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1 **Laboratory experiment of *Stigmatomyces majewskii* (Laboulbeniales:**

2 **Laboulbeniaceae) infection on *Drosophila suzukii* (Diptera: Drosophilidae)**

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4 Izumi Yamazaki¹, Moe Onuma¹, Tomohiko Ri^{1,2}, Izumi Okane³, Natsumi Kanzaki⁴,

5 Yousuke Degawa^{2,3}, Kyoichi Sawamura^{3,*}

6 ¹ Degree Programs in Life and Earth Sciences, Graduate School of Science and

7 Technology, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

8 ² Sugadaira Research Station, Mountain Science Center, University of Tsukuba, 1278-

9 294 Sugadaira Kogen, Ueda, Nagano 386-2204

10 ³ Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai,

11 Tsukuba, Ibaraki 305-8572, Japan

12 ⁴ Kansai Research Center, Forestry and Forest Products Research Institute, 68

13 Nagaikyutaroh, Momoyama, Fushimi, Kyoto, Kyoto, 612-0855 Japan

14

15 ORCID ID

16 I Yamazaki: <https://orcid.org/0000-0003-4697-2633>

17 M Onuma: <https://orcid.org/0000-0002-2971-7289>

18 T Ri: <https://orcid.org/0000-0002-8308-956X>

19 I Okane: <https://orcid.org/0000-0002-2365-9989>

1
2
3 20 N Kanzaki: <https://orcid.org/0000-0001-8752-1674>
4
5

6 21 Y Degawa: <https://orcid.org/0000-0002-5955-1187>
7

8
9 22 K Sawamura: <https://orcid.org/0000-0002-4229-3150>
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12
13
14 24 *Corresponding Author: Kyoichi Sawamura, Faculty of Life and Environmental Sciences,
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16
17 25 University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
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23 27 **Abstract**
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26 28 Originally endemic to Asia, *Drosophila suzukii* (Matsumura, 1931) is a serious
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29 29 agricultural pest that is rapidly spreading globally. To suppress the rising *D. suzukii*
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32 30 populations, several pest management programs have been implemented. Researchers
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35 31 have searched for natural enemies, such as predators, parasites, parasitoids, and
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38 32 pathogens. We discovered an obligate ectobiont fungus, *Stigmatomyces majewskii* H. L.
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41 33 Dainat, Manier & Balazuc, 1974, that is associated with *D. suzukii*. We conducted
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44 34 laboratory experiment of *S. majewskii* infection and revealed successful transmission via
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47 35 self-grooming and sexual contact during the courtship behavior of *D. suzukii*, although
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50 36 non-sexual contact through other behaviors may also be involved. We sought to measure
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53 37 the fitness of the fungus infected flies. The longevity of infected flies was comparable to
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56 38 non-infected flies, but further research is required to test other fitness traits.
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40 **Keywords**

41 Ectobiont fungus, Invasive species, Natural enemy, Pest control, Spotted wing

42 drosophila

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44 **Introduction**

45 The spotted wing drosophila, *Drosophila suzukii* (Matsumura, 1931), is one of the worst
46 agricultural pests in the world today; the females of this species have large, serrated
47 ovipositors that cause serious damage to ripening fruits and berries (Walsh et al. 2011;
48 Atallah et al. 2014; Asplen et al. 2015). Originally endemic to East and Southeast Asia,
49 the distribution of *D. suzukii* has since spread worldwide after its invasion of Europe and
50 the Americas in the late 2000s (Hauser 2011; Lee et al. 2011; Walsh et al. 2011; Calabria
51 et al. 2012; Cini et al. 2012; Rota-Stabelli et al. 2013; Deprá et al. 2014; Asplen et al.
52 2015; Haye et al. 2016; dos Santos et al. 2017). To suppress *D. suzukii* populations,
53 control measures, including insecticides, attract-to-kill traps, altered cultivation
54 environment, sterilized male release, and engineered gene drive systems have been
55 explored (Hamby et al. 2012; Mori et al. 2017; Buchman et al. 2018; Schetelig et al. 2018;
56 Tait et al. 2021), but have not succeeded. Introducing natural enemies (predators,
57 parasites, parasitoids, and pathogens) is an additional, clean strategy to combat invasive

