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1 **Overwintering Biology of the Pomegranate Fruit Moth *Apomyelois ceratoniae***
2 **(Pyralidae: Lepidoptera)**

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16 **Abstract**

17 The pomegranate fruit moth, *Apomyelois ceratoniae* (Zeller), is the most important pest of
18 pomegranate orchards in Iran, where infestations lead to 20-80 % fruit loss. *Apomyelois*
19 *ceratoniae* overwinters as larvae in several instars. The success in overwintering determines the
20 fruit loss in the following season, thus overwintering physiology of *A. ceratoniae* could provide
21 insights into population prediction. To this end, overwintering strategy and some seasonal
22 physiological and biochemical changes were investigated in the field-collected larvae of *A.*
23 *ceratoniae*. The lowest supercooling point was recorded in November (-14.6 ± 0.91 °C) and the
24 highest in both October and March (-10.2 ± 0.94 °C). The median lethal temperature (LT₅₀) of
25 larvae was higher than supercooling point, suggesting that *A. ceratoniae* is chill-susceptible. In
26 comparison with summer larvae, accumulation of glycerol and sorbitol in overwintering larvae
27 resulted in lower mortality when exposed to sub-zero temperatures. There were no significant
28 seasonal changes in body water content or hemolymph osmolality. Current winter temperatures
29 in Iranian orchards are higher than the cold tolerance thresholds of *A. ceratoniae*, suggesting that
30 overwintering mortality is not a key factor in determining *A. ceratoniae* populations.

31 **Key words:** cold hardiness, cryoprotectants, osmolality, supercooling point, water content

32

33 INTRODUCTION

34 The pomegranate fruit moth or carob moth, *Apomyelois ceratoniae* (Zeller) (Lepidoptera:
35 Pyralidae), is a polyphagous fruit pest in many tropical and subtropical countries (Gothilf 1984).
36 The larvae attack pomegranate, fig, and pistachio fruit in Iran (Hesami Fesharaki *et al.* 2011),
37 pomegranate in Iraq (Al-Izzi 1985); almond in Australia; fig, carob, mango, date and almond in
38 Mediterranean countries (Armstrong 2007); date in California, United States (Nay 2005); and
39 citrus and macadamia in South Africa (Gothilf 1984). *Apomyelois ceratoniae*, which originates
40 from Mediterranean countries, was first reported in Kashmar (Khorasan, Iran) pomegranate
41 orchards in 1974 and since 1980 it has been the major pest of pomegranate orchards in Iran
42 (Hashemi Fesharaki *et al.* 2011). Eggs are laid on or near the calyx, through which second or
43 third instar larvae enter the fruit, consuming the interior tissue and seeds. This feeding facilitates
44 entrance of saprophytic fungi resulting in fruit decay and leading to a 20-80 % reduction in fruit
45 yield (Hashemi Fesharaki Fesharaki *et al.* 2011). *Apomyelois ceratoniae* has 4-5 generations per
46 year (Shakeri 2004). In Iran, larvae of several instars enter dormancy in mid-autumn (November)
47 (Gothilf 1984; Al Izzi 1985; Shakeri 2004, Karami *et al.* 2011), and overwinter inside fruits
48 remaining on or under the trees, or under the bark of trees (Shakeri 2004). In early spring (mid-
49 April), the larvae pupate and adult emergence coincides with blossoming and fruiting of
50 pomegranate, continuing until the end of June or beginning of July (Shakeri 2004).

51 Overwintering can comprise half the life cycle of terrestrial organisms in temperate
52 environments (Williams *et al.* 2014). During overwintering, insects must survive low
53 temperature exposure, as well as desiccation and energy drain (Williams *et al.* 2014) that may
54 reduce overall physiological performance and population growth rate (Cárcamo *et al.* 2009). As
55 ectotherms, insects have limited ability to regulate their body temperature; thus, during winter it

56 is possible for their body fluids to freeze when exposed to freezing temperatures (Tattersall *et al.*
57 2012). The formation of internal ice can cause damage to tissues, cells and proteins through
58 mechanical damage or osmotic concentration and anoxia. In order to overcome freezing, many
59 temperate and polar insects seasonally enhance their cold-tolerance in preparation for winter
60 (Denlinger & Lee 2010).

