action" of *op* larvae

The video by Tabunoki *et al*. [9] was studied in detail; *op* and wild-type individuals were placed in separate boxes. However, one problem is that the density of *op* larvae (16 individuals; left box) was higher than that of the wild-type (9 individuals, right box). The conditions for the *op* larvae were so crowded that they touched each other. The three individuals indicated in the movie by black arrowheads clearly shook their heads. However, on inspection, it was revealed that these actions are no more than their reactions to being touched by other individuals. The total length of the video is 5 min and 38 s. From 2 min and 00 s to 2 min and 20 s, the middle individual, indicated by the arrowhead, shakes its head; however, this action is a reaction to the touch of the individual eating mulberry on its right side. At 1 min and 00 s from the beginning, the right individual, indicated by arrowhead, shakes its head several times, but these actions are reactions to being touched by the head of the middle individual (indicated by arrowhead). From 5 min and 10 s to 5 min and 20 s, the left individual, indicated by the arrowhead, shakes its head; again, this action is a reaction to the movement of the middle individual (indicated by arrowhead). Such head shaking is typical of the normal movement of silkworms, regardless of the strain. Thus, the judgment by Tabunoki *et al*. [9] that this is an "occasional unique action" is likely to be incorrect.

The possibility that the larvae of *op* strain would exhibit other "unique" actions that were not shown

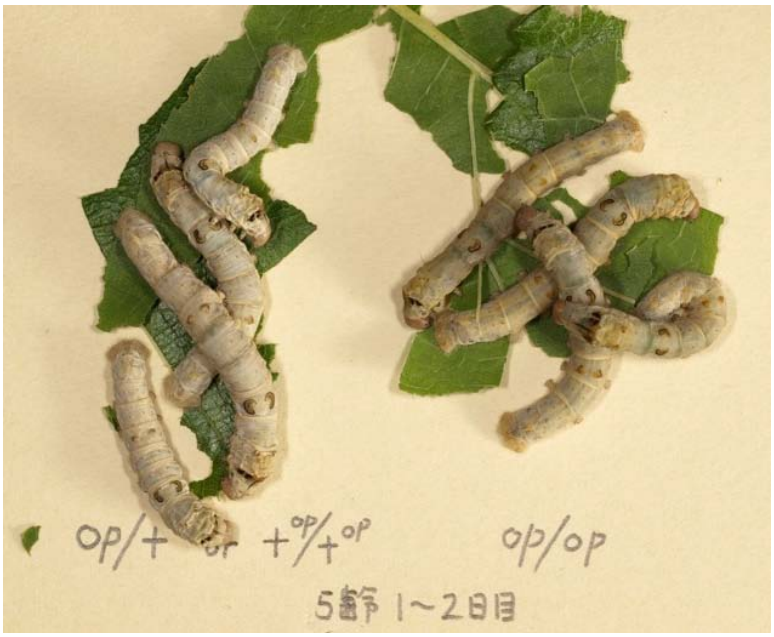

Figure 2. The 5th instar larvae of *op* (o751) strain 1 or 2 days after the 4th ecdysis. Larvae on the left are normal (*op*/+ or +/+). Larvae on the right are *op*/*op* individuals with translucent skin.

Figure 3. Head-shaking behavior of the silkworm larvae (p50 strain). The observer blew on the larvae. The larvae shook their heads immediately (blurring individuals).

in the movie was considered. Therefore, *op* strain larvae were reared, observed, and filmed for a long time. No actions or movements considered to be unique were observed in the *op* strain larvae.

Reactions of normal silkworm larvae to various stimuli

When an observer blew on the normal larvae, the larvae shook their heads (region from the

2nd abdominal segment to the head) to right and left (Figure 3). During head shaking, the larvae were fixing their four pairs of abdominal legs and one pair of caudal legs to the ground (or leaves). When a shock (bump) was given to the tray, some individuals of many larvae trembled for several seconds immediately after that (Figure 4, 5). In addition, the larva shook its head when touched by another larva (Figure 6).

Head-wagging behavior of *Arcte coerula* larva

As the best example of head-shaking behaviors, the threat display of *Arcte coerula* larva is demonstrated. The *A. coerula* larva grasps the food plant, *Boehmeria nivea* var. *nipononivea*. When a man touches the larva, it wags its head right and left rapidly and very intensely. Simultaneously, the food plant grasped by this larva shakes vigorously (Figure 7).

