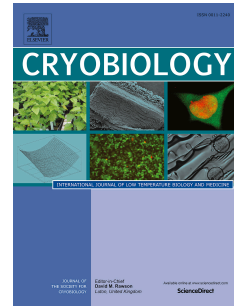# Journal Pre-proof

Cold tolerance and supercooling points of two ladybird beetles (Col.: Coccinellidae): Impact of the diet

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1   **Cold tolerance and supercooling points of two ladybird beetles (Col.: Coccinellidae):**

2   **impact of the diet**

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ABSTRACT

Ladybird beetles have successfully been used to control different pests. *Cheilomenes sexmaculata* (Fabricius) and *Oenopia conglobata contaminata* (Menetries) (Coleoptera: Coccinellidae) are two dominant and efficient predators of the common pistachio psylla, *Agonoscena pistaciae* (Hem.: Psyllidae) in Iran. In the current study, the impact of two diets, i.e., nymphs of *A. pistaciae* and eggs of *Ephestia kuehniella* Zeller (Lep.: Pyralidae), were investigated on the cold hardiness, supercooling point (SCP), and lethal temperature of different life stages of the coccinellids. The results suggested that the eggs of *E. kuehniella* are a suitable diet for both predators. In general, beetles of *O. conglobata contaminata* were more cold tolerant than those of *C. sexmaculata*. The SCP of the adults of *C. sexmaculata,* feeding on psyllid was about -15 ℃, whereas the SCP of the beetles, feeding on the eggs of flour moth, was about -19 ℃. However, the diets had no significant effects on the SCP of *O. conglobata contaminata*. For both ladybird beetles, eggs were the most sensitive stage, and adulthood was the most tolerant developmental stage. No eggs survived at zero and subzero temperatures. The survival of *C. sexmaculata* increased from 6.25% for adults fed on psyllids to 13.75% for those fed on flour moth eggs after 24 h exposure to cold at -4 ℃. The survival of *O. conglobata contaminata* adults after exposure to cold at -4 ℃ for 24 h raised from 28.75% for adults fed on psyllids to 42.50% for those fed on flour moth eggs. Regardless of the diet, both beetles were considered to be chill-intolerant insects as the most mortality occurred above the SCP.

Keywords: Coccinellids, Supercooling point, Cold hardiness, Lethal temperature, Factitious food.

1. Introduction

37

38   The ladybird beetle, *Cheilomenes sexmaculata* (Fabricius) (Coleoptera: Coccinellidae), is one

39 of the most important aphidophagous predators of the common pistachio psylla, *Agonoscena*

40 *pistaciae* (Hem.: Psyllidae) in the pistachio-growing regions of Iran. This coccinellid has also

41 been reported to be an efficient natural enemy of aphids coming from other Asian countries

42 [11,35]. The coccinellid beetle, *Oenopia conglobata contaminata* Menetries (Col.:

43 Coccinellidae) is another dominant and efficient predator of *A. pistaciae* in Iran [18]. Different

44 developmental stages of both of these predators, feed on different insect species, especially

45 aphids, scales, and psyllids [8]. Therefore, coccinellids have been considered as efficient

46 biological control agents in integrated pest management (IPM) programs in several

47 agroecosystems, including fields, gardens, and greenhouses [9]. *C. sexmaculata* is a potential

48 biocontrol agent of aphids and psyllids in forests and field crops in various parts of the world,

49 especially in East Asia. *O. conglobata contaminata* is another polyphagous coccinellid that has

50 been recorded to mainly prey on psyllids and aphids. Both predatory beetles are associated with

51 the common pistachio psylla, *Agonoscena pistaciae*, the major pistachio pest in Iran. Besides, as

52 *C. sexmaculata* and *O. conglobata contaminata* reared for consecutive generations in our

53 laboratory, therefore, eggs of *E. kuehniella* could support the reproduction and development of

54 these natural enemies. Subsequently, eggs of *E. kuehniella* were considered as a suitable diet for

55 mass-rearing of the coccinellid predators.

