Reprogramming of larval locomotory pattern during baculovirus-induced host manipulation

Hiroyuki Hikida* and Susumu Katsuma*

Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life
Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan

*Corresponding authors, Mailing address: Department of Agricultural and Environmental Biology,
Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi,
Bunkyo-ku, Tokyo, 113-8657, Japan
Phone: +81-3-5841-8994. Fax: +81-3-5841-8993.
E-mail: hikida@ss.ab.a.u-tokyo.ac.jp (H.H.) or katsuma@ss.ab.a.u-tokyo.ac.jp (S.K.)

16 **Abstract**

17 Many parasites manipulate host behaviour to enhance their transmission. Baculovirus induces
 18 enhanced locomotory activity (ELA) combined with subsequent climbing behaviour in lepidopteran
 19 larvae, which facilitates viral dispersal. Previously, larval locomotion during ELA was summarized
 20 as the distance travelled for a few minutes at several time points. However, as ELA continues for
 21 several hours, these methods are unlikely to evaluate larval locomotion precisely during ELA. We
 22 developed a novel method to continuously trace the locomotion of *Bombyx mori* larvae using
 23 time-lapse imaging. This method successfully quantified the locomotory activities of larvae infected
 24 with *Bombyx mori nucleopolyhedrovirus* (BmNPV) for 24 h. We found that both mock- and
 25 BmNPV-infected larvae periodically repeated moving and pausing with a similar frequency. In
 26 contrast, BmNPV-infected larvae showed fast and long-lasting locomotion compared with
 27 mock-infected larvae, which resulted in longer locomotory distances in infected larvae. Moreover,
 28 BmNPV-infected larvae exhibited biphasic behaviour. Initially, BmNPV-infected larvae showed
 29 longer locomotory distances, but the locomotory pattern was similar to mock-infected larvae.
 30 However, during the second phase, the locomotory pattern was drastically altered, with an extremely
 31 larger locomotory area. These results indicate that BmNPV reprograms host locomotory pattern,
 32 which is a turning point for the process of BmNPV-induced host manipulation.

33

34 **Keywords:** host manipulation, Baculovirus, hyperactivity, *Bombyx mori*

35

36

1. Introduction

Manipulation of host behaviour is a strategy commonly used by parasites to enhance their proliferation in the environment [1]. For example, hairworms cause crickets to jump into the water, where the hairworms can search for their mates [2], and *Ophiocordyceps* fungi make ants to bite onto vegetation at an elevated position, which is thought to enable the fungi to disperse their spores [3]. Baculovirus is an insect-specific large DNA virus, which mainly infects lepidopteran larvae and induces dramatic alternation to host behaviour, which is known as *Wipfelkrankheit* (tree-top disease in German) [4]. Baculovirus-infected larvae exhibit horizontal hyperactivity, which is called enhanced locomotory activity (ELA), followed by vertical movement, which is known as climbing behaviour (CB). These virus-induced host manipulations finally lead the larvae to the upper tree foliage, where they die. Baculovirus encodes cathepsin and chitinase in its genome, which cooperate to liquefy the larval body at a late stage of infection [5,6]. The combination of larval death at an elevated position and liquefaction of their body is thought to facilitate viral dispersal with rainfall or avian feeding [4,7].

Baculovirus-induced host manipulation was first quantified by Goulson in 1998 using *Mamestra brassicae* multiple nucleopolyhedrovirus (MbMNPV) and *Mamestra brassicae* larvae. The study demonstrated that MbMNPV-infected larvae show higher horizontal dispersal and die at an elevated position [4]. Recent studies identified baculovirus-encoding genes, *protein tyrosine phosphatase* (*ptp*) and *ecdysteroid UDP-glucosyl transferase* (*egt*), as factors for inducing larval locomotion during ELA and CB, respectively [8,9]. The deletion of *ptp* in *Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) impairs the horizontal locomotory activity of infected *Bombyx mori* and *Spodoptera exigua* larvae, respectively [8,10,11]. As *ptp* deletion affects ELA but not climbing height during AcMNPV infection, ELA and CB are thought to be governed by different mechanisms [12]. The deletion of *egt* in *Lymantria dispar* multiple nucleopolyhedrovirus and *Spodoptera exigua* multiple nucleopolyhedrovirus mitigates the climbing height of *Lymantria dispar* and *S. exigua* larvae, respectively, although the enhancement of larval activity by *egt* was not observed in BmNPV- and AcMNPV-infected larvae [9,13–15]. A recent study further showed that miRNA-mediated hormonal regulation affects the climbing height of *Helicoverpa armigera* larvae infected with *Helicoverpa armigera* single nucleopolyhedrovirus, indicating that hormonal cues trigger CB [16]. However, these studies assessed the larval locomotory level using a few sampling points of usually once per several hours. As ELA and CB are continuous events that occur for hours or days, these methods only evaluated a part of the baculovirus-induced host manipulation.