61 Two major strategies, freeze tolerance and freeze avoidance, are adopted by most
62 overwintering insects. The first group withstands the formation of internal ice and maintains a
63 high supercooling point (SCP), while the second die upon freezing and depress the SCP to
64 survive low temperatures. Chill-susceptible insects die within brief exposure to chill at moderate
65 to high sub-zero temperatures (Bale 1996). Both freeze-tolerant and freeze-avoidant insects
66 accumulate cryoprotectants to enhance their cold tolerance (Lee 2010). Accumulation of
67 cryoprotectants increases hemolymph osmolality results in a depression in hemolymph melting
68 point and prevention of ice-crystal formation (Lee 2010). Our knowledge of overwintering
69 biology of *A. ceratoniae* larvae is limited to three field studies in winter. Two of these
70 determined the overall survival of larvae (Khoshamadi & Baghestani 1987; Mehrnejad 1992),
71 reporting 92% and 77% larval survival respectively. Heydari and Izadi (2014) examined low
72 temperature biology of just the last larval instar at an Iranian location that experiences relatively
73 mild winters (Akbarkooh, mean minimum temperature in January: - 1.3 °C), identifying
74 mortality between -5 °C and -10 °C, and suggesting, based on the mortality occurrence at
75 temperature above the SCP that they are chill-intolerant. These authors also observed
76 accumulation of trehalose and myo-inositol in winter (November-February), suggesting that
77 these are the primary cryoprotectants in this species.

78 *Apomyelois ceratoniae* causes substantial fruit loss, even though there are several pest
79 management strategies such as collecting infested fruit in March, stamen elimination (Sheikali *et*
80 *al.* 2009), mating disruption (Zolfaghari *et al.* 2009), biological control (Karami *et al.* 2011)
81 and repellents (Peyrovi *et al.* 2011). Increased knowledge about the physiological performance
82 of different instars of overwintering larvae of *A. ceratoniae* is affected by winter could give the
83 insights in population prediction in spring. The objectives of this study were 1) to determine the
84 overwintering strategy and cold tolerance of all overwintering larval stages of field-collected *A.*
85 *ceratoniae*, at Chandab village, Semnan, Iran, where low winter temperatures are expected to be
86 important for survival, and 2) to identify some of the biochemical and physiological correlates of
87 seasonal changes in cold hardiness of this population.

88

89 MATERIALS AND METHODS

90 Animals

91 *Apomyelois ceratoniae* larvae were collected every month during autumn (October, November,
92 and December), winter (January, February, and March) and summer (June) of 2010-2011 and
93 2012-2013 by gathering infested pomegranate fruit from orchards located in Chandab village,
94 Semnan, Iran (35°25'N, 51°56'E, 1130 m above sea level). Infested pomegranates were
95 transferred to the Faculty of Agriculture, Tarbiat Modares University, and stored on the ground
96 covered with leaf litter. After one day, the larvae were removed and transferred to artificial diet
97 (bran 100g, yeast 5g, sucrose 20g, water 40ml, glycerol 30ml), and kept at ambient temperature
98 in the shade outdoors until use in experiments. We divided larvae into small (2nd and 3rd) and
99 large (4th and 5th) instars and kept them separately. Larvae were used in experiments within one
100 day of transfer to artificial diet.

101

102 Cold tolerance

103 To determine the SCP (n=13-23 per month), larvae were individually attached to NiCr-Ni
104 thermocouples (Type K, diameter 1.5 mm) using adhesive tape (Jason, Ariana Packing co.
105 Tehran, Iran), attached to a transparent plastic sheet and placed inside a programmable
106 refrigerated test chamber (Model MK53, Binder GmbH Bergstr., Tuttlingen, Germany). The
107 temperature of the test chamber was decreased from 15 °C to -30 °C at 0.5 °C/min. The
108 temperature of each insect was recorded every 30 s with a four-channel data logger (Testo Model
109 177-T4; Mehrkanaz Sanat Co.Tehran, Iran) and monitored using comsoft4 software (ComSoft

110 Basic Software; Mehrkanaz Sanat Co., Tehran, Iran). The SCP was recorded as the lowest
111 temperature prior to the start of the exotherm indicating the initiation of freezing.

