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1 **Effects of different *Philosamia ricini* (Lepidoptera: Saturniidae) cold storage periods on**
2 ***Ooencyrtus pityocampae* and *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae) rearing**

3
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10
11 **The effects of host cold storage on *O. pityocampae* and *O. kuvanae***

12
13 **Abstract:**

14 *Ooencyrtus pityocampae* and *Ooencyrtus kuvanae* are egg parasitoids that are considered
15 potential candidates for the control of different pest species through inundative release. The aim
16 of this study was to assess the effects of different cold-storage periods of *Philosamia ricini* eggs
17 (host) on the rearing parameters of *O. pityocampae* and *O. kuvanae*. Host eggs were stored at
18 3°C, and a factorial experiment involving two parasitoid species, nine host storage periods (1, 5,
19 10, 15, 30, 45, 60, 75 and 90 days) and a control, and two host ages (1 and 2 days) was
20 conducted, with 10 replications including 40-*P. ricini* eggs each. Adult emergence, development
21 time, longevity, and fecundity were investigated. The parasitoid adult emergence percentage
22 significantly varied with storage duration. These values were lower in *O. kuvanae* than in *O.*

23 *pityocampae*. The development time of *O. kuvanae* progeny increased in both host age groups
24 except in the 1-day storage period subgroup. However, the development times of the progeny of
25 *O. pityocampae* reared on one-day-old eggs stored for 5, 10, 60, and 75 days were increased, and
26 the development times of the progeny of *O. pityocampae* reared on 2-day-old eggs stored for 45
27 and 90 days were increased. The longevity of the F1 progeny of *O. kuvanae* was negatively
28 affected by storage time. There was no difference in the longevity of the F1 progeny of *O.*
29 *pityocampae* compared to that of the control. Additionally, the fecundities of the F1 progeny of
30 *O. pityocampae* and *O. kuvanae* were 54.7 and 47.0 offspring/female, respectively. These results
31 provide useful information for guiding the development of mass rearing methodologies for both
32 parasitoid species.

33

34 Key words: Cold storage, host, egg parasitoid, *Ooencyrtus pityocampae*, *Ooencyrtus kuvanae*,
35 *Philosamia ricini*

36

37 **Introduction**

38 *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encyrtidae) is an efficient biological control
39 agent of *Thaumetopoea pityocampa* (Den. & Schiff.) and *T. wilkinsoni* (Tams) (Lepidoptera:
40 Notodontidae), which are among the most important defoliators of pine forests throughout the
41 Mediterranean Basin (Buxton, 1983; Battisti et al., 1990; Masutti et al., 1993; Tiberi et al., 1994;
42 Hódar and Zamora, 2004; Zhang et al., 2005; Binazzi et al., 2013; Samra et al., 2015). Other
43 known hosts of *O. pityocampae* include *Nezara viridula* (Linnaeus) *Aelia rostrata* (Boh),
44 *Carpocoris* sp., *Dolycris baccarum* (Linnaeus), *Rhaphigaster nebulosa* (Poda), *Eurydema*
45 *ventrale* (Kolenati), *E. oleracea* (Linnaeus), *Graphosoma lineatum* (Linnaeus) *Halyomorpha*

46 *halys* (Stål) (Hemiptera: Pentatomidae) and *Eurygaster maura* (Linnaeus) (Hemiptera:
47 Scutelleridae) (Tiberi et al., 1991; Tiberi et al., 1993; Federico et al., 2016).

48 *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) is a primary egg parasitoid of
49 *Lymantria dispar* (L.) (Lepidoptera, Lymantriidae) (Howard, 1910; Tadic and Bincev, 1959;
50 Brown, 1984; Hofstetter and Raffa, 1998; Wang et al., 2013) but it can also parasitize other hosts,
51 such as *Dendrolimus spectabilis* Butler (Lepidoptera: Lasiocampidae), *Malacosoma americana*
52 (Fabricius), *M. neustria tartacea* (Motschulsky), *Euproctis chrysorrhoea* (Linnaeus),
53 *Hemerocampa leucostigma* (Abbot & Smith), *Hemerocampa definata* (Packard), *Lymantria*
54 *fumida* (Butler), *Nygmia phaeorrhoea* (Donovan), *Stilpnotia salicis* (Linnaeus) *Eriogyna*
55 *pyreterom* (Westwood) (Lepidoptera: Saturniidae), *Lycorma delicatula* (White) (Hemiptera:
56 Fulgoridae) and *H. halys* (Stål) (Hemiptera: Pentatomidae) (Huang and Noyes, 1994; Hofstetter
57 and Raffa, 1998; Liu, 2019; Tunca et al., 2019).