72 We developed a novel method to continuously observe the locomotion of BmNPV-infected *B. mori*
73 larvae using short-interval time-lapse recording for 24 h, followed by a video analysis to trace larval
74 locomotion. *B. mori* has been highly domesticated for silk production, so the species is generally
75 inactive [17–19]. In contrast, they exhibit vigorous locomotion during BmNPV infection, which
76 makes the species an optimal model to measure the change in locomotory activity following
77 baculovirus infection [8]. We quantified the horizontal locomotion of BmNPV- and mock-infected
78 larvae in cell culture dishes. Our method successfully traced larval locomotion at a 3 s resolution. It
79 revealed that BmNPV-infected larvae periodically showed moving and pausing activity at a similar
80 frequency to mock-infected larvae, but their locomotion was faster and longer-lasting than that of
81 mock-infected larvae. We also found that the locomotion of both mock- and BmNPV-infected larvae
82 was restricted around food at the early phase of observation. In contrast, the locomotory pattern of
83 BmNPV-infected larvae was altered drastically at the late phase of observation, and their locomotory
84 area became extremely larger. These results provide novel insight into the mechanisms of
85 BmNPV-induced host manipulation system.

86

87

88 **2. Materials and Methods**

89 **(a) Insects, cell lines, and viruses**

90 *B. mori* larvae (Kinshu × Showa) were reared as previously described [20]. BmN-4 cells were
91 cultured in TC-100 medium supplemented with 10% fetal bovine serum at 26°C. The T3 strain was
92 used as the wild-type BmNPV [21] and propagated in BmN-4 cells. Viral titre was determined by a
93 plaque assay method using BmN-4 cells.

95 **(b) Locomotion assays**

96 Fourth-instar larvae were starved for several hours and then injected intrahaemocoelically with a
97 viral suspension containing 1×10^5 plaque forming units (PFU) or a control medium with 5 mg/ml
98 Kanamycin (Wako). From 72 h post-infection, each larva ($n = 6$) was placed in a 100 mm cell
99 culture dish, round shape of which does not hinder larval locomotory activities and allows the larvae
100 to move continuously. A portion of artificial diet and a piece of black paper were set at the bottom of
101 each dish (Figure 1A). Locomotion was recorded at a 3 s interval using a time-lapse camera
102 (TLC200-pro, Bruno) under light conditions (Figure 1A). We conducted the assay twice as assays #1
103 and #2.

105 **(c) Video processing**

106 After 24 h of recording, the video was converted to grayscale (Figure 1B). The background image
107 was created as the average intensity. The background was subtracted from all frames in the grayscale
108 video, and then binarization was conducted (Figures 1C and 1D). The processed video was used to
109 determine the larval positions at each frame by calculating the centre of mass of detected particles.
110 These image-processing steps were conducted in Image J software [22].

112 **(d) Data analysis**

113 The obtained data containing X- and Y-coordinates of the centre of mass were analysed using custom
114 Python scripts. First, we plotted the coordinates onto the original video and checked whether the
115 larval positions were determined appropriately. Based on this analysis, apparently inappropriate
116 points (i.e. dots outside the dish) were identified and removed from the data. Moreover, we found
117 that the positions of some mock-infected larvae were inappropriately mapped because the larvae
118 were so inactive that the method could not create an appropriate background image for these larvae.
119 We excluded the data for these larvae from further analysis. Consequently, we excluded one larva
120 from each assay. The modified data were used for calculating the larval travel distance between two
121 adjacent frames. When multiple points were assigned to a larva in a single frame, we adopted the
122 average of coordinates as the larval position. When the larval position was missing in a frame, the
123 position at its previous frame was used for calculation.