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3 58 species, which can cause pandemics as they may be free from the natural enemies present
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6 59 in their original habitat (Roy et al. 2011; Schulz et al. 2019). Natural enemies applicable
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9 60 to *D. suzukii* have been sought in their original distribution range as well as among
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12 61 commercial products of biopesticides (Kasuya et al. 2013; Renkema et al. 2015;
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15 62 Cuthbertson and Audsley 2016; Becher et al. 2018; Garriga et al. 2018; Girod et al. 2018;
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18 63 Foye and Steffan 2020; Bing et al. 2021; Sario et al. 2021). In this paper, we introduce an
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21 64 ectobiont fungus, *Stigmatomyces majewskii* H. L. Dainat, Manier & Balazuc, 1974, which
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24 65 was recently discovered on *D. suzukii* in Japan, the original distribution range of the fly
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27 66 (Yamazaki et al. 2023).

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29 67 The genus *Stigmatomyces* s. str. (Laboulbeniales: Laboulbeniaceae) comprises
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32 68 171 species, the majority of which are obligate ectobionts found on Dipteran cuticles
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35 69 (Benjamin 1973; Tavares 1985; Haelewaters et al. 2020a, 2021). Among them, ten are
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38 70 known to be associated with drosophilid flies (Index Fungorum 2022; Yamazaki et al.
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41 71 2023). *S. majewskii* was originally described by Dainat et al. (1974), and six drosophilid
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44 72 host species have been reported in Europe, Africa, and Asia (Dainat et al. 1974; Christian
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47 73 2001; Rossi et al. 2010, 2013; Haelewaters et al. 2012). We recently added two host
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50 74 species from Japan, *D. suzukii* and *D. rufa* Kikkawa & Peng, 1938 (Yamazaki et al. 2023).
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53 75 The infection frequency is high in wild *D. suzukii* populations in Japan: 16.5% in males
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3 76 and 3.3% in females (Yamazaki et al. 2023). Thus, we hypothesize that *S. majewskii* may
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6 77 affect native *D. suzukii* populations.
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9 78 The first step in understanding the host-ectobiont relationship is to conduct the
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11 79 laboratory experiment of infection. Laboulbeniales infection has been analyzed in several
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14 80 host-ectobiont systems. Classical observations indicate that transmission between host
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17 81 individuals primarily occurs via direct contact (Richards and Smith 1955; Whisler 1968).
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20 82 However, in the case of infection on carabid ground beetle, indirect transmission via the
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23 83 substrate (soil) has also been suggested (De Kesel 1995). Several insects have been
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26 84 analyzed to study the parasitic effects on the host, such as fitness outcomes of the
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29 85 infections and parasitic manipulations of behaviors (Strandberg and Tucker 1974; Csata
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32 86 et al. 2014; Báthori et al. 2015, 2017; Konrad et al. 2015; Haelewaters et al. 2020b;
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35 87 Szentiványi et al. 2020; Tartally et al. 2021; Awad et al. 2023).
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38 88 An extremely advanced study of Laboulbeniales infection was carried out on the
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41 89 ladybird *Harmonia axyridis* (Pallas, 1773), examining the system of a parasitic
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44 90 *Hesperomyces* species (reviewed by Riddick et al. 2009; Ceryngier and Twardowska
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47 91 2013; Haelewaters et al. 2017, 2022). This Asian ladybird was introduced to the USA and
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50 92 Europe as a biocontrol agent against aphids and scale insects. However, these introduced
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53 93 populations have now expanded worldwide causing several negative impacts, threatening
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56 94 native prey species as well as agricultural production and human health; the introduced
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95 control agents have themselves become invasive pests (Kock and Galvan 2008; Roy et
96 al. 2016; Brown and Roy 2017). Therefore, in search for biological controls, parasites and
97 pathogens of *H. axyridis* were studied. Previous analyses have indicated that a number of
98 behavioral and life history traits of the ladybird promote the spread of the parasite within
99 the population and that infection mostly occurs during sexual contact in the
100 mating/feeding season (Riddick and Schaefer 2005; Riddick 2006; Harwood et al. 2006;
101 Nalepa and Weir 2007; Riddick and Cottrell 2010; Cottrell and Riddick 2012). The
102 parasite was observed to have negative effects on host activity and survival (Nalepa and
103 Weir 2007; Riddick 2010), especially when an entomopathogenic fungus is co-infected
104 in the American native ladybird species tested (Haelewaters et al. 2020b).