58 To reduce the costs associated with biological control programmes and to ensure the supply of
59 high-quality natural enemies at times of high demand, it is important to improve parasitoid mass-
60 rearing techniques (Spínola-Filho, 2014). The encyrtid egg parasitoids *O. pityocampae* and *O.*
61 *kuvanae* can be successfully raised on the host *Philosamia ricini* (Donovan) (Lepidoptera:
62 Saturniidae) (Tunca et al., 2016, 2017). *Philosamia ricini* can be reared on *Ligustrum vulgare* (L.)
63 (Amaranthaceae) and *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) (Venard, 2016), and
64 rearing is straightforward and inexpensive. In addition, *P. ricini* oviposits more often, and its
65 eggs are larger than those produced using two other known laboratory hosts, *N. viridula* and *H.*
66 *halys*. Although methods for breeding this host are well known and have been optimized, they
67 can be affected by external problems, such as the natural contamination of host plants by
68 bacterial species. Consequently, there are occasional problems related to the production of *P.*
69 *ricini* eggs due to high larval mortality, and the rearing of the two parasitoids can be affected.

70
71 When inundative release is used as a biological pest control strategy, large numbers of parasitoids
72 are required to rapidly reduce the damaging pest population. However, the major challenge
73 associated with this biological control strategy is the production of a large number of parasitoids
74 of adequate quality (Orr, 1988). Several techniques have been used to optimize the large-scale
75 mass production of parasitoids in laboratories. Some of these approaches include, the in vitro
76 development of parasitoids (Strand et al., 1988; Nettles Jr,1990; Consoli and Vinson, 2004;
77 Shirazi, 2006; Paladino et al. 2010; Kim et al. 2018), cold storage of parasitized hosts (Noble,
78 1937; Dass and Ram, 1983; Gautam, 1986; Ganteaume et al. 1995 a, b; Bayram et al., 2005;
79 Tunca et al., 2014; Liu et al., 2014; Kidane et al., 2015), cold storage of parasitoids as pupae or
80 adults (Gautam, 1986; Foerster et al., 2004; Foerster and Doetzer, 2006; Yılmaz et al. 2007;
81 Mousapour et al., 2014; Afshari and Fandokht, 2019; Cira et al., 2021) and cold storage of
82 unparasitized hosts (Corrêa-Ferreira and Moscardi, 1993; Kıvan and Kılıç, 2005; Mahmoud and
83 Lim, 2007; Alim and Lim, 2010; de Carvalho Spínola-Filho et al., 2014; Singhamuni et al., 2015;
84 Wong et al., 2020).

85
86 Storing a host at a low temperature can arrest its development at the desired stage and contribute
87 to the rearing of parasitoids. The cold storage technique allows the synchronization of parasitoid
88 release with outbreaks of insect pests (Leopold, 1998; Pitcher et al., 2002; Colinet and Boivin,
89 2011). Cold storage of *P. ricini* eggs is important, as it allows for a sufficient number of egg hosts
90 for the rearing of *O. pityocampae* and *O. kuvanae*. The objective of this study was to investigate
91 the optimum cold storage conditions of host eggs and to assess the performance of *O.*
92 *pityocampae* and *O. kuvanae* reared on stored *P. ricini* eggs.

93

94

95

96 **Materials and Methods**

97 This study was conducted at the INRAE-PACA Mediterranean Forest and Entomology Unit,
98 Laboratory of Biological Control, Antibes, France. The experimental trials were conducted under
99 laboratory conditions at $25 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity (RH), with a photoperiod of 18:6
100 h L:D. *P. ricini* and *O. pityocampae* were reared according to Tunca et al. (2016). *O. kuvanae*
101 was reared according to Tunca et al. (2017).

