

11-7-2015

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## Citation of this paper:

Ahmadi, Banafsheh; Moharramipour, Saeid; and Sinclair, Brent J, "Overwintering biology of the carob moth *Apomyelois ceratoniae* (Lepidoptera: Pyralidae)" (2015). *Biology Publications*. 94.  
<https://ir.lib.uwo.ca/biologypub/94>

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

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*“This is an Accepted Manuscript of an article published by Taylor & Francis in [International Journal of Pest Management] in [2016], available online at: <http://www.tandfonline.com/doi/full/10.1080/09670874.2015.1102984>”*

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 ( $-14.6 \pm 0.91$  °C) and the  
24 highest in both October and March ( $-10.2 \pm 0.94$  °C). The median lethal temperature (LT<sub>50</sub>) 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 (2<sup>nd</sup> and 3<sup>rd</sup>) and  
99 large (4<sup>th</sup> and 5<sup>th</sup>) 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 3<sup>rd</sup> 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<sup>-1</sup> 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 (2<sup>nd</sup> and 3<sup>rd</sup> instars) and large (4<sup>th</sup> and 5<sup>th</sup> 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 (2<sup>nd</sup> and 3<sup>rd</sup> instars) and  
160 large larvae (4<sup>th</sup> and 5<sup>th</sup> 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  $-14.6 \pm 0.91$  °C respectively),  
162 whereas the highest SCP was recorded in March 2013 (-6 °C). The highest mean SCPs of  
163 overwintering larvae ( $-10.2 \pm 0.94$  °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 ( $1.87 \pm 0.13$  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 LT<sub>50</sub> 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 LT<sub>50</sub> 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

**246 ACKNOWLEDGMENTS**

247 This research was financially supported by Iran National Science Foundation (project number  
248 91003418).



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355

356 **Figure captions**

357 **Figure 1** The median lethal temperature (LT<sub>50</sub>) and supercooling point (SCP) (mean ± 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<sub>50</sub>.  
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 (2<sup>nd</sup> and 3<sup>rd</sup> instars) and large (4<sup>th</sup> and  
364 5<sup>th</sup> 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)