112 We determined survival of low temperatures each month in both years by measuring survival of
113 field-collected larvae after 3 h exposure to -7, -10 and -12 °C. For each of the temperature
114 treatments, three replicates of seven larvae were placed in glass Petri dishes (100 mm ×15 mm),
115 the bottom of which was covered by dry tissue paper. The Petri dishes were then placed in the
116 programmable refrigerator and cooled at 0.5 °C/min from 10 °C to the test temperature (-7, -10
117 or -12 °C). After 3 h, the larvae were rewarmed at 0.5 °C/min until reaching 10 °C, and survival
118 (ability to walk in a coordinated fashion) was assessed after 24 h.

119

120 **Haemolymph composition and body water content**

121 Haemolymph was collected from 20 individuals for osmometry. Ten microliters of hemolymph
122 was collected with a pipette after cutting the 3rd leg of a larva. Hemolymph was frozen at -80 °C
123 in microcentrifuge tubes (the cap of which was sealed with parafilm) until osmometry was
124 performed. Measurements of hemolymph osmolality were performed with a nanolitre osmometer
125 (Clifton Technical Physics, Hartford, NY; Košťál *et al.* 2011). To measure water content of the
126 larvae, the whole fresh body (FW) was weighed to the nearest 0.1 µg and then dried at -40 °C in
127 a freeze dryer (48 h) to constant mass (DW). Water content (WC) was expressed as mg mg⁻¹ DW
128 (Košťál & Simek 2000).

129

130 **Cryoprotectants**

131 Van Handel's (1965) method was performed to extract the sugars and polyols from whole larvae.
132 Each larva (n=3/month) was weighed and homogenized in a few drops of methanol and 0.05 ml

133 of saturated sodium sulfate solution (for details see Van Handel 1965). The extracts were dried at
134 35 °C in vacuum drying oven (model VO400, Germany) and resuspended in 200 µl of high
135 performance liquid chromatography (HPLC) grade water. The samples were filtered using a 0.45
136 µm syringe filter (Millex, Tokyo, Japan) and 30 µl of each sample was injected into an HPLC
137 (Waters 600 Controller, Milford, MA, USA) and separated on a carbohydrate column
138 (Supelcolgel™, Ca HPLC column) with 9 µm particle size (300 mm long × 7.8 mm ID). The
139 solvent was water and flow rate was kept constant at 0.5 ml/min. Separation was performed at
140 room temperature and all data were acquired and processed with Empower chromatography
141 software (Waters) compounds were identified and quantified from retention time of carbohydrate
142 standards (Fulka, Bush, Switzerland).

143

144 **Statistics**

145 Mean osmolality and cryoprotectants were compared among months by one-way analysis
146 of variance (ANOVA) followed by Tukey's (differences were considered significant at $P <$
147 0.05). Water content was compared with ANCOVA (with dry mass as covariate). A two-way
148 mixed-model ANOVA followed by Tukey's *post hoc* test was performed to compare the
149 differences among SCP in both years. The lethal temperature at which 50% of the population
150 died after a 3 h exposure to subzero temperatures (LT_{50}) was determined using binary logistic
151 regression (Berkvens *et al.* 2010). Comparisons of LT_{50} values were based on non-overlapping
152 95% confidence intervals. Because neither SCP nor cold tolerance was affected by larval instar
153 ($P > 0.05$) the data for both small (2nd and 3rd instars) and large (4th and 5th instars) larvae were
154 pooled. Analyses were conducted using SPSS for Windows (v. 20.0; IBM, Armonk, NY, USA)

155

156 RESULTS

157 Supercooling point and lethal temperatures were compared to determine the cold
158 tolerance strategy. SCP was measured in a total of 250 larvae in 2010-2011 and 2012-2013. The
159 distribution of SCP values in different months (Fig. 2) and among small (2nd and 3rd instars) and
160 large larvae (4th and 5th instars; Fig. 3) was unimodal. The lowest absolute and mean SCP of *A.*
161 *ceratoniae* larvae was recorded in November 2010 (-21.4 °C and -14.6 ± 0.91 °C respectively),
162 whereas the highest SCP was recorded in March 2013 (-6 °C). The highest mean SCPs of
163 overwintering larvae (-10.2 ± 0.94 °C) were recorded in October 2010 and March 2013 (Fig. 1).
164 There was no significant month \times year interaction ($F_{5,238} = 1.344$, $P = 0.24$), however there was a
165 significant main effect of month in both years ($F_{5,235} = 4.712$, $P < 0.001$). Seasonal fluctuations
166 in SCP pattern in different month was equal in followed the same pattern in both years. None of
167 the larvae survived freezing at the SCP.