Injection of XO into the *op* larvae

Whether the *op*/*op* phenotype could be rescued by the injection of XO was assessed. XO was injected into the *op* and og^z larvae, with the og^z larvae serving as a positive control. The translucent skin of og^z individuals gradually whitened and had turned almost completely white and opaque after 100 h (Figure 8). In contrast, the translucent skin of *op* individuals never changed, even at 96 h after injection (Figure 9).

DISCUSSION

Reconsideration of the "occasional unique action" shown by *op* larvae

In sericulture, the population density of larvae in the rearing bed is generally high. Mulberry feed is provided to the larvae once or twice a day. Because the mulberry leaves gradually dry or are eaten, the larvae enter a fasting state until the next mulberry provision. The larvae remain still, except for lifting of the head (including the thorax), without displaying any body movement. In normal rearing, several racks are placed in rooms with many trays on each rack. When providing mulberry leaves, the trays are pulled out. At this time, a shock (bump) is given to the tray. Immediately, the larvae begin to tremble, lasting for several seconds (Figures 4, 5). According to the observations, this trembling action of the larvae occurs frequently, regardless of the silkworm strain. Thus, it was suspected that Tabunoki *et al*. [9] might have interpreted this trembling action as the "occasional unique action". However, "occasional unique action" shown by them is not this trembling action. According to the observations, it is likely that Tabunoki's "occasional unique action" is a reaction to the touch of other individuals.

Surprisingly, the uric acid content in the integument of wild-type individuals (*op*/+ or +/+) measured by Tabunoki *et al*. [9] was extremely low. Usually, the uric acid content in the integument of wild-type (non-translucent) individuals is 5%-10% dry weight [18, 19]. However, in their study, the uric acid content in the *op*/+ or +/+ (wild-type) individuals was 0.01% [9]. Therefore, the uric acid content of the *op*/*op* individuals, is lower than that of *op*/+ or +/+. According to this data, the o751 strain (*op* strain) is extremely unique. Therefore, these *op*/*op* individuals might exhibit unique behaviors or movements. However, no behavior or movement specific to *op*/*op* individuals was observed.

When the silkworm larvae feel air currents (as from the breath of an observer), the vibration of their rearing bed, or the touch of other larvae, they shake or wag their heads (region from the 2nd abdominal segment to the head) right and left. During shaking or wagging of the head, four pairs of abdominal legs and one pair of caudal legs are fixing to the ground (or leaves). There is nothing unusual about these actions. One of the main symptoms of Parkinson's disease is tremor, which is the uncontrollable shaking of the hand or arm. If a silkworm were to show symptoms similar to those of Parkinson's disease, the thoracic, abdominal, and caudal legs would shake, preventing maintenance of its posture. In contrast, *op*/*op* individuals maintain their posture by grasping plants with their abdominal and caudal legs, can move, and can shake their heads normally.

op larvae response to XO injection

The translucent character of *oq*, *og*, og^k, og^t, and og^z mutants is caused by a deficiency in XDH activity [20]. XDH catalyzes the oxidation of hypoxanthine to xanthine and then xanthine to uric acid [17]. The causative gene in the *oq* mutant is BmXDH1. An 8-bp deletion in BmXDH1

Figure 4. Trembling behavior of silkworm larva (Kuroshima strain). When a shock (bump) was given to the tray, the individual shown in the center trembled for several seconds.

Figure 5. Trembling behavior of silkworm larva (Kuroshima strain). When a shock (bump) was given to the tray, the white individual shown in the center trembled for several seconds.

Figure 6. Head-shaking behavior of the silkworm larva (o351 strain) in response to touch by another larva. (A) The unmoving larva. (B) When the unmoving larva was touched several times by another, it shook its head (blurring individual).

Figure 7. Head-wagging behavior of *Arcte coerula* larva. (A) The unmoving larva. (B) Larva stimulated by touch wagged its head to the right and left rapidly and intensely (shown by blurring).

Figure 8. Effect of xanthine oxidase injection into the 5th instar *og*^{z} larvae on skin translucency. Three larvae on the left side shown in each figure were injected with 3 mg of xanthine oxidase dissolved in Grace's insect medium. The three larvae on the right side shown in each figure were injected with Grace's insect medium without xanthine oxidase. A, 24 h; B, 48 h; C, 79 h, and D, 100 h after injection.

Figure 9. Effect of xanthine oxidase injection into the 5th instar *op* larvae on skin translucency of their color. Three larvae on the left side shown in each figure were injected with 3 mg xanthine oxidase dissolved in Grace's insect medium. The three larvae on the right side shown in each figure were injected with Grace's insect medium without xanthine oxidase. A, 24 h; B, 48 h; C, 72 h; and D, 96 h after injection.

