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Effect of different feeding sources on lifespan and fecundity in the biocontrol agent *Torymus sinensis*

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1 Effect of different feeding sources on lifespan and fecundity in the biocontrol agent *Torymus*
2 *sinensis*

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11

12 Compliance with ethical standards

13 All the insect rearing and experiments were conducted in accordance with the legislation and
14 guidelines of the European Union for the protection of animals used for scientific purposes
15 (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). All experimental
16 protocols using insects were approved by the *ad-hoc* Committee of DISAFA of the University of
17 Torino.

18

19 Conflict of interest

20 The authors declare that they have no conflict of interest.

21

22 Author contribution statement

23 CF, LP, and AA conceived and designed research. CF and LP carried out field and laboratory assays.
24 All authors contributed to the writing of the manuscript and approved the final manuscript.

25

26 **Abstract**

27 *Torymus sinensis* Kamijo (Hymenoptera, Torymidae) is a biocontrol agent released to control
28 outbreaks of the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera,
29 Cynipidae). This wasp is mass reared in controlled conditions in order to be released on a large scale
30 in chestnut orchards and coppices, thus factors such as food quality may deeply influence the
31 effectiveness of parasitoids. To identify different diets that could be used to increase mass rearing,
32 performance, longevity and fecundity of *T. sinensis* were assessed using honey, honey plus pollen,
33 pollen, and water as diet, compared to unfed wasps in laboratory experiments.

34 In the present study diet quality greatly influenced survival and reproductive output in *T. sinensis*.
35 Adults using honey plus pollen as food had the longest life span, and significantly increased fecundity
36 compared to pollen alone, water, and unfed wasps. Median lifespan (day at which 50% of the initial
37 number of wasps are still alive) was 31, 31, 2.5, 4, 1.5 days, in the honey, honey plus pollen, pollen,
38 water, control, respectively.

39 The egg production in wasps fed with honey (diet 1) and honey plus pollen (diet 2) observed over
40 lifetime showed the newly emerged specimens with an average of 2.40 ± 0.21 and 0.14 ± 0.12
41 respectively at day 0. The number of mature eggs increased rapidly reaching in diet 1 the highest
42 average number, 26.60 ± 2.48 , at day 6, and 30.2 ± 1.59 at day 7 in diet 2. No significant difference
43 in the mean number of mature eggs between diets 1 and 2 was observed during all 13 weeks except
44 for week 11. The mean number of reabsorbed eggs was 0 both in diet 1 and 2 at day 0. The mean
45 number increased till 2.80 ± 0.66 at day 10 and 1.40 ± 0.24 at day 14 in diet 1 and 2 respectively.

46 The cumulative lifetime mean egg load was 1012.67 and 1095.82 when feeding honey and honey
47 plus pollen, respectively, which was significantly higher than 32.40 for pollen, 58.40 for water and
48 43.57 for control.

49 Based on our studies, feeding the female wasps with honey and pollen increased lifespan by 33-fold,
50 and fecundity by 2.3-fold at day 4 over unfed wasps.

51 This work contributes to a better understanding of the influence of different diets on lifespan and
52 fecundity in the parasitoid wasp *T. sinensis*, to the refinement of mass rearing in controlled
53 conditions, and to the optimization of classical biocontrol programs. Prior to the field release, it
54 seems advisable to supply insects with sugar-rich diets to improve their performances in fields and
55 to enhance parasitoid egg expenditure.

56 Key words: parasitoid feeding; honey-based diets; oosorption; mass rearing; Asian chestnut gall
57 wasp.

58 **Introduction**

59 Life history traits of insects in nature, namely survival, development, and fecundity, are deeply
60 influenced by food quality. Food sources of adult parasitoids include host food, host hemolymph and
61 tissues. However, host-feeding female parasitoids can also use non-host foods to prolong their
62 lifespan and increase their reproductive potential, as they usually contain carbohydrates, proteins, and
63 other trace elements (Liu et al., 2015).

64 Proteins are often regarded as important nutritional elements for insect reproduction, enhancing
65 offspring production (Bong et al., 2014). Conversely, the energy obtained from a carbohydrate-rich
66 diet is mainly focused on insect life maintenance, thus prolonging life span. Potential sugar sources
67 for parasitoids include nectar and homopteran honeydew. These sugar-meals carried over from
68 juvenile stage can be used immediately to generate energy for metabolic purposes and even stored
69 (Olson et al., 2000). Furthermore, pollens contain a number of nutrients and often high levels of free
70 amino acids, instead of most nectars, there included all 10 essential amino acids. Compared to nectars,
71 pollens have higher level of protein, lipid and polysaccharides (Thompson et al., 1999; Zhang et al.,
72 2004).