56   Winter may be cold enough for pistachio trees to complete their dormancy. Different cultivars

57 of pistachio trees (*Pistacia vera* L.) need about 750 to 1400 accumulated hours of temperatures

58 below or at 7°C to break their dormancy. In addition, the regular development of this plant is

59 substantially enhanced in cold months of winter [25]. Moreover, in temperate and cold regions,

60　survival against harsh winter conditions is crucial for the reproduction, abundance and

61　distribution of insects. Cold hardiness or cold tolerance is an insect's ability to prevent cold

62　injuries during unfavorable winter conditions. Cold hardiness in insects can be achieved by

63　maintaining a supercooled condition colligatively, i.e., synthesis and accumulation of

64　cryoprotectants [1,7,13,29,32], or non-colligatively, i.e., enhancement of the supercooling point

65　(SCP) without any further changes in cryoprotectant concentrations. In the latter group, the

66　changes in SCP values do not follow a particular seasonal trend, and the limit of cold tolerance is

67　regulated by the SCP [20,23]. When the ambient temperature is lower than the freezing point of

68　insects, supercooling is said to occur, where the body fluids still remain unfrozen in a liquid

69　state. This phenomenon is usually adopted to evaluate cold tolerance strategies [15,30].

70　Measuring SCP is a prerequisite for determining the given strategies [20,27]. Based on these

71　strategies, Sinclair et al. [27] divided insects into three major categories: 1- freeze-intolerant

72　(insects whose mortality mostly occurs at SCP); 2- freeze-tolerant (insects whose mortality

73　mostly occurs below SCP), and 3- chill-intolerant (insects whose mortality mostly occurs above

74　SCP).

75　　The lethal temperature has been defined as the point at which x% of individuals die after a

76　specific period of exposure. Therefore, LT_{50} is a temperature where 50% of a population dies

77　after a specific exposure time. The lower lethal temperature (LLT) is the point where the

78　identified percentage of individuals of a population is killed after a certain exposure time [27].

79　However, this temperature normally correlates with acclimation. Thus, both upper and lower

80　lethal temperatures may decrease under low-temperature acclimation [33].

81　　In natural conditions, predators and parasitoids solely rely on their prey as a source of energy

82　for reproduction and development, but the availability of suitable non-prey food can impact their

83   efficiency as biological control agents of pests. Therefore, in mass rearing of these biological

84   control agents, the availability of suitable non-prey food, as a part of their diet, must be

85   considered [34]. Beside, several biotic or abiotic factors can hamper or limit the action of these

86   powerful control agents. Some understanding of these factors is fundamental to design biological

87   control strategies. In this regard, in the current study, cold hardiness and SCPs of two ladybird

88   beetle species, i.e. *C. sexmaculata* and *O. conglobata contaminata*, were compared at different

89   developmental stages using two diets: a natural diet (nymphs and adults of *A. pistaciae*) and a

90   factitious diet (eggs of *E. kuehniella*). Results of this study can help to predict the development

91   of the coccinellids in the field and offer valuable basic information for the use of these native

92   predators in mass release biological control programs.

93   **2. Materials and methods**

94   *2.1. Laboratory culture and rearing conditions*

95   The laboratory colonies of *C. sexmaculata* and *O. conglobata contaminata* were established

96   in 2015 with individuals previously collected from pistachio gardens near Rafsanjan, Iran. The

97   stock colonies were maintained on the common psylla, *A. pistaciae*, and eggs of the

98   Mediterranean flour moth, *E. kuehniella*. The insects were reared in a growth chamber at 26 ± 1

99   °C, in 65 ± 5 % RH, for a photoperiod of 16:8 (L:D) h. The predators used in the experiments

100   were in the third generation of laboratory rearing at our facilities. One-day old of each

101   developmental stage, without starvation prior to measurement of the SCPs and cold hardiness,

102   were used.

103   *2.2. Measurement of the supercooling point (SCP)*

104   The SCPs of both predators at different developmental stages were measured using a

105   thermocouple (testo, model 177-T4, German). The insect (n= 6) was fixed to the end of the

106 sensor (Nickel-Worm) of the thermocouple by a strip of adhesive tape and moved to a

107 programmable chamber device (GT-7005, Geotech, Taiwan). The temperature dropped from 25

108 °C to the desired temperature with a rate of 0.5 °C/min. The data were read using Comsoft 3

109 Software. Any decrease in the temperature was recorded. The temperature at which the latent

110 heat of freezing released was shown as an abrupt jump in the diagram and was considered to be

111 the SCP.

112 *2.3. Cold tolerance assay and assessment of lethal temperatures (LT$_{30,50,99}$)*

113   Different developmental stages of both predators were transferred (n=20) to a programmable

114 chamber. The temperature was lowered from 20 °C to the desired temperature with a rate of 0.5

115 °C/min. The eggs, larvae, pupae, and adults of the beetles were held at each temperature for 24 h,

116 and were then heated slowly (0.5 °C/min) to 25 °C. The survival of the eggs, larvae, pupae, and

117 adults of both predators were counted after 24 h. The lethal temperatures of different

118 developmental stages of *C. sexmaculata* and *O. conglobata contaminata*, including immature

119 and adult stages, were assessed after 24 h exposure to subzero temperatures. This was performed

120 in the same way as was done for the cold tolerance assessment.