102 Experimental Setup

103 To determine the effect of cold storage of unparasitized *P. ricini* eggs on *O. pityocampae* and *O.*
104 *kuvanae* rearing parameters, an experiment was carried out in a completely randomized 2 (1- and
105 2- day old hosts) \times 10 (1, 5, 10, 15, 30, 45, 60, 75, and 90 days cold storage and control) \times 2 (2
106 parasitoids, *O. pityocampae* and *O. kuvanae*) factorial design, with 10 replicates of each
107 treatment combination. One- and two-day-old eggs were kept at 3°C in a refrigerator during the
108 cold storage period and 40 host eggs were placed in a test tube (1 x 7 cm) with a single mated *O.*
109 *pityocampae* or *O. kuvanae* female for 24 h for oviposition. Five-day-old mated females of *O.*
110 *pityocampae* and *O. kuvanae* were fed honey and used for the oviposition experiments. After
111 oviposition, *O. pityocampae* and *O. kuvanae* females were removed from the test tubes. The
112 tubes were incubated at $25 \pm 1^\circ\text{C}$, with a RH of $65\% \pm 5\%$ and a 16:8 h L:D photoperiod until
113 parasitoid offspring emerged. Host larvae that hatched from unparasitized eggs were removed,
114 and parasitized eggs were left in the tube.

115 Exposed eggs were monitored on a daily basis, and the number of emerged adults was recorded.
116 Similarly, the time that elapsed from the exposure until adult emergence of the parasitoids was
117 recorded to account for the developmental time. The emergence rate was calculated as the

118 proportion of parasitized eggs in the tube and expressed as a percentage. To determine adult
119 longevity, the parasitoids were placed in a test tube (1 x 7 cm) with a drop of bio-honey.
120 Longevity was recorded daily until all of the parasitoids died. The fecundities of *O. pityocampae*
121 and *O. kuvanae* were determined using one-day-old *P. ricini* eggs. For this analysis, *O.*
122 *pityocampae* and *O. kuvanae* females (8 and 7 females, respectively) that emerged from stored
123 eggs were chosen randomly. Thirty eggs were supplied on a daily basis to each newly emerged
124 female until the females died, and the parasitoids that emerged from the parasitized eggs were
125 counted every day.

126 The emergence rate, the development time and adult longevity were analysed using a general
127 linear model (GLM). Percentage data were normalized using an arcsine transformation (Zar,
128 1999). The means were compared with Duncan's test at a significance level of $\alpha = 0.05$
129 (McKenzie and Goldman, 2005; Minitab Release 14, SAS Institute, 2003).

130 **Results**

131 The cold storage period \times parasitoid species interaction showed a significant ($P < 0.001$) effect on
132 the emergence rates of *O. pityocampae* and *O. kuvanae* (Table 1). Increasing cold storage periods
133 significantly reduced the emergence rate in both parasitoid species ($F = 8.76$, $df = 9$, $P < 0.001$). The
134 emergence rates of *O. pityocampae* were higher than those of *O. kuvanae* at 10, 15, 30, 45, 60, 75
135 and 90 days of storage (Table 2). The development times of the parasitoids were significantly
136 affected by the interaction of three factors: cold storage period, host age, and parasitoid species
137 ($F = 3.58$, $df = 9$, $P < 0.001$) (Table 3). The development times of both parasitoid species increased
138 compared to those of the corresponding controls for some storage periods (Table 4). The
139 longevity of the parasitoids was affected by the interaction of the F1 condition and parasitoid
140 species ($F = 13.68$, $df = 1$, $P < 0.001$) (Table 5). The longevity of *O. pityocampae* (43.6 days) was
141 significantly longer than that of *O. kuvanae* (36.6 days) when reared on stored eggs. Compared

142 with the life span of *O. kuvanae* reared on unstored eggs (49.5 days), of *O. kuvanae* reared on
143 stored eggs was shorter (36.6 days) (Table 6). The fecundities of *O. pityocampae* and *O. kuvanae*
144 were 54.7 (progeny/per female) and 47.0 (progeny/per female), respectively. The pre-oviposition
145 times of *O. pityocampae* and *O. kuvanae* were 1.37 days and 1.28 days, respectively. At the same
146 time, both have a post-oviposition periods. The post-oviposition times of *O. pityocampae* and *O.*
147 *kuvanae* were 20.25 days and 14.71 days, respectively.