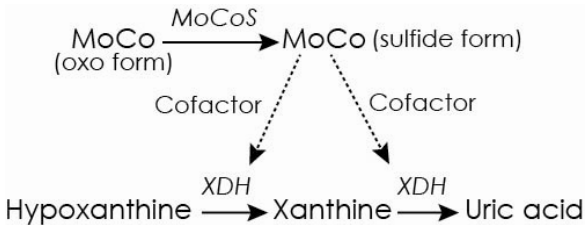

Figure 10. Pathways for uric acid biosynthesis from hypoxanthine. MoCo, molybdenum cofactor; MoCoS, molybdenum cofactor sulfurase; XDH, xanthine dehydrogenase.

generates a premature stop codon [12]. XDH requires the sulfide form of molybdenum cofactor (MoCo) for its enzymatic activity [21]. The causative gene of the *og* mutants is the MoCo sulfurase (MoCoS) gene. MoCoS is responsible for converting the oxo form of MoCo to its sulfide form [22, 23] (Figure 10). In the og^k mutant, a deletion from exon 5 to exon 6 generates a premature stop codon [13]. In the og^t mutant, a premature stop codon was generated by the insertion of a miniature inverted-repeat transposable element (MITE) into exon 5 [13]. In the og^Z mutant, a deletion from the upstream of exon 1 to exon 3 prevents transcript generation [15]. In the original *og* mutant, several amino acid-substitutions are likely to decrease the activity of MoCoS. However, the molecular mechanism of the *og* remains unknown [13]. Tamura *et al*. [18] clearly showed that the injection of XO derived from milk, an enzyme equivalent XDH, changed the translucent skin of *oq*, *og*, and og^t mutants to the normal opaque skin and increased the amount of uric acid in the integument. Thus, mutants induced by a deficiency in XDH activity can be rescued by the injection of XO [18, 24]. Tabunoki *et al*. [9] found a novel uric acid synthesis-modulating pathway, from DJ-1 to XO based on microarray analysis. Human DJ-1 is believed to be a causative gene in PRK7-linked familial Parkinson's disease [16] and may be a key pathway in uric acid synthesis [9]. XDH1 and XDH2 mRNAs are significantly down-regulated in the *op* mutant [9]. If the cause of *op* phenotype is the down-regulation of XDH, it should be possible to rescue the phenotype by XO injection. However, XO injection did not rescue the *op* phenotype in the present study. Therefore, it may be necessary to reconsider the uric acid synthesis-modulating pathway from DJ-1 to XDH reported by Tabunoki *et al*. [9], which will be best addressed after cloning of the causative gene in *op*.

Abnormal behaviors of the silkworm larva and moth and normal lepidopteran larvae behavior

Most lepidopteran larvae resemble those of the silkworm morphologically. The larvae grasp a food plant with their abdominal legs and caudal legs and frequently move and wag while searching for food or shaking off an enemy. One extreme example of these wagging behaviors is the threat display of *Arcte coerula* larvae. These larvae grasp the food plant, *Boehmeria nivea* var. *nipononivea* and respond to stimulation from the outside by rapid and vigorous head-wagging (Figure 7).

Several silkworm strains show clearly abnormal behavior or movements. The non-preference strain that eats plant leaves other than mulberry exhibit a food-habit abnormality [25]. The *spli* strain has larvae that are soft and pliable [26]. Overturned *spli* larvae cannot readily get up. Moreover, the full courtship behavior of *spli* male moths is evoked by the pheromone "bombykal" alone but not by "bombykol" [27]. The ZZ_1 male moths are flapless because of abnormal indirect flight muscle dystrophy [28]. The infected larvae with nuclear polyhedrosis virus (NPV) keep wandering intensely on the rearing bed (tray) [29]. Thus, these behaviors are clearly abnormal.

In addition, Tabunoki *et al*. [9] reported *op*/*op* male individual's infertility. The expression levels of *B. mori* DJ-1 protein decreased and DJ-1 changed to the oxidized form in the testis of fifth instar *op* mutant larvae. However, the fertilized eggs when using *op*/*op* male moths can be obtained (details not shown).

CONCLUSION

Occasional unique actions of *op*/*op* larvae reported by Tabunoki *et al*. [9] were simply reactions to the touch of other larvae. Xanthine oxidase injection did not rescue the phenotype of *op* mutant larvae.

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CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

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