168 Overwintering individuals were more resistant to sub-zero temperatures than summer-
169 collected larvae. Overwintering individuals survived 3 h exposures to sub-zero temperatures as
170 low as -12 °C, whereas summer-collected individuals only survived 3 h exposures as low as -7
171 °C (Fig. 5). As exposure temperature to sub-zero temperatures decreased, survival of the
172 overwintering larvae decreased (Fig. 4). Cold tolerance did not differ among months during
173 autumn and winter in either year (2010-2011: $F_{5,83} = 1.18$, $P = 0.327$; 2012-2013: $F_{5,63} = 1.008$, P
174 $= 0.420$).

175 The median lethal temperature (LT_{50}) was higher than the SCP of overwintering larvae
176 during sampling months. In 2010-2011 the highest and the lowest LT_{50} were observed in March
177 (-8.9 °C) and February (-12.4 °C) respectively. In 2012-2013, however, the highest and lowest
178 LT_{50} occurred in January (-8.6 °C) and December (-10.7 °C) (Fig. 1). The LT_{50} of larvae in each

179 month was higher than their corresponding SCP (Fig. 1), which suggests the larvae are chill-
180 susceptible. There was no correlation between SCP of overwintering larvae and their respective
181 LT_{50} in sampling months ($r_s=0.096$, $P=0.081$).

182 There was no significant difference between the water content of overwintering ($1.73 \pm$
183 0.18 mg water/mg DW) and summer-collected larvae (1.87 ± 0.13 Water/mg DW; $F_{7,70} = 1.913$,
184 $P=0.098$). Hemolymph osmolality ranged from 413 mOsmol kg^{-1} in March to 443 mOsmol kg^{-1}
185 in January, but there were no significant differences across the year (Fig. 6 A). Glycerol, glucose
186 and sorbitol were detected by HPLC. Glucose levels did not change during the year (0.025 - 0.03
187 mg/g fresh weight); whereas glycerol increased steadily during autumn, peaked in January (2.5
188 mg/g fresh weight), declined in February and reached its lowest point (0.02 mg/g fresh weight)
189 in March. Sorbitol went up in November (2 mg/g fresh weight) decreased to 1 mg/g fresh weight
190 in February and reached its lowest point in March (Fig. 6 B).

191

192 **DISCUSSION**

193 The larvae of *A. ceratoniae* consistently died at temperatures above the supercooling
194 point, suggesting that they are chill-susceptible. In addition, the lack of relationship between SCP
195 and cold hardiness confirms that SCP is not indicative of cold hardiness in this species. Heydari
196 and Izadi (2014) similarly concluded that another (Akbarkooh, Iran) population of *A. ceratoniae*
197 are chill-intolerant (a synonym of chill-susceptibility, Denlinger & Lee 2010), however they did
198 not expose the larvae to temperatures lower than -10 °C. Chill-susceptibility has also been
199 reported for overwintering adults of *Alphitobius diaperinus* (Panzer) (Colinet *et al.* 2011), larvae
200 of *Drosophila melanogaster* (Košťál *et al.* 2012) pupae of *Helicoverpa zea* (Boddie) (Morey *et*

201 *al.* 2012), and the larvae of *Thaumatotibia leucotreta* (Meyrick) (Boardman *et al.* 2012). We

202 found that the lower lethal temperature and LT₅₀ of both small and large overwintering *A.*
203 *ceratoniae* larvae decreased in November 2010 (-11.8 °C), February 2011 (-12.4 °C) and
204 December 2012 (-10.7 °C). This winter decrease in lower thermal limits was previously reported
205 in last instar *A. ceratoniae* (Heydari & Izadi 2014), and is typical of insects overwintering in
206 temperate regions (Khani & Moharramipour 2007; Denlinger & Lee 2010; Crosthwaite *et al.*
207 2011). However LT₅₀ of *A. ceratoniae* was not determined in Akbarkooh population.