121 *2.4. Statistical analysis*

122   The effects of the different diets on the SCPs of *C. sexmaculata* and *O. conglobata*

123 *contaminata* were evaluated in a factorial experiment based on the completely randomized

124 design with six replications. The first factor consisted of two kinds of diets (eggs of *E. kuehniella*

125 and feeding stages of *A. pistaciae*). The second factor included the predatory ladybirds (*C.*

126 *sexmaculata* and *O. conglobata contaminata*) and the third factor was the developmental stage of

127 the predators. Moreover, the effects of the diets on cold tolerance of the predators were evaluated

128 in factorial experiments based on the completely randomized design with four replications for

129   each predator, separately. In the experiment that was designed for *C. sexmaculatus* the first,

130   second and third factors were two kinds of diets (*E. kuehniella* and *A. pistaciae*), developmental

131   stages (egg, larva, pupa, and adult) and different temperatures (0. -1, -2, -3 and -4°C),

132   respectively. In another experiment that was designed for *O. conglobata contaminata* the first,

133   second and third factors were two kinds of diets, developmental stages and different

134   temperatures (0. -3, -5, and -7°C), respectively.

135   **3. Results**

136   *3.1. Effects of the diets on the SCPs of C. sexmaculata and O. conglobata contaminata*

137   Analysis of variance showed a significant interaction between the diets, predators and

138   developmental stages (F=34.24; df= 5, 180; p<0.01) for SCPs. Therefore, the results of the

139   means comparison using slicing interactions were presented in Figures 1-4.

140   The SCP of *C. sexmaculata* ranged from -14.5 to -19.3 °C when fed *A. pistaciae*. The highest

141   and the lowest SCPs were recorded in the fourth instar larvae and pupae, respectively (*F*=14.20;

142   df=5,30; *p*<0.05). The mean SCP of the larvae (feeding stage) was -16.6 °C. For the ladybird

143   beetle *O. conglobata contaminata,* the SCPs were a little bit lower than those of *C. sexmaculata,*

144   but the differences were not significant in most of the cases, following the same trend (*F*=9.42;

145   df=5,30; *p*<0.05). The mean SCP of the larvae (feeding stage) was -17.4 °C.

146   When the ladybird beetle *C. sexmaculata* was fed on the eggs of *E. kuehniella,* the SCPs of

147   the second instar larvae and pupae were at the lowest levels (ca. -21 °C), while the SCP of the

148   fourth instar larvae (ca. -14 °C) was at the highest level (*F*=14.93; df=5,30; *p*<0.05). The mean

149   SCP of the larvae (feeding stage) was -17.08 °C. For *O. conglobata contaminata* the SCPs were

150   significantly higher than those of *C. sexmaculata*. In this coccinellid, the lowest SCP (ca. -19 °C)

151   was recorded in pupae. The mean SCP of the feeding stage (all larval instars) was -17.4 °C.

152   The data indicate that the type of diet significantly influenced the SCP of *C. sexmaculata*.

153   When this coccinellid fed on *A. pistaciae*, the SCPs of the second instar larvae, pupae, and adults

154   were about -18, -19, and -15 ºC, respectively. These supercooling temperatures significantly

155   decreased and reached about -21, -21, and -19 ºC, respectively for the beetles feeding on the eggs

156   of *E. kuehniella*. For *O. conglobata contaminata*, the changes in the SCPs, under different food

157   regimes, were not significant. The effects of the diets on the SCPs of two ladybird species were

158   shown in Figures 1-4. For species feeding on *A. pistaciae,* the SCPs of the fourth instar larvae

159   and adult females of *O. conglobata contaminata* were significantly lower than those of *C.*

160   *sexmaculata*. When these coccinellids, were fed on the eggs of *E. kuehniella,* the SCPs of all

161   developmental stages of *O. conglobata contaminata* were significantly lower than those of *C.*

162   *sexmaculata*.