73 Insects, like many animals, have the ability to respond plastically to environmental stress, resorbing
74 oocytes that are not oviposited (Moore and Attisano, 2011). Sugar-feeding may help synovigenic
75 species to mature additional eggs, and can prevent parasitoids from resorbing eggs (Lee et al., 2004).
76 Honey has long been used as an alternate food source to sucrose solution for insects such as
77 parasitoids mass reared in controlled conditions, but little is known about its actual properties in
78 influencing reproduction and lifespan (Harvey et al., 2012). Honey is presumably the most studied
79 artificial diet for parasitoids since it is palatable and cheap. Provision of honey solutions boosts
80 mymarid and trichogrammatid parasitoid lifespan similarly to floral and extrafloral nectars, and even
81 more than honeydew (Benelli et al., 2017). A lack of sugar availability may temporarily or
82 permanently limit the reproductive success of parasitoids in agricultural system (Heimpel et al.,
83 1997).

84 Pollen is a food for numerous insects, including many species of natural enemies, and parasitic
85 Hymenoptera feed on pollen to varying degrees (Lundgren and Wiedenmann, 2004). It is one of the
86 most nutritious non-prey food sources for parasitoids based on its protein levels, but pollen-feeding
87 by parasitoids has been studied much less frequently than sugar-feeding (Lundgren, 2009).

88 *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) is the biocontrol agent of the Asian chestnut
89 gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) (Moriya et al., 2003), and
90 is considered as one of the most successful examples of recent European classical biocontrol
91 programs (Avtzis et al., 2018; Ferracini et al., 2018a; Matošević et al., 2017). Although research on
92 the reproductive biology and fecundity of this synovigenic parasitoid was already carried out (Picciau
93 et al., 2017), the impact of diet on its life history has never been studied. In the present work, we
94 investigated if lifespan and fecundity of *T. sinensis* are affected by different sugar diets. Since factors
95 such as food quality are also likely to determine the effectiveness of parasitoids, wasps were allowed
96 to feed on honey, honey plus pollen, pollen, and water. Depending on the different diet, lifespan, egg
97 load and egg resorption of *T. sinensis* individuals were compared with unfed wasps in laboratory
98 experiments. The results were expected to deepen knowledge about the factors affecting the
99 biocontrol efficacy of this species in controlled conditions, thus improving mass rearing by insectaries
100 and private companies.

101 **Material and Methods**

102 *Insect culture*

103 Galls were randomly collected during winter 2016-2017 according to the method described by
104 Ferracini et al. (2015; 2018b) in chestnut-growing areas of NW Italy with a stable population of *T.*
105 *sinensis*. Galls were individually isolated in plastic vials (120mm in length by 25mm in diameter),
106 and kept outdoor until *T. sinensis* emergence.

107 *Food sources*

108 Insects were provided *ad libitum* one of the following different diets: 1) undiluted honey, 2) undiluted
109 honey plus organic grinded pollen grains, 3) organic grinded pollen grains, 4) distilled water. The

110 diets have been compared to a control, with no food source provided. For the first diet we used a total
111 of 0.02 g of honeydew honey, disposing few droplets on a cardboard, using a pipette. For the second
112 diet, a stock mixture was prepared adding 1 g of organic pollen to 30 g of honeydew honey; few
113 droplets were placed on a cardboard, for a total of 0.02 g. For the third diet, 0.02 g of organic pollen
114 grain was weighted, grinded with a pestle and directly inserted in the plastic tube. For the fourth diet,
115 the cotton plug of the tube was imbedded with 3 ml of distilled water. In the control, no source of
116 food and water was provided. Each sugar source was replaced once a week, while the moistened
117 cotton plug was changed twice a week.

118 *Parasitoid lifespan*

119 Three newly emerged wasps for each sex, for a total of six individuals, were placed into plastic vials
120 (120 mm in length by 25 mm in diameter), and provided with one of four different sugar diets
121 throughout their adult life. Twenty replications were performed for each food source. The vials were
122 maintained in climatic chamber under controlled conditions [15 ± 1 °C, 60 ± 5 % RH, and a
123 photoperiod of 16:8 (L:D) h], and checked twice a day for dead wasps every 10 a.m. and 16 p.m. each
124 day until all individuals had died.