105 In the present study, we conducted laboratory experiments on *S. majewskii*
106 infection in live *D. suzukii* flies and measured the fitness of *S. majewskii*-infected
107 individuals. This may provide fundamental knowledge in managing invasive populations
108 of *D. suzukii* in the future.

109
110 **Materials and Methods**

111 **Flies**

112 We used a standard strain of *D. suzukii* (SGD001) which was derived from a female
113 collected at Sugadaira highland, Ueda, Nagano Pref. in 2017. Wild flies were constantly

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114 collected from three localities – i.e., Kyoto (34.94°N, 135.77°E), Sugadaira (36.52°N,
115 138.35°E), and Tsukuba (36.12°N, 140.10°E) (Yamazaki *et al.*, 2023) and *S. majewskii*-
116 infected *D. suzukii* were used for the infection experiments.

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118 **Infection Experiments**

119 In the first experiment, an infected fly (a wild-caught male; donor) and non-infected flies
120 (SGD001; recipient) were confined in a vial (diameter, 3 cm; height, 10 cm) containing
121 the fly medium (cornmeal/glucose/yeast extract/agar); number of recipient flies depends
122 on trials as mentioned below. The flies were aspirated weekly from the vial into a Petri
123 dish (diameter, 3.6 cm; height, 1.0 cm), examined under a binocular stereo microscope
124 (SMZ-U, Nikon, Japan), and transferred to a fresh vial. Dead flies were collected from
125 the vial, preserved in 70% ethanol, and later examined for infected body parts. We
126 conducted the experiments under different conditions (temperature, lightning, and
127 number and sex of recipient flies), which are summarized in Table 1. After the pilot tests,
128 further experiments were conducted at 18 °C under constant light, and the infected flies
129 ($n = 16$) were photographed weekly using a camera (EOS Kiss X10, Canon, Japan)
130 connected to the binocular microscope. For the second experiment, we used a laboratory-
131 infected male donor (SGD001) and an uninfected, virgin female recipient (SGD001).

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132 They were confined in a vial for 4 days and reared separately thereafter. This experiment
133 was replicated five times.

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135 **Longevity Experiments**

136 An infected fly (wild-caught or SGD001; donor) and five non-infected flies (SGD001;
137 recipient) were confined in a vial; the latter were 6–8 days old, with unmated males or
138 females (Table S1). One week later, the donor flies were sacrificed and preserved in 70%
139 ethanol, and the recipient flies were individually reared transferring into a new vial at 1-
140 week intervals. The recipient flies were examined every 1–3 days to check if they were
141 alive, and preserved in 70% ethanol after death. The presence/absence of the *S. majewskii*
142 infection was also checked. The experiment was conducted at 18 °C under constant light.

143

144 **Results**

145 **Infection succeeded in laboratory**

146 In a pilot test, we successfully transmitted *S. majewskii* to three male and two female *D.*
147 *suzukii* flies (Table 1). The infection frequency in males was 4.3% (2/47) at 18 °C under
148 constant light, and 2.1% (1/47) at 25 °C under a light/dark cycle (L:D=14h:10h). The
149 infection frequency in females was 8.3% (2/24) and 0% (0/27) in respective conditions.

150 The positions of *S. majewskii* in the donors and recipients are summarized in Figure 1. As

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151 the frequency was higher in the former condition, we used that condition (at 18 °C under
152 constant light) in the following experiments. We successfully identified three infection
153 patterns here: (1) male → male, (2) male → female, and (3) female → male. Furthermore,
154 by using newly infected *D. suzukii* as the secondary donor, we were able to achieve up to
155 three successive passages of *S. majewskii* transmission on *D. suzukii*: female → male →
156 female. Infection was detected (thalli were just observed irrespective of maturity) as early
157 as 2 weeks after confinement in a female host.