124

125 **(e) Quantification of larval locomotion**

126 The total travel distance was calculated as the sum of travel distance between two adjacent frames
 127 for 24 h. The accumulated travel distance was also calculated at each time point. Sums of travel
 128 distance for each 3 h segment were also calculated. We assumed that a larva exhibited “locomotion”
 129 when the average travel distance for 30 s exceeded 0.5 pixels. Moreover, we defined locomotory
 130 “continuity” as the number of serial 30 s periods in which a larva exhibited “locomotion.” When the
 131 distance of the larval position between two adjacent frames exceeded 0.5 pixels, we assumed the
 132 larvae was “moving”; otherwise, the larva was assumed to be “pausing.”

133

134 **(f) Statistical analysis**

135 A rank-sum test was used to compare the total travel distance, median locomotory duration,
 136 frequency of locomotion, and median travel distance during larval movement between mock- and
 137 BmNPV-infected larvae. The sums of 3 h travel distance were compared between mock- and
 138 BmNPV-infected larvae using a rank-sum test, followed by Benjamini–Hochberg correction.

139

140

141 **3. Results**

142 **(a) Overview of larval locomotion over 24 h**

143 Our method successfully traced the locomotion of mock- and BmNPV-infected larvae for 24 h
144 (Supplementary video and Figure 1). Mock-infected larvae generally remained close to a piece of
145 artificial diet showing little locomotion, whereas the locomotory area of BmNPV-infected larvae
146 covered almost the entire area of the dish in both assays #1 and #2 (Figures 1E and 1F). The
147 accumulated travel distance of BmNPV-infected larvae increased continually and more rapidly
148 compared with mock-infected larvae (Figures 2A and 2C). As a consequence, the total travel
149 distance of BmNPV-infected larvae over 24 h was significantly longer than that of mock-infected
150 larvae in two independent assays (Figures 2B and 2D).

152 **(b) Larval locomotion with 3 s resolution**

153 Larval locomotion of mock- and BmNPV-infected larvae were investigated at a 3 s resolution. We
154 found that mock-infected larvae periodically repeated moving and pausing as described previously
155 [17–19] (Figures 3A, 3C, S1, and S2). This periodic pattern of moving and pausing was also
156 observed in BmNPV-infected larvae, but they were more active than mock-infected larvae (Figures
157 3B, 3D, S1, and S2).

159 **(b) Quantitative analysis of larval locomotion**

160 The duration of larval locomotion was extended in BmNPV-infected larvae compared with that of
161 mock-infected larvae (Figures S3 and S4). The medians of locomotory duration in BmNPV-infected
162 larvae were ~700 s, which were significantly longer than those of mock-infected larvae (Figures 4A
163 and 4B). In contrast, there was no significant difference between the frequency of continuous
164 locomotion over 24 h of mock- and BmNPV-infected larvae (Figures 4C and 4D). Furthermore, we
165 found that BmNPV-infected larvae moved slightly faster than mock-infected larvae (Figures S5 and
166 S6). The medians of larval locomotory speed during movement were significantly higher in
167 BmNPV-infected larvae than those of mock-infected larvae (Figures 4E and 4F).

169 **(c) Temporal change in larval locomotory patterns**

170 To examine temporal change in larval locomotory pattern, 24 h data were divided into eight 3 h
171 segments and separately analysed. In all 3 h segments, mock-infected larvae stayed in the centre of
172 the dish, where an artificial diet was placed (Figures 5A, 5C, S7, and S8). BmNPV-infected larvae
173 also exhibited food-oriented distribution with slightly larger diffusion during the early time segments
174 (Figures 5B, 5D, S7, and S8). In contrast, BmNPV-infected larvae moved along the edge of the dish
175 and rarely stayed in the centre of the dish in later segments (Figures 5B, 5D, S7, and S8). The sums
176 of travel distance of BmNPV-infected larvae in the segments were generally longer than those of

177 mock-infected larvae, regardless of the infection stages (Figures 5E and 5F).