125 *Parasitoid fecundity*

126 Emerging females only, used for egg load survey, were collected every day in number of 1,800
127 specimens originating 30 age-cohorts for each feed-test for a total of 180 cohorts.

128 The insects were reared in plastic vials (120 mm in length by 25 mm in diameter) arranged in 5 groups
129 each fed with different food combination: 1) honey, 2) honey plus pollen, 3) pollen, 4) water and 5)
130 no food source (control). All of them were kept in a climatic chamber under controlled conditions (15
131 ± 1 °C, 60 ± 5 % RH, and a photoperiod of 16:8 L:D) according to Picciau et al. (2017) and maintained
132 till dissection.

133 *Egg load and oosorption quantifying*

134 Five females for each age cohort were collected daily and killed with ethyl acetate. The specimens
135 were placed on a microscope slide ($25 \times 75 \times 1$ mm) into a phosphate buffered saline (PBS) solution

136 for ovary dissection. Egg load was assessed by dissecting each female under a stereomicroscope at
137 $25 \times$ magnification. The abdomen was separated from the rest of the body using a couple of needles.
138 Ovaries were kindly removed from the abdomen by means of micro-pin needles and spread on a slide
139 for egg load counts. Mature eggs were defined as described in Picciau et al. (2017). Egg resorption
140 (oosorption) was also investigated by counting those eggs close to the lateral oviducts which
141 displayed a collapsed pattern indicating a reabsorption process (Figure 1). The number of mature and
142 reabsorbed eggs was counted under microscope at $80 \times$ magnification.

143 *Statistical analysis*

144 The data on the female longevity of the different diets were $\log_{10}(x+1)$ transformed and transformed
145 using analysis of variance (ANOVA). We used the Kaplan-Meier estimator (Kaplan and Meier, 1958)
146 to visualize survivorship curves in each diet, and a log-rank test was used to determine differences in
147 survival rates. The significance of each term in the model was analyzed using Wald χ^2 statistic.

148 After testing for homogeneity of variance (Levene test), data were analyzed by T test independent
149 samples ($P < 0.05$) to compare the egg load and the oosorption records comparing wasps fed with
150 honey and with honey and pollen through 13 weeks. Furthermore, all data were analyzed within the
151 first 8 days by a Generalized Linear Model (GLM) with Poisson distribution and Log link function.
152 Averages among the diets were separated by a Bonferroni *post hoc* when significant difference were
153 present. All analyses were performed using the software SPSS version 20.0 (SPSS, Chicago, IL,
154 USA). The cumulative mean egg load was analyzed with a Kruskal Wallis test, and averages were
155 separated by a Bonferroni *post hoc* when significant difference were present.

156 **Results**

157 The results concerning the effects of different food sources on lifespan of the parasitoid *T. sinensis*
158 are shown in Figure 2. Diet significantly affected lifespan, in particular wasps fed with honey (diet
159 1) and honey plus pollen (diet 2), increased lifespan to the greatest extent. This lifespan, in fact, was
160 more than 20 times as long as that of individuals starved (control) (Figure 2). Furthermore, lifespan
161 of the wasps was significantly reduced when they were fed with pollen (diet 3), and water (diet 4) as

162 well. Even if the average lifespan was higher when adults were provided with honey plus pollen, no
163 significant difference was found between this source and honey alone.

164 Median lifespan (day at which 50% of the initial number of wasps are still alive) was 31, 31, 2.5, 4,
165 1.5 days, in the honey, honey plus pollen, pollen, water, and control, respectively. Furthermore,
166 according to the diet all wasps were dead after 88, 108.5, 6, 7, 4 days in honey, honey plus pollen,
167 pollen, water and control, respectively. The median survival time differed between female and male
168 parasitoids, with females living longer (log rank test, $\chi^2 = 5.42$, $df = 1$, $P = 0.0199$) (Figure 3).

169 The comparison of egg load and oosorption was performed through 13 weeks, considering only honey
170 (diet 1) and honey plus pollen (diet 2), basing this choice on the diets with the longest lifespan. The
171 egg production observed over lifetime showed that the newly emerged specimens had an average of
172 2.40 ± 0.21 mature eggs in specimens fed with diet 1 and 0.14 ± 0.12 mature eggs in specimens fed
173 with diet 2 at day 0. In the following days the number of mature eggs increased rapidly reaching in
174 diet 1 the highest average number, 26.60 ± 2.48 , at day 6 with a maximum value per individual of 34
175 eggs. For this diet the highest number per individual was 40 and reached at day 17 when the average
176 was 20.8 ± 5.21 . The highest average number reached in diet 2 was 30.2 ± 1.59 at day 7 with a
177 maximum value per individual of 34 eggs. For this diet the highest number per individual was 43 and
178 reached at day 9 when the average was 26.5 ± 3.12 . No significant difference in the mean number of
179 mature eggs between diets 1 and 2 was observed during all 13 weeks except for week 11 ($t = -0.452$,
180 $df = 7.842$, $P = 0.663$) (Figure 4).