158

159 **Monitoring of *S. majewskii* on *D. suzukii***

160 From our observations of 16 infected-*D. suzukii* flies, we collected sufficient data on
161 visual changes in *S. majewskii* in 12 cases (Table 2). The following events were observed:
162 (1) maturation (before completion of the appendage), (2) elongation of matured thalli, (3)
163 propagation (offspring production), (4) no growth, (5) decrease of immature thalli, and
164 (6) decrease of matured thalli. The developmental staging of *S. majewskii* has been
165 described in Yamazaki *et al.* (2023).

166 When we started observation (day 1), host F (male) exhibited multiple mature
167 thalli on the right foreleg femur. While resting, the tip of the thalli contacted the posterior
168 region of the right compound eye (Figure S1a). He groomed his head with his forelegs,
169 and the neck of the thalli, which is where the ascospores were released, was rubbed around

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170 the right eye. No new *S. majewskii* were detected by day 7 (Figure S1b), but immature
171 thalli were observed on the margin of his right eye on day 13 (Figure S1c), which matured
172 by day 22 (Figure S1d). In host B (male), multiple mature thalli were observed on the
173 dorsal abdomen on day 1 (Figure S2a). The thalli elongated (Figure S2b), and the size
174 almost doubled by day 25 (Figure S2c). However, the number of thalli decreased by day
175 32 (Figure S2d).

176 In contrast, we also observed cases in which the infected thalli stopped growing.
177 Host A (female) exhibited an immature thallus in front of the head on day 1 (Figure S3a,
178 b). The thallus did not develop by day 15 (Figure S3c) and was lost by day 20 (Figure
179 S3d). Host G (male) exhibited an immature thallus on the tarsus of the left foreleg (Figure
180 S4a). The thallus did not develop by day 36 (Figure S4b) and was lost by day 39 (Figure
181 S4c).

182 We observed a case of male-to-female transmission in one of the five pairs. In the
183 donor male, multiple thalli were restricted to the position between the margin of the left
184 compound eye and maxillary pulp (Figure 2a). After 18 days of isolation, we observed an
185 immature thallus on the dorsal abdomen (right side between segments T4 and T5) of the
186 recipient female (Figure 2b). This process is schematically shown in Fig. 2c.

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188 **Effect of *S. majewskii* on *D. sukuzii* longevity**

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189 We measured the longevity of 80 female and 63 male *D. suzukii* flies (Table S1). Among
190 the recipient flies five males (7.9%) and no females were infected with *S. majewskii*. As
191 shown in Figure 3, uninfected females ($n = 80$) survived longer than uninfected males (n
192 = 58); however, the difference was not statistically significant (log-rank test: $p = 0.09$).
193 Similarly, infected males ($n = 5$) survived longer than uninfected males ($n = 58$); however,
194 the difference was not statistically significant (log-rank test: $p = 0.5$). Each experimental
195 batch included five recipient flies, and longevity was compared between them (Figure
196 S5). Three of the five infected males were ranked in the top two in terms of longevity,
197 suggesting that *S. majewskii* infection did not reduce male longevity under the given
198 conditions.

200 **Discussion**

201 To understand biology of Laboulbeniales, infected sites on the hosts collected from the
202 wild have been investigated (e.g., Hedström 1994; Nalepa and Weir 2007; Sundberg et
203 al. 2018; Doorenweerd et al. 2020; Szentiványi et al. 2020), and laboratory experiments
204 of infection have been conducted (e.g., Richards and Smith 1955; Whisler 1968; De Kesel
205 1995; Haelewaters et al. 2020b). In the present study, we examined the transmission of *S.*
206 *majewskii* among *D. suzukii* individuals via laboratory experiments (Table 1; Figure 1).

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207 Based on data obtained from continuous monitoring (Table 2), we hypothesized the
208 infection mechanisms.