163   *3.2. The effects of the diets on cold tolerance of C. sexmaculata and O. conglobata contaminata*

164   The effects of two different diets (*A. pistaciae* and *E. kuehniella*), were evaluated on the cold

165   hardiness of different developmental stages of *C. sexmaculata*. Analysis of variance indicated a

166   significant interaction among the diets, temperatures and developmental stages (F=34.19; df= 24,

167   210; p<0.01) for survival of *C. sexmaculata*. The results of the means comparison using slicing

168   interactions were presented in Table 1. When female *C. sexmaculata* was fed on *A. pistaciae*, the

169   survival of their eggs was negligible even at 0C/24 h, and at temperatures below 0C/24 h no eggs

170   hatched. For other developmental stages, the cold hardiness significantly decreased as the

171   temperature lowered and reached the lowest level at -3 ºC/24 h. However, among different

172   instars of the larvae, the cold tolerance of the fourth instar was substantially lower than that of

173   the others. In immature stages, the cold tolerance of the pupa was at the lowest level, but in

174   general, the adults of the beetle showed the highest cold tolerance (Table 1). When *C.*

8

175 *sexmaculata was* fed on the eggs of *E. kuehniella*, the cold tolerance of different developmental

176 stages other than the eggs significantly increased. However, the changes in the cold tolerance of

177 the beetle generally followed the same trend when *A. pistaciae* was used as food (Table 1). Also,

178 the effects of two different diets, consisting of *A. pistaciae* and *E. kuehniella*, were studied on the

179 cold hardiness of different developmental stages of *O. conglobata contaminata*. Analysis of

180 variance showed a significant interaction between the diets, temperatures and developmental

181 stages (F=7.03; df= 18, 168; p<0.01) for survival of *O. conglobata contaminata*. The results of

182 the means comparison using slicing interactions were presented in Table 2. When *A. pistaciae*

183 was provided as food for this beetle, the egg and adult stages of development were found to be

184 the most sensitive and cold-tolerant ones, respectively. No egg hatch was observed even after 24

185 hours of exposure to 0 ºC, but the survival of other stages was at the highest levels. Nonetheless,

186 the survival of the larvae, pupa, and adults decreased by decreasing the exposure temperature and

187 reached the lowest levels at -7 ºC/24 h (Table 2). When *O. conglobata contaminata* was fed on

188 *E. kuehniella* eggs, the changes in the cold hardiness followed the same trend as those in the

189 beetles feeding on psyllids, but the cold hardiness significantly enhanced (Table 2). For example,

190 at -7 ºC/24 h, the cold hardiness of the adult beetles feeding on psyllids was 28.8%, while it

191 reached 42.5% in the beetles feeding on the flour moth eggs.

192 *3.3. Effects of food on lower lethal temperatures of the ladybird beetles*

193   The data in Figures 5 and 6 indicate that, for the beetles of *C. sexmaculata* feeding on

194 psyllids, the LTs of the non-feeding stage (pupae) were higher than those of the feeding stages

195 (larvae and adults). The lower lethal temperature (LLT$_{99-24h}$) of the pupae, i.e. the temperature at

196 which 99% of the insects die after 24 hours of exposure, was -1.6 ºC, whereas this temperature

197 was found to be -3.5 and -4.7ºC for the larvae and adult, respectively. For the beetles of *C.*

9

198 *sexmaculata* feeding on moth eggs, the $LLT_{99\text{-}24h}$ of the first instar larva was similar to that of the

199 beetles fed on the psyllids, while for other developmental stages, the $LLT_{99\text{-}24h}$ decreased and

200 reached the lowest level of -6.1℃ in the feeding stages. The LLTs of different developmental

201 stages of *O. conglobata contaminata* in both diets were lower than those of *C. sexmaculata*. For

202 both beetles of this species, regardless of psyllids or flour moth eggs as a diet, the lowest

203 $LLTs_{99\text{-}24h}$ were recorded for the adults, i.e. -1.2 and -13.8 ℃, respectively.

204 **4. Discussion**

205   The results of the current study indicated the following findings. Regardless of the diet, the

206 eggs of *C. sexmaculata* and *O. conglobata contaminata* were found to be the most sensitive

207 developmental stage to cold. No eggs hatched when the eggs were exposed to zero and subzero

208 temperatures for 24 h. In the feeding stages (larvae) of *C. sexmaculata* feeding on psyllids, the

209 cold tolerance of the fourth instar larvae was significantly lower than that of the first, second, and

210 third instar larvae, whereas, for the larvae of the *C. sexmaculata* feeding on flour moth eggs, the

211 cold tolerance increased as larvae grew up to reach the highest level in the fourth instar larvae.

212 When the larvae were fed on eggs of the Mediterranean flour moth, the survival of the fourth

213 instar larvae increased up to 4.8 times. For the different larval instars of *O. conglobata*

214 *contaminata*, the cold hardiness was similar, but the cold tolerance of the larvae feeding on flour

215 moth eggs was significantly higher than that of the larvae feeding on psyllids. The larval survival

216 of *O. conglobata contaminata* feeding on *A. pistaciae* at -5 ℃/24 h, was lower than that of

217 conspecifics feeding on eggs of *E. kuehniella* (15% on *A. pistaciae* vs. 40% on flour moth eggs).