178

179

180 4. Discussion

181 We developed a long-term observation system using a time-lapse camera and continuously
182 quantified the locomotory activity of BmNPV-infected larvae over 24 h. Our method enabled us to
183 clarify the difference between the locomotory activity of mock- and BmNPV-infected larvae using a
184 single recording (Figures 1 and 2). Compared with previous methods in which larval locomotion was
185 recorded at multiple time points [11,14,23,24], this method provides a much easier way to
186 quantifying larval locomotory activities during baculovirus infection.

187
188 During the continuous observation, mock-infected larvae periodically repeated moving and pausing,
189 which coincides with previous studies (Figures 3A and 3C) [17–19]. BmNPV-infected larvae showed
190 a similar periodic pattern, but the activity level was different from that of mock-infected larvae
191 (Figures 3B and 3D). Quantitative analysis revealed that BmNPV-infected larvae exhibited
192 significantly longer locomotory duration and faster locomotion than mock-infected larvae, whereas
193 the frequency of moving was similar between mock- and BmNPV-infected larvae (Figure 4). These
194 results indicate that BmNPV infection does not induce larval locomotion but increases the
195 locomotory duration and speed, which causes infected larvae to move extremely long distance.
196 Furthermore, we found that both mock- and BmNPV-infected larvae moved close to a piece of
197 artificial diet at the early stage of infection, which indicates that BmNPV-infected larvae maintain a
198 naive locomotory pattern in this infection stage (Figures 5A–D). In contrast, at the late stage of
199 infection, BmNPV-infected larvae moved around the peripheral area of the dish and were rarely
200 found in the centre, (Figure 5B and 5D). This behaviour was not observed in mock-infected larvae
201 (Figures 5A and 5C). These data indicate that BmNPV reprograms the locomotory pattern of its host
202 at a certain time point during the late stage of infection. As larval travel distance was generally
203 longer throughout the late stage of infection, fast and extended locomotion is precedent to the
204 reprogramming of host locomotory pattern and continues independently of this reprogramming
205 (Figures 5E and 5F).

206
207 In summary, our study provides a novel insight into BmNPV-induced host manipulation in which
208 BmNPV first enhances the speed and duration of host locomotion, and then reprograms host
209 locomotory pattern, inducing distinct larval behaviour (Figure 6). Currently, baculovirus-induced
210 host manipulation is thought to be biphasic, in which the two phases are divided by whether larval
211 locomotion is horizontal or vertical. These two types of locomotion correspond to ELA (also known
212 as hyperactivity) and CB (also known as tree-top disease), respectively [8,10,12]. Recent studies
213 showed that CB is evoked by phototaxis and that a light signal just before the onset of CB fixes the
214 locomotory direction of the larvae during CB [25,26]. It has also been shown that the larval
215 locomotory direction is irreversible after the critical time point [26], implicating that a

216 reprogramming point of the larval locomotory pattern detected in this study may correspond to the
 217 transition point from ELA to CB. As this study did not evaluate vertical movement or phototaxis of *B.*
 218 *mori* larvae, further analysis is required to determine whether the biphasic behaviour observed here
 219 is simulates the ELA/CB model. Tentatively, we designate the two phases of the biphasic behaviour
 220 “marathon behaviour (MB)” and “lost-child phase (LCP),” which represent the fast and long-lasting
 221 locomotion and the reprogramming of the larval locomotory pattern, respectively (Figure 6).

222

223 In this study, BmNPV and *B. mori* larvae were used to assess larval activity during baculovirus
 224 infection, but *B. mori* is a highly domesticated insect that exhibits a distinct behavioral pattern
 225 compared with its putative ancestor, *B. mandarina* [17]. *B. mandarina* exhibits fast-moving but
 226 long-term pausing, whereas *B. mori* repeats moving and pausing over a short period [17]. As
 227 BmNPV can infect both *B. mori* and *B. mandarina*, it should be elucidated whether MB and LCP are
 228 observed in *B. mandarina* larvae infected with BmNPV, as well as in other host-baculovirus
 229 combinations. The continuous observation system developed here is applicable to other insects,
 230 although equipment size and observation period should be optimized for the particular insect species.
 231 The comparison of host locomotion in various host-baculovirus combinations may clarify the
 232 conserved range of MB/LCP model and whether the model is identical to the ELA/CB model, which
 233 will highlight underlying mechanisms for the baculovirus-induced host manipulation system.

234

235

236 **Authors' contributions**

237 H.H. conceived the study and conducted the experiments. H.H. and S.K. analysed the data and wrote
238 drafts of the manuscript.