208 We found that *A. ceratoniae* accumulated glycerol and sorbitol during the dormant
209 period, and it appears that this accounts for the observed increase in cold tolerance in
210 overwintering larvae. Glycerol is the most widely-distributed metabolite reported in cold-hardy
211 (Denlinger & Lee 2010) insects (e.g.; chill-susceptible bark beetle *Pityogenes chalcographus*
212 (L.); Košťál *et al.* 2014 while sorbitol is a common secondary cryoprotectant in other
213 overwintering insects (Khani *et al.* 2007; Williams & Lee 2011). By contrast, Heydari and Izadi
214 (2014) reported accumulations of trehalose and myo-inositol (but not glycerol) in the last instar
215 larvae. This discrepancy could be because of differences in population as divergent selective
216 pressure in local environments can result in differentiation in thermal biology (Sinclair *et al.*
217 2012). The difference in the cryoprotectants may reflect differences in the cryoprotectant
218 physiology of the populations studied, since the present study was conducted in Chandab where
219 the duration of sub-zero temperatures is longer in winter, so the colder weather could have
220 triggered sorbitol production (e.g. *Eurosta solidaginis* produces sorbitol only when exposed in
221 temperatures below 5 °C (Storey & Storey 1990)). Further exploration and direct comparison
222 will be necessary to determine the underlying cause of the apparently divergent cryoprotectant
223 strategies in these two populations.

224 Although partial dehydration may increase cold tolerance (Ring and Danks 1994; Block
225 1996); insects can rapidly change their cuticular permeability and resist dehydration (Bazinet *et*
226 *al.* 2010). In the case of *A. ceratoniae*, the difference in water content between overwintering and
227 active larvae (c. 6 %) was probably not biologically-significant. Furthermore hemolymph
228 osmolality (October-March) was similar or only slightly higher in the January. *Apomyelois*
229 *ceratoniae* larvae overwinter inside the fruit, a protected site that buffers against low ambient
230 temperatures, reduces the risk of ice damage, and likely protects the larvae from water loss.

231 Although overwintering larvae of *A. ceratoniae* are chill-susceptible, the minimum air
232 temperature in pomegranate-growing areas of Iran normally does not decrease below -10 °C, and
233 -12 °C was the lowest temperature recorded (<http://www.irimo.ir/>); otherwise the pomegranate
234 tree encounters serious injuries. Thus, low temperatures are unlikely to cause significant
235 overwinter mortality, allowing a substantial population to persist through to the following
236 season. However we studied the effect of only a single short time exposure to cold and only
237 measured survival. Nevertheless during winter *A. ceratoniae* encounters prolonged and repeated
238 cold exposures with sub-lethal effects on reproductive fitness as they might invest energy to
239 repair chilling injuries as well as changing pathways to produce heat shock proteins (Marshall &
240 Sinclair 2012). The first pomegranate flowers (which lead to the highest-quality fruit) emerge on
241 late April (Shakeri 2008); and seem to be the host for the first generation of *A. ceratoniae*. Thus,
242 future work understanding the timing of cold exposure (and cold tolerance of non-dormant life
243 stages) and termination of dormancy may improve the timing of integrated pest management
244 interventions aimed at preventing egg-laying by this first generation.

245

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356 **Figure captions**

357 **Figure 1** The median lethal temperature (LT_{50}) and supercooling point (SCP) (mean \pm SE) of
358 overwintering and summer larvae of *Apomyelois ceratoniae* in 2010-2011 and 2012-2013. In
359 October (2010) and February (2013), the number of larvae was too small to calculate LT_{50} .
360 Means with the same letter are not significantly different (Tukey's *post-hoc* tests, $P \leq 0.05$).

361 **Figure 2** Frequency of supercooling point (SCP) in overwintering larvae of *Apomyelois*
362 *ceratoniae* in autumn and winter 2010-2011 and 2011-2012.

363 **Figure 3** Frequency of supercooling point (SCP) in small (2nd and 3rd instars) and large (4th and
364 5th instars) larvae of *Apomyelois ceratoniae*.

365 **Figure 4** Mortality and cumulative supercooling point (SCP) in overwintering larvae of
366 *Apomyelois ceratoniae* in different sub-zero temperatures in autumn and winter 2010-2011 and
367 2012-2013. In October (2010) and February (2013), the number of larvae was too small to
368 calculate mortality.

369 **Figure 5** Mortality and cumulative supercooling point (SCP) of larvae of *Apomyelois ceratoniae*
370 in different sub-zero temperatures in June 2012.

371 **Figure 6** Hemolymph osmolality (A) and whole-body cryoprotectants (B) of field-collected
372 overwintering larvae of *Apomyelois ceratoniae* in 2010-2011.

373

Month

2010-2011

2012-2013

Oct Nov Dec Jan Feb Mar

Jun Oct Nov Dec Jan Feb Mar

Temperature (°C)