218 In the non-feeding stage (pupa) of *C. sexmaculata* fed on psyllids, the cold tolerance was at the

219 lowest level, but in *C. sexmaculata* feeding on eggs, the cold tolerance increased up to 4.5 times.

220 When the larvae of *O. conglobata contaminata* were fed on the eggs of flour moth, the cold

221 tolerance of the pupa, at -7 ℃/24 h, was 11 times higher than that of the pupa of the larvae fed on

222 psyllids. In *C. sexmaculata,* the cold tolerance in the adulthood stage was significantly higher

223 than that in other developmental stages in both diets. In addition, the cold tolerance of the adults

224 was affected by the diets and increased 1.14 times when the beetles were fed on the eggs of the

225 flour moth.

226   In *O. conglobata contaminata* at 0 ℃/24 h, no significant differences were detected between

227 the adult, the larval and the pupal stages, but with a decrease in the temperature, the cold

228 tolerance in the adult stage decreased less than that of the pupa and larvae. Sex had no effect on

229 the SCPs of *C. sexmaculata* and *O. conglobata contaminata* adults. This finding was in line with

230 those found by Koch et al. [14] regarding *H. axyridis*. However, the findings of the current study

231 did not support the previous research done by Hefty et al. [6] who found that, in *Pityophthorus*

232 *juglandis* (Col.: Scolytidae), the SCP of the male was lower than that of the female. It is worth

233 mentioning that, in these coccinellids, similar to most other insects [14, 20], the SCP was

234 affected by the developmental stage of the beetles.

235   A limiting factor in the augmentation programs is relatively unaffordable mass-rearing

236 operations of natural enemies. In these operations, maintenance necessity of three trophic levels

237 (i.e., biological control agent, its host, and host's food plant) may raise the rearing costs.

238 Factitious diets are unnatural foods that facilitate mass-production of insect's natural enemies by

239 reducing these expenditures. Hence, determination of the value of natural and factitious diets is a

240 prerequisite for mass-rearing of natural enemies and also, planning a biological control program

241 [2, 4, 10]. Nevertheless, the result of this study highlighted the substantial effects of diet on the

242 cold tolerance and the survival of the coccinellids. Given the cold tolerance of the ladybird

243 beetles, the eggs of the Mediterranean flour moth were found to be the most efficient and

244   suitable diet in comparison with the common pistachio psylla. This finding strongly suggests that

245   the nutrition requirements of the ladybird beetles are different, and the two diets had different

246   nutritional values. Several studies have documented the impacts of diets on biological parameters

247   of different insects. Soares et al. [28] studied the influences of two aphid species on the fitness of

248   *H. axyridis* adults and demonstrated the effects of the differences in the suitability of the prey on

249   the growth and development of the predator. Omkar and James [24] demonstrated the influences

250   of six aphid species on some biological parameters of *Coccinella transversalis* Fabricius (Col.,

251   Coccinellidae) and found that the prey significantly affected the required time for the

252   development of immature species, wet weight, and adult longevity. Significant effects of five

253   different diets on the developmental time of the predatory pentatomids, *Picromerus bidens* L.,

254   and *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae) were reported by Mahdian et al.

255   [17]. Facon et al. [5] reported that mating and reproduction decreased the cold tolerance of the

256   ladybird beetle, *H. axyridis*. The present findings seem to be consistent with other studies [16, 5,

257   21] finding that several factors, such as food type, mating, and reproduction, could greatly

258   influence the physiological status of the insects and might subsequently affect the cold hardiness.

259   Our results corroborate the findings of Maes et al. [16] who showed that the cold tolerance of the

260   predatory bug, *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), was affected by the diet.

261   The cold hardiness of those bugs feeding on an artificial diet, based on egg yolk, was less than

262   that of those feeding on the eggs of *E. kuehniella.* Mohammadzadeh and Izadi [21] indicated that

263   the quality of food might affect the SCPs and the cold hardiness of *Trogoderma granarium*

264   Everts (Coleoptera: Dermestidae). Santacruz et al. [26] found no significant difference between

265   the cold tolerance of two geographical populations of the predatory wasps *Trissolcus japonicus,*

266   and *T. cultratus* (Hym.: Scelionidae). However, between the two predators, different stages

267 (except for eggs) of *O. conglobata contaminata* were found to be more cold-tolerant than those

268 of *C. sexmaculata*.