239

240 **Competing interests**

241 We declare we have no competing interests.

242

243 **Funding**

244 H.H. is a recipient of a fellowship from the Japan Society for the Promotion of Science (19J13438).

245 This work was supported by JSPS KAKENHI grant numbers 16H05051 and 19H02966 to S.K.

246

247 **Acknowledgement**

248 We thank Takashi Kiuchi and Kenta Tomihara for useful comments. The authors would like to thank
249 Enago (www.enago.jp) for the English language review.

250

251

252 References

- 253 1. Moore J. 2002 Parasites and the Behavior of Animals. New York: Oxford University Press.
- 254 2. Thomas F, Schmidt-Rhaesa A, Martin G, Manu C, Durand P, Renaud F. 2002 Do
255 hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial
256 hosts? *J. Evol. Biol.* **15**, 356–361. (doi:10.1046/j.1420-9101.2002.00410.x)
- 257 3. Andersen SB, Gerritsma S, Yusah KM, Mayntz D, Hywel-Jones NL, Billen J, Boomsma JJ,
258 Hughes DP. 2009 The life of a dead ant: the expression of an adaptive extended phenotype.
259 *Am. Nat.* **174**, 424–433. (doi:10.1086/603640)
- 260 4. Goulson D. 1997 *Wipfelkrankheit*: modification of host behaviour during baculoviral
261 infection. *Oecologia* **109**, 219–228. (doi:10.1007/s004420050076)
- 262 5. Hawtin RE, Zarkowska T, Arnold K, Thomas CJ, Gooday GW, King LA, Kuzio JA,
263 Possee RD. 1997 Liquefaction of *Autographa californica* Nucleopolyhedrovirus-Infected
264 Insects Is Dependent on the Integrity of Virus-Encoded Chitinase and Cathepsin Genes.
265 *Virology* **238**, 243–253. (doi:10.1006/viro.1997.8816)
- 266 6. Ohkawa T, Majima K, Maeda S. 1994 A cysteine protease encoded by the baculovirus
267 *Bombyx mori* nuclear polyhedrosis virus. *J. Virol.* **68**, 6619–6625.
- 268 7. Entwistle PF, Forkner AC, Green BM, Cory JS. 1993 Avian Dispersal of Nuclear
269 Polyhedrosis Viruses after Induced Epizootics in the Pine Beauty Moth, *Panolis flammea*
270 (Lepidoptera: Noctuidae). *Biol. Control* **3**, 61–69. (doi:10.1006/bcon.1993.1010)
- 271 8. Kamita SG, Nagasaka K, Chua JW, Shimada T, Mita K, Kobayashi M, Maeda S,
272 Hammock BD. 2005 A baculovirus-encoded protein tyrosine phosphatase gene induces
273 enhanced locomotory activity in a lepidopteran host. *Proc. Natl. Acad. Sci. U. S. A.* **102**,
274 2584–2589. (doi:10.1073/pnas.0409457102)
- 275 9. Hoover K, Grove M, Gardner M, Hughes DP, McNeil J, Slavicek J. 2011 A Gene for an
276 Extended Phenotype. *Science* **333**, 1401. (doi:10.1126/science.1209199)
- 277 10. van Houte S, Ros VID, Mastenbroek TG, Vendrig NJ, Hoover K, Spitzen J, van Oers MM.
278 2012 Protein Tyrosine Phosphatase-Induced Hyperactivity Is a Conserved Strategy of a
279 Subset of BaculoViruses to Manipulate Lepidopteran Host Behavior. *PLoS One* **7**, e46933.
280 (doi:10.1371/journal.pone.0046933)
- 281 11. Katsuma S, Koyano Y, Kang W, Kokusho R, Kamita SG, Shimada T. 2012 The
282 Baculovirus Uses a Captured Host Phosphatase to Induce Enhanced Locomotory Activity
283 in Host Caterpillars. *PLoS Pathog.* **8**, e1002644. (doi:10.1371/journal.ppat.1002644)
- 284 12. van Houte S, Ros VID, van Oers MM. 2014 Hyperactivity and tree-top disease induced by
285 the baculovirus AcMNPV in *Spodoptera exigua* larvae are governed by independent
286 mechanisms. *Naturwissenschaften* **101**, 347–350. (doi:10.1007/s00114-014-1160-8)
- 287 13. Han Y, van Houte S, Drees GF, van Oers MM, Ros VID. 2015 Parasitic Manipulation of