209 *Stigmatomyces majewskii* propagated within *D. suzukii* (host F; Figure S1). This
210 is presumably an auto-infection caused by the self-grooming behavior of *D. suzukii*
211 (Yamazaki et al. 2023), and a similar infection has been suggested in other host-ectobiont
212 systems: *Herpomyces* species on cockroaches (Richards and Smith 1955), *Stigmatomyces*
213 species on lesser house fly (Whisler 1968), *Hesperomyces* species on ladybird (Nalepa
214 and Weir 2007), and *Rickia* species on *Myrmica* ants (Haelewaters et al. 2015). Mature
215 *S. majewskii* thalli exhibited elongation, but most of them disappeared later (host B;
216 Figure S2). Grooming might also cause thalli to be removed from the exoskeleton,
217 although this can be an active process of *S. majewskii*. The development of *S. majewskii*,
218 in some cases, ceased (hosts A and G; Figure S3, S4). This might depend on the host
219 condition, paucity of nutrients for the parasite, or difference in resistance against the
220 parasite.

221 In the case of a male-female pair of *D. suzukii*, *S. majewskii* seemed to be
222 transmitted from the front of the male's head to the dorsal abdomen of the female (Figure
223 2). These two parts were in contact owing to the courtship behavior of *D. suzukii* (see
224 Figure 1 of Revadi et al. 2015). This strongly suggests that *S. majewskii* can be transmitted
225 from *D. suzukii* males to females via sexual contact. Transmission during sexual contact

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226 has been suggested in several host-ectobiont systems: *Laboulbenia* species on *Benbidion*
227 ground beetle (Benjamin and Shanor 1952), *Stigmatomyces* species on lesser house fly
228 (Whisler 1968), *Filariomyces* species on predatory earwig (Strandberg and Tucker 1974),
229 *Stigmatomyces* species on guava fruit flies (Hedström 1994), *Laboulbenia* species on
230 *Pogonus* ground beetle (De Kesel 1995), and *Coreomyces* species on aquatic Hemiptera
231 (Sundberg et al. 2018). Another possible mechanism of *S. majewskii* transmission on *D.*
232 *suzukii* is through aggressive behavior, where legs are used for fencing and males perform
233 flying attachments in some social situations (Belenioti and Chaniotakis 2020). As males
234 are more aggressive than females, such behavior may lead to male-to-male transmission.
235 We do not have evidence of such a mechanism; however, it is worth examining this
236 possibility in the future.

237 In the aforementioned male-female pair, we detected immature thalli in the
238 recipient female 18 days after isolation. As it takes at least 9 days for the *S. majewskii*
239 maturation (as seen in the case of host F), the life cycle of *S. majewskii* is hypothesized
240 to be approximately 27 days, which is similar to previous reports (2–3 weeks) for other
241 Laboulbeniales fungi (Richards and Smith 1955; Whisler 1968; Haelewaters et al. 2021).
242 This is much shorter than the longevity of *D. suzukii* (Figure 3; see also Cloutier et al.
243 2021). Although *S. majewskii* can breed on a single host individual, *S. majewskii* must be
244 transmitted to a new host for the continuation of generations. The rate of successful

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245 transmission is still low in the laboratory (maximum 8%), and it must be improved in the
246 future by searching for suitable conditions for transmission. It should also be noted that
247 the infected flies used as donors do not always carry *S. majewskii* at the reproductive
248 stage. The term of donor/recipient confinement (7 days for the longevity experiment)
249 might be too short for *S. majewskii* maturation on the donor.

250 The sample size of the longevity experiment was apparently too small; $n = 5$ for
251 the infected flies. But the present experiment is important because most of the previous
252 reports concerning longevity used wild-collected, already-infected individuals (Riddick
253 2010; Csata et al. 2014; Konrad et al. 2015; Szentiványi et al. 2020; Tartally et al. 2021).
254 This could have led to sampling bias, as researchers may have selectively collected
255 infected survivors. The exceptions were *Stigmatomyces* species on lesser house fly
256 (Whisler 1968) and *Hesperomyces* species on ladybirds (Haelewaters et al. 2020b), where
257 laboratory-infected materials were used. Our preliminary data (Figure 3, S5) do not
258 suggest that *S. majewskii* infection reduced the longevity of *D. sukuzii*. Although *S.*
259 *majewskii*-infected *D. sukuzii* produced offspring both in males and females (data not
260 shown), it is essential to measure other fitness traits, such as reproductive activity and the
261 degree of fertility. This is important because some behaviors are suppressed in heavily
262 infected individuals (Strandberg and Tucker 1974; Nalepa and Weir 2007; Riddick 2010).
263 It is also possible that *S. majewskii* infection has an effect on the immune system of *D.*