148

149 **Discussion**

150 It is important to rear egg parasitoid species on suitable hosts to ensure the success of biocontrol
151 programmes (Consoli et al., 2010; Masry and El-Wakeil, 2020). Additionally, the ability to mass
152 produce a large number of parasitoids is required storing host eggs for different periods could
153 contribute positively to the mass rearing of parasitoids (Bigler, 1986; Vieira and Tavares, 1995;
154 Lalitha et al., 2010; Masry and El-Wakeil, 2020).

155 However, longer storage times may result in a decrease in the nutritional quality of host eggs;
156 therefore the performance of parasitoids reared on refrigerated eggs may be reduced (Flanders,
157 1938; Kostal et al., 2004, 2006). For this reason, it is important to take into consideration the host
158 storage period during the rearing of parasitoids (Wong et al., 2020). Our results showed that the
159 storage of *P. ricini* eggs caused adverse effects on in the biological parameters of the F1
160 generation of *O. pityocampae* and *O. kuvanae* adults, represented by the rate of adult emergence,
161 development time, longevity and fecundity. These results can be explained as follows: first, lethal
162 effects occur during parasitoid development in low-quality stored eggs, and second parasitoids
163 fail to accept stored eggs as hosts (Wong et al., 2020).

164 Lethal effects lead to decreased parasitoid progeny emergence (Wong et al., 2020; Mahmoud and
165 Lim, 2007; Mainali and Lim, 2013; McIntosh et al., 2019). In this study, exposure of *P. ricini*

166 eggs at different ages to low temperatures led to a reduction in the emergence rates of the both
167 parasitoids. Siam et al. (2019) reported that low-temperature storage of host eggs for different
168 periods had an effect on the efficiency of *Trichogramma* parasitoids. After 10, 15, 20 and 30 days
169 of storage at 5°C *T. evanescens* emergence percentages decreased by 84.91, 80.48, 61.17, and
170 50.73%, respectively. Bradely et al. (2004) and Ozder (2004) noted that the prolongation of cold
171 storage led to a reduction in the efficiency of F1 female parasitoids. In our study, all the cold
172 storage periods except for 1 day of cold storage (5, 10, 15, 30, 45, 60, 75 and 90 days) affected
173 adult emergence in both parasitoids. In another study, when refrigerated *H. halys* eggs were
174 stored at 8°C for up to two months, the emergence rate of *Trissolcus japonicus* (Ashmead)
175 (Hymenoptera: Scelionidae) decreased significantly (Wong et al., 2020). Similar results have
176 been reported in other studies for *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae)
177 (Chen and Leopold, 2007), *Trichogramma acacioi* (Brun), *T. atopovirilia* (Oatman & Platner), *T.*
178 *benneti* (Nagaraja & Nagarkatti), *T. brasiliensis* (Ashmead), *T. bruni* (Nagaraja) , *T. demoraesi*
179 (Nagaraja), *T. galloi* (Zucchi), *T. pretiosum* (Riley), *T. soaresi* (Nagaraja) (Hymenoptera:
180 Trichogrammatidae) (de Carvalho Spínola-Filho et al., 2014), *T. chilonis* and *T. achaeae*
181 (Singhamuni et al., 2015). Tunca et al. (2014) noted that *Venturia canescens* (Gravenhorst)
182 (Hymenoptera: Ichneumonidae) could not develop on *Ephestia kuehniella* (Zeller) or *Plodia*
183 *interpunctella* (Hübner) (Lepidoptera: Pyralidae) stored at 5°C for 5, 7 and 15 days. Additionally,
184 they did not develop on *P. interpunctella* larvae stored at 10°C for 15 days. The low rate of
185 emergence can additionally be explained by the low rate of parasitism by *O. pityocampae* and *O.*
186 *kuvanae*. Neither species may not accept stored *P. ricini* eggs for parasitization. There were
187 significant reductions in the emergence rates of *O. pityocampae* and *O. kuvanae* after 5- days of
188 host storage.