269 According to Sinclair et al. [27], as for both diets, the rate of mortality of *C. sexmaculata* and

270 *O. conglobata contaminata* mostly increased above the SCP, these coccinellids were considered

271 as chill-intolerant insects. These findings were consistent with those obtained by Koch et al. [14]

272 who found the multicolored Asian ladybird beetle, *Harmonia axyridis* (Pallas) (Col.:

273 Coccinellidae), to be a freeze-intolerant insect. These findings further support the idea proposed

274 by Tian et al. [31] who indicated that freeze avoidance was the primary strategy used by the

275 Japanese pine sawyer, *Monochamus alternatus* (Col.: Cerambycidae). However, it is noteworthy

276 that the beetles were reared at 26 ℃ and long photoperiod. Under these conditions most insects,

277 even those that are very cold tolerant at other times, are likely to be chill-intolerant. If the

278 predators cold acclimated or collected from the natural overwintering sites the results could be

279 quite different.

280 Although the lowest SCP was recorded in the second instar larva and pupa of *C. sexmaculata*

281 feeding on both psyllids and flour moth eggs, the SCP of the beetle fed on flour moth eggs was

282 significantly lower than that of the beetle fed on psyllids. In contrast to *C. sexmaculata*, however,

283 no evidence was found indicating the significant effects of diet on the SCP of *O. conglobata*

284 *contaminata*. Few literatures are available concerning the effects of diets on SCPs of coccinellid

285 in various developmental stages. The mean SCPs of the pupae, larvae, and adults of *H. axyridis*

286 were reported to be -21.3,-14.2, and -11.9 ℃, respectively [14]. However, it is notable that most

287 freeze avoiding insects cease feeding prior to winter cold exposure. So, that ingested food (that

288 often contains ice nucleators) is not present in the gut to induce lethal freezing. Since the beetles

289 in this study were not starved for a few days prior to testing, this leaves open the possibility that

290 the resulting SCPs were due to nucleators in the food ingested. The non-feeding pupae typically

291 had the lowest SCPs. Therefore, while the SCP results could be due to endogenous variations in

292 the beetles resulting from the diet, it is also possible that SCP differences could result from

293 exogenous ice nucleators in the food. Further qualitative and/or quantitative investigations on

294 enzymatic differences may exist between the various developmental stages or between the

295 species could uncover this ambiguity.

296 The mean LLT of the *C. sexmaculata* feeding on psyllids was -3.4 ℃, whereas the mean LLT

297 of the *O. conglobata contaminata feeding on* psyllids was -7.4 ℃. The mean LLT of the *C.*

298 *sexmaculata* feeding on the flour moth eggs was about -5.3 ℃, whereas the LLT of the *O.*

299 *conglobata contaminata feeding on the* flour moth eggs was about -9.3 ℃. However, it is

300 possible that some of the mortality in the LLT studies resulted from the freezing of the insects.

301 Although LLTs were higher than the SCPs, it should be remembered that supercooled water is in

302 a metastable state. As such, the time has an effect on the likelihood of freezing. While mean

303 SCPs measured with a cooling rate of 0.2C/min might be -15C it is possible that an insect held in

304 a supercooled state at a higher temperature (-2 to -7C in these experiments) above its actual

305 freezing point (probably ~ -0.5 to -0.8C) for 24 hours might freeze. The potential effects of ice

306 nucleators in the food ingested, as mentioned above, holds here as well. Another possibility that

307 could result in the freezing of an insect above its measured SCP, especially over a 24 hour

308 period, is that as the temperature in the chamber enclosing the insect is decreased water

309 condenses out onto the surface of the insect, then freezes and inoculates the insect across the

310 cuticle. While this is possible during the short time frame over which the usual SCP is measured,

311 it is more probable over a 24-hour period. Of course the higher the relative humidity is during the

312 measurements, the more likely the probability of inoculative freezing. However, this does not

313 affect the lethal effects of lower lethal temperatures.

314   In general, our data indicate that the flour moth eggs are the more suitable diet for the ladybird

315 beetles compared with the psyllid nymphs, and could subsequently be considered as a factitious

316 food for the larvae and adults of *C. sexmaculata* and *O. conglobata contaminata*. Several studies

317 introduced the eggs of *E. kuehniella* to be a factitious food for the larvae and adults of *Adalia*

318 *bipunctata* (Coleoptera: Coccinellidae) [4,10,12], *H. axyridis* (Pallas) (Coleoptera:

319 Coccinellidae) [2], nymphs and adults of *Nesidiocoris tenuis* (Reuter) *M. pygmaeus* Rambur

320 (Hemiptera: Miridae) [22], and the anthocorid predators *Orius thripoborus* (Hesse) and *Orius*

321 *naivashae* (Poppius) (Hemiptera: Anthocoridae) [3]. Mirhosseini et al. [19] found that the eggs

322 of *E. kuehniella* were more suitable for the development of the beetles than for the reproduction

323 of *C. sexmaculata* and *O. conglobata contaminata*.