181 Concerning the oosorption, the number of reabsorbed eggs observed over lifetime showed that the
182 newly emerged specimens had an average of 0 collapsed eggs both in diet 1 and 2. Subsequently the
183 number increased, reaching in diet 1 the highest average number, 2.80 ± 0.66 , at day 10 with a
184 maximum value per individual of 5. For this diet the highest number per individual was 6 and reached
185 only at day 14 when the average was 2.60 ± 0.87 . The highest average number reached in diet 2 was
186 1.40 ± 0.24 at day 14 with a maximum value per individual of 2 reabsorbed eggs. For this diet the
187 highest number per individual was 2 and reached for the first time at day 11 when the average was

188 0.40 ± 0.40. Significant differences were found only in weeks 1, 3, 12 and 13 (week 1: $t = -1.364$, $df = 8.053$, $P = 0.209$; week 3: $t = -4.418$, $df = 6.075$, $P = 0.004$; week 12: $t = -0.926$, $df = 1.010$, $P =$
189 0.523 ; week 13: $t = -3.000$, $df = 3.000$, $P = 0.058$) (Figure 5).

191 The comparison among all the diets was performed only through the first 8 days when only the
192 specimens provided with diet 1 and 2 remained alive (Figure 6). The unfed wasp, with the shortest
193 lifespan, showed a peak at the third day with an egg load mean of 17.70. The specimens fed with
194 pollen had the higher egg load during the last three days before dying reaching an average egg load
195 of 8.53. In case of water, the egg load followed a normal distribution with the highest mean value of
196 18.20 eggs at day 2. The mean egg load for wasps provided with honey and honey plus pollen showed
197 a positive trend with lower values until day 3 with a subsequent increase in the following days
198 overcoming the other feeding sources (Figure 6). The cumulative lifetime mean egg load was 1012.67
199 and 1095.82 when feeding honey and honey plus pollen, respectively, which was significantly higher
200 than 32.40 for pollen, 58.40 for water and 43.57 for control.

201 **Discussion**

202 Egg maturation in parasitoids depends on the diet provided to adult females (Onagbola et al., 2007).
203 It is considered a fitness-related parameter, which may influence the reproductive potential of
204 biocontrol agents and thus the population dynamics of host. However, different diets may have an
205 effect on the egg maturation patterns through lifetime of synovigenic parasitoids species (Jervis et al.,
206 2001). In fact, lipids and proteins, from either the diet and teneral reserves, are important for
207 production of the eggs (Ellers and Van Alpen, 1997), and the balanced acquisition of nutrients has
208 great impact on the size and number of eggs produced (Harvey et al., 2012; Wheeler 1996). However,
209 egg production is also improved by various artificial diets based on carbohydrate solutions, and
210 exploitation of sugar sources or pollen is widespread in a broad range of orders, including
211 Hymenoptera (Wackers et al., 2008). Food quality greatly influences life history traits such as
212 survival, development, and fecundity of insects. Its availability over time can affect fitness-related
213 traits and may alter egg maturation (Benelli et al., 2017) in synovigenic species, there included *T.*

214 *sinensis* (Picciau et al., 2017). Carbohydrate-rich diet access, even as intermittent feeding and without
215 additional proteins, has a strong effect on life span being able to increase longevity of about 5 times
216 compared to water alone as demonstrated by Ellers et al. (2011). Among the different sugar source,
217 honey is considered the most effective food to promote egg maturation (Hogervorst et al., 2007), and
218 improve fecundity by prolonging parasitoid lifespan (Mutitu et al., 2013). Moreover, pollen can also
219 be exploited as food source to improve fecundity when used in addition to honey (Zhang et al., 2004).
220 In the present study diet quality equally influenced the survival and the reproductive output in *T.*
221 *sinensis*. Adults fed with honey plus pollen had the longest life span, and this diet significantly
222 increased fecundity compared to control. In particular, *T. sinensis* responded to a honey supplement,
223 increasing the mean survival up to 49 days, considering female wasps provided with honey plus
224 pollen, confirming previous data by Picciau et al. (2017). Conversely, when only pollen or water were
225 provided similar effects to control were recorded. In fact, wasps reached a mean survival of 2.5, 4,
226 and 1.5 days, respectively. Significant reduction in longevity, with no significant difference between
227 sexes, was recorded highlighting how parasitoids wasps are especially sensitive to sugar deprivation
228 as adults. As already pointed out by Olson et al. (2000), laboratory lifespan of many parasitoids
229 species is typically less than 5 days in the absence of sugar but exceeds 2 to 3 weeks when sugar
230 meals are provided, confirming our results.