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264 *suzukii*, which might enhance or suppress resistance to other parasites and pathogens.
265 Changes in mortality in doubly infected hosts have been observed in some host–parasite
266 systems (Konrad et al. 2015; Haelewaters et al. 2020b; Tartally et al. 2021). Introducing
267 a second parasite or pathogen into *S. majewskii*-*D. suzukii* system is needed in future
268 studies.

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512 Japan. Fly submitted

514 **Figure legends**

515 **Fig. 1**

516 Sites of *Stigmatomyces majewskii* infection on donor and recipient of *Drosophila suzukii*
517 (a) at 25 °C under light/dark cycle (L:D=14h:10h). (b)–(e) at 18 °C under constant light.
518 (a)–(c) male → male; (d) and (e) male → female. Left, donor. Right, recipient. Arrow in
519 (c) indicates scattered, black dots (truncated *S. majewskii*); see also Figure S7b’ of
520 Yamazaki *et al.* (2023). The infection on the thorax of the recipient in (c) is ventral.

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522 **Fig. 2**

523 A case of *S. majewskii* transmission in a pair of *D. sukuzii* (male → female). (a) the male
524 on day 1. (b) the female at day 19; arrow indicates the position of *S. majewskii*. (c)
525 schematic drawing.

526

527 **Fig. 3**

528 Survival curves of *D. sukuzii*. Red, uninfected females ($n = 80$). Blue, uninfected males
529 ($n = 58$). Black, males infected with *S. majewskii* ($n = 5$).

530

531 **Fig. S1**

532 *S. majewskii* infection on host F (male) of *D. sukuzii*. (a) day 1. (b) day 7. (c) day 13. (d)
533 day 22.

534

535 **Fig. S2**

536 *S. majewskii* infection on host B (male) of *D. sukuzii*. (a) day 1. (b) day 8. (c) day 25. (d)
537 day 32.

538

539 **Fig. S3**

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540 *S. majewskii* infection on host A (female) of *D. suzukii*. (a), (b) day 1. (c) day 15. (d) day
541 20. (b) frontal view; (a), (c), (d) top view. Arrow indicates the position of *S. majewskii*.

542

543 **Fig. S4**

544 *S. majewskii* infection on host G (male) of *D. suzukii*. (a) day 1. (b) day 36. (c) day 39.

545

546 **Fig. S5**

547 Longevity of five *D. suzukii* males from the same batch. Blue, uninfected. Orange,
548 infected with *S. majewskii*. Data from three batches are shown. 5-1, 5-2 etc. are individual
549 flies.