189

190 There are two important factors that influence the parasitism of stored host eggs: one is the ability
191 to recognize chemical signals in host eggs, and the other is the level of tolerance to changes in
192 physical characteristics, such as the colour, size and shape of the eggs (Stoepler et al., 2011).
193 Female parasitoids may refuse cold-stored eggs with modified chemical and physical
194 characteristics (Soares et al., 2009; Goubault et al., 2011; Penaflor et al., 2011; Stoepler et al.,
195 2011) Relatedly, Conti et al. (1996) reported that low-temperature storage modified egg shape
196 and affected host recognition by parasitoids. The parasitism rates of *T. semistriatus* were
197 decreased when exposed to *Dolycoris baccarum* (L.), *Graphosoma lineatum* (L.) and *Eurydema*
198 *ornatum* (L.) (Heteroptera: Pentatomidae) eggs stored for three months at 6°C (Kıvan and Kılıç,
199 2005). Chen and Leopold (2007) reported that parasitism by *Gonatocerus ashmeadi* Girault
200 (Hymenoptera: Mymaridae) on eggs of *Coagulata homalodisca* (Say) (Hemiptera: Cicadellidae)
201 decreased with an increasing cold storage period. Similarly, Karaborklu and Ayvaz (2007) noted
202 that the emergence rate of and parasitism by *T. evanescens* adults that emerged from stored host
203 eggs decreased depending on the storage period at 4°C. A similar result was also obtained for
204 *Trichogramma olea* reared on *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs stored
205 at 4°C (Gharbi, 2014).

206
207 Compared with those reared on unstored eggs, *O. pityocampae* and *O. kuvanae* reared on eggs
208 subjected to cold storage for different periods had longer development times. Chen and Leopold
209 (2007) reported that after 70 days of *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae) egg
210 storage, the developmental time of the parasitoid *G. ashmeadi* was delayed. Similarly, the
211 development time of *V. canescens* was negatively affected by low temperature and storage time
212 (Tunca et al., 2014). Wong et al. (2020) noted that the development time of *T. japonicus*

213 increased when eggs were refrigerated for long periods. Relatedly, the development time of
214 parasitoids obviously decreased when reared on eggs of *H. halys* refrigerated for short periods.

215
216 The longevity of adult *O. pityocampae* that emerged from cold-stored eggs did not differ from
217 that of the adult control group. However, the longevity of *O. kuvanae* was significantly reduced.
218 Kidane et al. (2015) reported that the longevity of *Encarsia sophia* (Hymenoptera: Aphelinidae)
219 that emerged from host pupae stored for one week at 12 and 8°C was not affected, although
220 longevity decreased to 66-72% with increasing storage period. The longevity of adult *T.*
221 *evanescens* decreased significantly with increased host storage time (Ozder and Saglam 2004).
222 Similarly, Gharbi (2014) reported that the longevity of *T. oleae* adults that emerged from stored
223 pupae decreased significantly with increasing cold storage duration. Siam et al. (2019), showed
224 that the longevity of female *T. evanescens* decreased with prolonged *Sitotroga cerealella*
225 (Olivier) (Lepidoptera: Gelechiidae) cold storage periods.

226 The cold storage of *P. ricini* eggs creates unfavourable conditions for the development of both *O.*
227 *pityocampae* and *O. kuvanae*. *Ooencyrtus pityocampae* and *O. kuvanae* showed decreases in
228 fecundity of 18.1% and 31.7%, respectively, when reared on cold-stored *P. ricini* eggs compared
229 to those reared on fresh eggs (Tunca et al., 2019; Tunca et al., 2017). Similar results were
230 obtained for *T. cacaoeciae*, *T. brassicae*, *T. evanescens* (Ozder and Saglam, 2004), *Gonatocerus*
231 *ashmeadi* (Hymenoptera: Mymaridae) (Chen and Leopold, 2007) and *T. evanescens* (Siam et al.,
232 2019).

233
234 The healthy storage of host insects is extremely important for mass production. However, storing
235 *P. ricini* eggs did not lead to the successful rearing of parasitoids. *Ooencyrtus kuvanae* was more
236 sensitive than *O. pityocampae* in terms of development on stored eggs. However, the results of

237 this study showed that one- and two- day-old *P. ricini* eggs could be stored for up to 30 days for
238 the rearing of *O. pityocampae* and that those stored for up to 10 days at 3°C could be used for
239 rearing *O. kuvanae* for the sustainable production of these parasitoids. These results should be
240 considered in the mass production of these two parasitoid species during autumn and winter and
241 for their release in the field during the critical periods of natural pest hosts outbreaks.

242

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246

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