231 Honey has long been used in controlled conditions as an alternate food source to sucrose solution for
232 insects such as parasitoids, but its actual properties in influencing parasitoid longevity and
233 reproduction have been little explored. The content of honey is dominated by sugars, although it also
234 contains trace amounts of vitamins or minerals, with tiny amounts of several compounds thought to
235 function as antioxidants (Harvey et al., 2012). Based on our studies, feeding the female wasps with
236 honey plus pollen increased lifespan by 33-fold, and fecundity by 2.3-fold at day 4 over unfed wasps.
237 Moreover, pollen of plants is known to be a source of food for a large number of insect species in
238 nature, but pollen feeding by parasitoids has been studied less frequently than sugar feeding. Some
239 groups of insects have been reported feeding on pollen (e.g., Bombyliidae, Eulophidae, Mutillidae,

240 Scoliidæ, Trichogrammatidæ) (Jervis et al., 2008; Patt et al., 1997; Zhang et al., 2004). In our
241 experiment, pollen alone did not increase lifespan but a higher longevity was recorded when it was
242 provided in addition to honey.

243 In case of scarcity of food, insects, like many animals, have the ability to adapt to environmental
244 stress, resorbing oocytes that are not oviposited (Moore and Attisano, 2011). In our experiments the
245 presence of egg deterioration related to oosorption was verified in *T. sinensis*, as previously
246 hypothesized in Picciau et al. (2017). The comparison of egg load and egg maturation trend recorded
247 in the specimens fed with honey and honey plus pollen through their lifetime suggest that the addition
248 of pollen in the diet does not heavily affect the number of egg production. On the contrary it seems
249 to have a strong impact on egg resorption probably due to the increased nutritional value of the food
250 provided. Compared to males, females were in fact able to reallocate resources to somatic
251 maintenance as evidenced by no reduction in life span, especially in their first month of life.
252 Comparing the mean cumulative egg load recorded with all different diets to the unfed wasps, a
253 significant increase in fecundity was detected. In particular feeding with honey increased the total
254 egg load by 23.2-fold, and with honey plus pollen by 25.1-fold, whilst with only water only 1.4-fold
255 and pollen alone showed no significant difference. As already stated by Zhang et al. (2004) for
256 *Trichogramma* spp., honey and pollen should be considered a complete diet for *T. sinensis* as well
257 conversely to pollen and water.

258 This knowledge about feeding sources influence on parasitoid life-history traits is essential to improve
259 mass-rearing techniques. In particular, *T. sinensis*, being a biocontrol agents employed in classical
260 biocontrol programs in Europe, may be positively affected by diet in laboratory conditions. A
261 balanced acquisition of nutrients has indeed great influence on the size and number of eggs produced
262 (Harvey et al., 2012; Wheeler 1996).

263 Modern agriculture has simplified the agro-ecosystem landscape, increasing the pest population
264 density and reducing the alternative non-crop sources for natural enemies (Benelli et al., 2017). In a
265 frame of a biocontrol programs based on conservative and augmentative actions, nectar and

266 honeydew accessibility in the field is very important to improve parasitoid effectiveness, as already
267 pointed out in case of scarce flower resources (Pinheiro et al., 2015; Wäckers et al., 2008). Moreover,
268 providing parasitoids with sugar-rich diets before their release to the field is recommendable to
269 improve their performance and optimize the egg laying (Hougardy and Mills, 2007).
270 The current findings have offered some important insights into the life history traits of *T. sinensis*,
271 providing new cues to enhance methods of mass rearing for biocontrol. To overcome occurring field
272 limitations, the employment of artificial sugar sources, flowering cover crops and the management of
273 natural food resources may be helpful to improve natural populations and to enhance the fitness of
274 parasitic wasps in both conservative and augmentative biocontrol programs.

275

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282

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