288 Host Behaviour: Baculovirus SeMNPV EGT Facilitates Tree-Top Disease in *Spodoptera*
289 *exigua* Larvae by Extending the Time to Death. *Insects* **6**, 716–731.
290 (doi:10.3390/insects6030716)

291 14. Katsuma S, Shimada T. 2015 The killing speed of *egt*-inactivated *Bombyx mori*
292 nucleopolyhedrovirus depends on the developmental stage of *B. mori* larvae. *J. Invertebr.*
293 *Pathol.* **126**, 64–70. (doi:10.1016/j.jip.2015.01.012)

294 15. Ros VID, van Houte S, Hemerik L, van Oers MM. 2015 Baculovirus-induced tree-top
295 disease: How extended is the role of *egt* as a gene for the extended phenotype? *Mol. Ecol.*
296 **24**, 249–258. (doi:10.1111/mec.13019)

297 16. Zhang S, An S, Hoover K, Li Z, Li X, Liu X, Shen Z, Fang H, Ros VID, Zhang Q, Liu X.
298 2018 Host miRNAs are involved in hormonal regulation of HaSNPV-triggered climbing
299 behaviour in *Helicoverpa armigera*. *Mol. Ecol.* **27**, 459–475. (doi:10.1111/mec.14457)

300 17. Kômoto N. 2017 Behavior of the larvae of wild mulberry silkworm *Bombyx mandarina*,
301 domesticated silkworm *B. mori* and their hybrid. *J. Insect Biotechnol. Sericology* **86**,
302 17–20. (doi:10.11416/jibs.86.1_017)

303 18. Nagata S, Nagasawa H. 2006 Effects of diet-deprivation and physical stimulation on the
304 feeding behaviour of the larvae of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **52**,
305 807–815. (doi:10.1016/j.jinsphys.2006.04.008)

306 19. Nagata S, Morooka N, Asaoka K, Nagasawa H. 2011 Identification of a Novel
307 Hemolymph Peptide That Modulates Silkworm Feeding Motivation. *J. Biol. Chem.* **286**,
308 7161–7170. (doi:10.1074/jbc.M110.176016)

309 20. Nakanishi T, Goto C, Kobayashi M, Kang W, Suzuki T, Dohmae N, Matsumoto S,
310 Shimada T, Katsuma S. 2010 Comparative Studies of Lepidopteran Baculovirus-Specific
311 Protein FP25K: Development of a Novel *Bombyx mori* Nucleopolyhedrovirus-Based
312 Vector with a Modified *fp25K* Gene. *J. Virol.* **84**, 5191–5200. (doi:10.1128/JVI.00099-10)

313 21. Maeda S, Kawai T, Obinata M, Fujiwara H, Horiuchi T, Saeki Y, Sato Y, Furusawa M.
314 1985 Production of human α -interferon in silkworm using a baculovirus vector. *Nature*
315 **315**, 592–594. (doi:10.1038/315592a0)

316 22. Schneider CA, Rasband WS, Eliceiri KW. 2012 NIH Image to ImageJ: 25 years of Image
317 Analysis. *Nat. Methods* **9**, 671–675. (doi: 10.1038/nmeth.2089)

318 23. Kokusho R, Kawamoto M, Koyano Y, Sugano S, Suzuki Y, Shimada T, Katsuma S. 2015
319 *Bombyx mori* nucleopolyhedrovirus actin rearrangement-inducing factor 1 enhances
320 systemic infection in *B. mori* Larvae. *J. Gen. Virol.* **96**, 1938–1946.
321 (doi:10.1099/vir.0.000130)

322 24. Kokusho R, Zhang CX, Shimada T, Katsuma S. 2011 Comparative analysis of budded
323 virus infectivity of *Bombyx mandarina* and *B. mori* nucleopolyhedroviruses. *Virus Genes*

- 324 **43**, 313–317. (doi:10.1007/s11262-011-0623-7)
- 325 25. van Houte S, van Oers MM, Han Y, Vlak JM, Ros VID. 2014 Baculovirus infection
- 326 triggers a positive phototactic response in caterpillars to induce ‘tree-top’ disease. *Biol.*
- 327 *Lett.* **10**, 20140680. (doi:10.1098/rsbl.2014.0680)
- 328 26. Han Y, van Houte S, van Oers MM, Ros VID. 2018 Timely trigger of caterpillar zombie
- 329 behaviour: Temporal requirements for light in baculovirus-induced tree-top disease.
- 330 *Parasitology* **145**, 822–827. (doi:10.1017/S0031182017001822)
- 331
- 332

Figure legends

Figure 1

The summary of the behavioural monitoring system.