324   In conclusion, the results of our study suggest that the eggs of the predators were the most

325 sensitive stage to cold, and adulthood was the most cold-resistant stage of *C. sexmaculata* and *O.*

326 *conglobata contaminata*. Both of the species were found to be chill-intolerant, and the eggs of

327 the *E. kuehniella* were proven to be suitable for the ladybird beetles. In a practical point of view,

328 mass-rearing is a fundamental stage in classical biological control and providing natural enemies

329 with a suitable diet is a prerequisite in mass-rearing programs. Therefore, with emphasis on our

330 results, eggs of *E. kuehniella* can be used as a reliable diet in mass-rearing of the predatory

331 beetles. Besides, our results provided a reliable cold tolerance strategy for rearing and

332 management of the predatory beetles.

335

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439

440 **Fig 1**

441 Mean comparison of the supercooling point of different developmental stages of *C. sexmaculata* and *O.*
442 *conglobata contaminate* fed on *A. pistaciae* (Means in each column fallowed by similar letters are not
443 significantly different (Tukey's test, P > 0.05)

444 **Fig 2**

445 Mean comparison of the supercooling point of different developmental stages of *C. sexmaculata* and *O.*
446 *conglobata contaminate* fed on *E. kuehniella* (Means in each column fallowed by similar letters are not
447 significantly different (Tukey's test, P > 0.05)

448 **Fig 3**

449 Mean comparison of the supercooling point of different developmental stages of *C. sexmaculata* fed on
450 different diets (*A. pistaciae* and *E. kuehniella*) (Means in each column fallowed by similar letters are not
451 significantly different (Tukey's test, P > 0.05)

452 **Fig 4**

453 Mean comparison of the supercooling point of different developmental stages of *O. conglobata*
454 *contaminate* fed on different diets (*A. pistaciae* and *E. kuehniella*) (Means in each column fallowed by
455 similar letters are not significantly different (Tukey's test, P > 0.05)

456 **Fig 5**

457 Effect of diet on lethal temperatures of each life stage of *Cheilomenes sexmaculata*

458 For each diet lethal temperatures with different letter in a column are considered significantly different if
459 their 95% confidence interval (CI) did not overlap.

460

461 **Fig 6**

462 Effect of diet on lethal temperatures of each life stage of *O. conglobata contaminate*

463 For each diet lethal temperatures with different letter in a column are considered significantly different if
464 their 95% confidence interval (CI) did not overlap.

465

Table 1. Mean comparison of survival (%) of different developmental stages of *C. sexmaculata* fed on different diets under different temperatures