550

551 **Table 1**

552 Tests of *S. majewskii* infection on *D. suzukii*

553

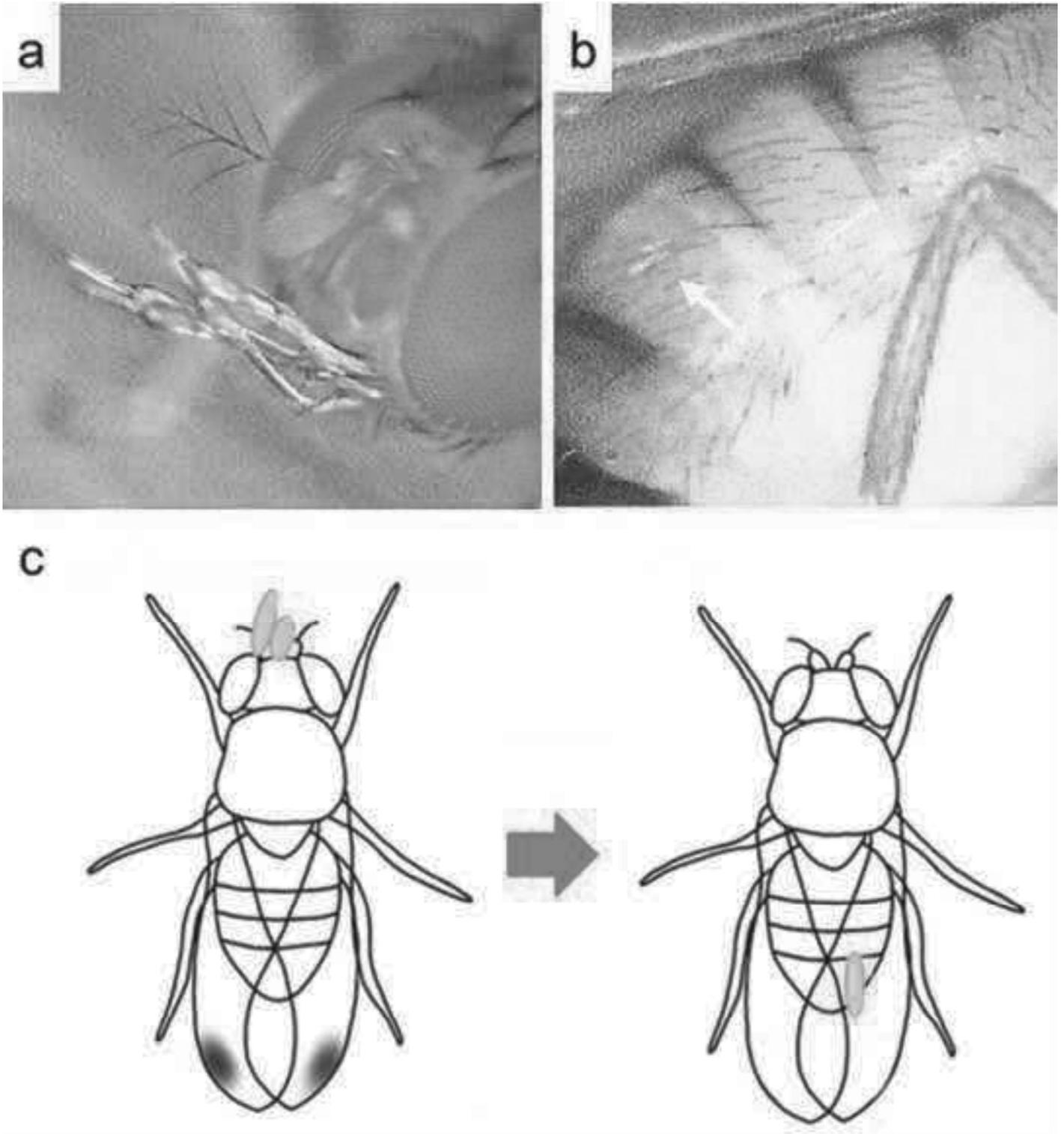
554 **Table 2**

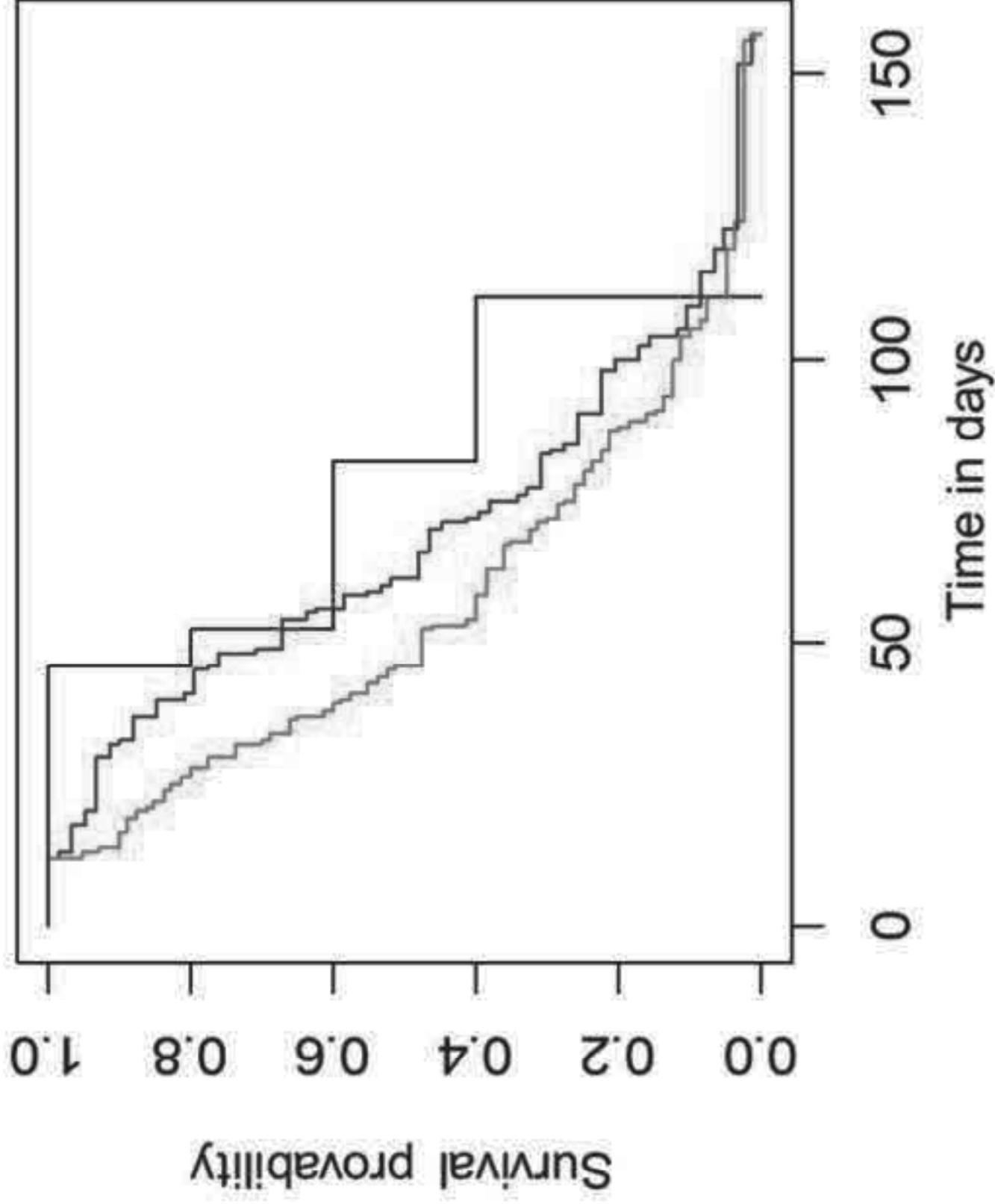
555 Events observed in *S. majewskii* on individual *D. suzukii*

556

557 **Table S1**

558 Combinations of donors and recipients in the longevity experiment





Table

Table 1 Tests of *S. majewskii* infection on *D. sukuzii*

Temperature	Light (L : D)	No. recipient flies per test	No. replicate	No. males tested	No. males infected	No. females tested
25°C	14h : 10h	3-5 males	10	39	1	-
		3-5 females	5	-	-	20
		2 males + 2 females	4	8	0	7
18°C	24h : 0h	3-5 males	10	39	2	-
		3-5 females	4	-	-	16
		2 males + 2 females	4	8	0	8

No. females
infected

-

0

0

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Table 2 Events observed in *S. majewskii* on individual *D. sukuzii*

Host	Sex	Maturation	Elongation	Propagation	No growth	Decese of immatured	Decrease of matured
A	Female				✓	✓	
B	Male		✓				✓
D	Male	✓		✓			
E	Male			✓		✓	
F	Male	✓		✓			
G	Male				✓	✓	
I	Female				✓	✓	
J	Female				✓	✓	
K	Female			✓			
L	Male		✓	✓			
M	Male		✓	✓			✓
O	Male				✓		