(A) Schematic image of the equipment. The circle and green quadrant represent a cell culture dish and a portion of artificial diet, respectively.

(B–D) Image processing. The obtained image was converted to grayscale (B), and the background was removed (C), followed by binarization (D).

(E, F) Distribution of larvae for 24 h observation in assays #1 (E) and #2 (F). Each dot indicates the larval position in a frame. Green and orange colours indicate mock- and BmNPV-infected larvae, respectively.

Figure 2

(A) The accumulated travel distance of an individual larva in assay #1. The X- and Y-axes show hours post infection (hpi) and accumulated travel distance at each time point, respectively. The travel distance was measured in pixels.

(B) The total travel distance for 24 h in assay #1 shown as a box-and-whisker plot. Boxes show the quartiles, and the whiskers extend to show the rest of the distribution. $**p < 0.01$ by rank-sum test.

(C) and (D) show the accumulated and total travel distance of assay #2, respectively.

Green and orange colours indicate mock- and BmNPV-infected larvae, respectively.

Figure 3

Travel distance between two adjacent frames, which corresponds to 3 s. A representative larva was selected from mock-infected larvae of assays #1 (A) and #2 (C) and the BmNPV-infected larvae of assays #1 (B) and #2 (D). Black lines indicate travel distance equal to 0.5 pixels in 3 s, which correspond to the threshold of moving and pausing. The rectangular diagram below the scatter plots indicates “continuity” of the locomotion, as defined in this study. Green and orange colours indicate mock- and BmNPV-infected larvae, respectively. Data of all individuals are shown in the Supplementary Materials (Figures S1 and S2).

Figure 4

(A, B) Medians of the locomotory duration of assays #1 (A) and #2 (B).

(C, D) Frequency of locomotion of assays #1 (C) and #2 (D).

(E, F) Medians of travel distance between two adjacent frames during larval movement. Assays #1 (E) and #2 (F).

All boxes and whiskers show the quartiles and 1.5 inter quartile range, respectively. Dots indicate values of each larva. $*p < 0.05$ and $**p < 0.01$ by rank-sum test, respectively.

369

370 **Figure 5**

371 (A–D) Distribution of individual larva for 3 h segments. The time range is shown above the boxes.
 372 Each dot indicates the larval position in a frame. A representative larva was selected from
 373 mock-infected larvae of assays #1 (A) and #2 (C) and the BmNPV-infected larvae of assays #1 (B)
 374 and #2 (D). The individuals are identical to those shown in Figure 3. Data of all individuals are
 375 shown in the Supplementary Materials (Figures S7 and S8).

376 (E, F) Sum of travel distance for each 3 h segment for assays #1 (E) and #2 (F). The data are shown
 377 as a box-and-whisker plot. Boxes show the quartiles, and the whiskers extend to show the rest of the
 378 distribution. Outliers distributed outside of the 1.5 inter-quartile range are indicated as black dots.
 379 Coloured dots indicate values of each larva. *Adjustment $p < 0.05$ by the rank-sum test
 380 with Benjamini–Hochberg correction.

381 Green and orange colours correspond to mock- and BmNPV-infected larvae, respectively.

382

383 **Figure 6**

384 A proposed model for BmNPV-induced host manipulation.

385 The circles and green quadrants represent the cell culture dishes and portions of artificial diet,
 386 respectively. Arrows indicate the virtual tracks of larval locomotion.

387

A

B

C

D

E

F

Before host manipulation

Similar locomotion with healthy larvae

Marathon behaviour

Fast and long-lasting locomotion

Lost-child phase

Fast and long-lasting locomotion

+
Reprogramming of
host locomotory pattern