Developmental stages	Temperature	Diet	
		A. pistaciae	*E. kuehniella*
Egg	0 ℃	0.0 ± 0.0 k	0.0 ± 0.0 q
	-1 ℃	0.0 ± 0.0 k	0.0 ± 0.0 q
	-2 ℃	0.0 ± 0.0 k	0.0 ± 0.0 q
	-3 ℃	0.0 ± 0.0 k	0.0 ± 0.0 q
	-4 ℃	0.0 ± 0.0 k	0.0 ± 0.0 q
First instar	0 ℃	45.0 ± 4.1 bc	40.0 ± 2.9 hi
	-1 ℃	8.8 ± 2.4 g-k	28.7 ± 2.4 j-l
	-2 ℃	7.5 ± 1.4 g-k	13.7 ± 2.4 m-o
	-3 ℃	5.0 ± 0.0 i-k	0.0 ± 0.0 q
	-4 ℃	0.0 ±0.0 k	0.0 ± 0.0 q
Second instar	0 ℃	40.0 ± 3.5 cd	31.2 ± 3.7 i-k
	-1 ℃	33.7 ± 2.4 d	27.5 ± 2.5 j-l
	-2 ℃	10.0 ± 2.0 g-j	18.7 ± 2.4 l-n
	-3 ℃	0.0 ±0.0 k	8.7 ± 1.2 n-q
	-4 ℃	0.0 ±0.0 k	3.7 ± 1.2 o-q
Third instar	0 ℃	52.5 ± 4.3 b	61.2 ± 3.7 ef
	-1 ℃	32.5 ± 1.4 de	28.7 ± 2.4 j-l
	-2 ℃	16.2 ± 1.2 fg	22.5 ± 1.4 k-m
	-3 ℃	0.0 ±0.0 k	18.7 ± 1.2 l-n
	-4 ℃	0.0 ±0.0 k	11.2 ± 1.2 n-p
Fourth instar	0 ℃	15.0 ± 2.0 f-h	72.5 ± 1.4 cd
	-1 ℃	13.7 ± 2.4 g-i	53.7 ± 2.7 fg
	-2 ℃	8.7 ± 1.2 g-k	43.7 ± 2.4 gh
	-3 ℃	0.0 ± 0.0 k	25.0 ± 2.0 kl
	-4 ℃	0.0 ± 0.0 k	11.2 ± 1.2 n-p
Pupa	0 ℃	12.5 ± 2.5 g-i	67.5 ± 1.4 de
	-1 ℃	1.2 ± 1.2 jk	36.2 ± 3.1 h-j
	-2 ℃	1.2 ± 1.2 jk	18.7 ± 1.2 l-n
	-3 ℃	0.0 ±0.0 k	6.2 ± 1.2 o-q
	-4 ℃	0.0 ± 0.0 k	2.5 ± 1.4 pq
Adult	0 ℃	78.7 ± 2.4 a	100.0± 0.0 a
	-1 ℃	71.2 ± 3.1 a	81.2 ± 2.4 bc
	-2 ℃	23.7 ± 1.2 ef	83.7 ± 1.2 b
	-3 ℃	12.5 ± 1.4 g-i	63.7 ± 2.4 d-f
	-4 ℃	6.2 ± 1.2 h-k	13.7 ± 1.2 m-o

Means in each column not fallowed by the same letters are significantly different (Tukey's test, $P > 0.05$)

Table 2. Mean comparison of survival (%) of different developmental stages of *O. conglobata contaminate* fed on different diets under different temperatures

Developmental stages	Temperature	Diet	
		A. pistaciae	*E. kuehniella*
Egg	0 ℃	0.0 ± 0.0 i	0.0 ± 0.0 i
	-3 ℃	0.0 ± 0.0 i	0.0 ± 0.0 i
	-5 ℃	0.0 ± 0.0 i	0.0 ± 0.0 i
	-7 ℃	0.0 ± 0.0 i	0.0 ± 0.0 i
First instar	0 ℃	100.0 ± 0.0 a	100.0 ± 0.0 a
	-3 ℃	48.7 ± 2.4 de	70.0 ± 4.1 c-e
	-5 ℃	16.2 ± 3.7 g	42.5 ± 4. 8 h
	-7 ℃	0.0 ± 0.0 i	3.7 ± 2.4 i
Second instar	0 ℃	100.0 ± 0.0 a	100.0 ± 0.0 a
	-3 ℃	38.7 ± 3.7 ef	58.7 ± 3.1 e-g
	-5 ℃	15.0 ± 3.5 gh	35.0 ± 2.0 h
	-7 ℃	0.0 ± 0.0 i	0.0 ± 0.0 i
Third instar	0 ℃	100.0 ± 0.0 a	100.0 ± 0.0 a
	-3 ℃	46.2 ± 3.7 de	67.5 ± 1.4 d-f
	-5 ℃	16.2 ± 3.1 g	35.0 ± 3.5 h
	-7 ℃	0.0 ± 0.0 i	2.5 ± 1.4 i
Fourth instar	0 ℃	100.0 ± 0.0 a	100.0 ± 0.0 a
	-3 ℃	43.7 ± 3.1 de	60.0 ± 2.9 e-g
	-5 ℃	13.7 ± 2.4 gh	47.5 ± 4.3 gh
	-7 ℃	0.0 ± 0.0 i	6.2 ± 2.4 i
Pupa	0 ℃	87.5 ± 2.5 b	100.0 ± 0.0 a
	-3 ℃	53.7 ± 3.7 d	82.5 ± 4.3 bc
	-5 ℃	3.7 ± 2.4 hi	56.2 ± 2.4 fg
	-7 ℃	0.0 ± 0.0 i	11.2 ± 3.7 i
Adult	0 ℃	100.0 ± 0.0 a	100.0 ± 0.0 a
	-3 ℃	98.7 ± 1.2 ab	88.7 ± 2.4 ab
	-5 ℃	68.7 ± 5.1 c	80.0 ± 2.0 b-d
	-7 ℃	28.7 ± 2.4 f	42.5 ± 4.3 h

Means in each column not fallowed by the same letters are significantly different (Tukey's test, P > 0.05)