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1 **Studies on chill coma recovery in the ladybird, *Harmonia axyridis*: ontogenetic profile,**  
2 **effect of repeated cold exposures, and capacity to predict winter survival**

3

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20

21 **Abstract**

22 The harlequin ladybird, *Harmonia axyridis*, is one of the most successful invasive insect  
23 species worldwide. We investigated whether (i) chill coma recovery time (CCRt) changes  
24 during the ontogenetic development of this species, (ii) CCRt varies in response to repeated  
25 cold shocks, and (iii) CCRt could be a good predictor of winter survival ability in adults.  
26 CCRt decreased during larval development, the lowest CCRt values were observed in teneral  
27 adults (one and four days old), and significantly higher values were observed for older adults  
28 (16 and 32 days old). Repeated cold shocks (two hours at -3 °C), interrupted by short (30  
29 minutes) warm periods (22 °C) resulted in decreased CCRt after the second cold shock,  
30 probably depicting an acclimation response, but then CCRt increased with additional cold  
31 shocks, likely revealing the accumulation of chill injuries. The CCRt of pre-overwintering  
32 individuals was not correlated with their winter survival. This indicates that CCRt is not a  
33 reliable measure of cold tolerance in *H. axyridis*. However, this result could be partially  
34 affected by the experimental setup – the use of laboratory-reared individuals who experienced  
35 standardized conditions and thus the variability in CCRt of tested animals could be much  
36 lower than the variability present in nature. The substantial variation observed over the  
37 ontogenetic development of *H. axyridis* poses important methodological implications for  
38 future studies, as animals of the same stage/age should be compared with each other. The  
39 observed U-shaped response to repeated cold shocks indicates that the expectation of linearity  
40 between cold exposure and insect response is an oversimplification of real situations.

41 **Highlights:**

- 42 - chill coma recovery time (CCRt) varies significantly during ladybird ontogeny
- 43 - repeated cold shocks result in a U-shaped CCRt response
- 44 - pre-overwintering CCRt was not related to winter survival in this species
- 45 - the relevance of CCRt as a measure of cold tolerance needs to be evaluated in other
- 46 species

47

48 **Key Words:**

49 Ageing, chill injury, cold tolerance, invasive species, ontogenetic stage, overwintering

## 50 **1. Introduction**

51 Because of their ectothermic nature and small body sizes, insects are particularly prone to  
52 rapid changes in their body temperature and have had to develop a wealth of behavioural and  
53 physiological responses to survive at low temperatures (Teets and Denlinger, 2013). As a  
54 result, thermal tolerance is a crucial physiological trait that is significantly related to the  
55 distribution of insect species on Earth (Araujo et al., 2013). A range of techniques are  
56 available for assessing the cold tolerance of insects; while some of them directly measure  
57 mortality (e.g., estimation of survival capacities, measurements of lower lethal limits), other  
58 techniques are only proxies of insect tolerance to the deleterious effects of low temperatures  
59 (e.g., supercooling point, chill coma onset and recovery; see Terblanche et al., 2011 and  
60 Sinclair et al., 2015 for a review).

61 Chill coma onset and recovery have been described as reliable metrics of thermal  
62 plasticity and tolerance in insects (Sinclair et al., 2015). Chill coma is initiated when  
63 neuromuscular function is disrupted as a result of the organism's inability to maintain proper  
64 ion balance due to depolarization of the resting membrane potential (MacMillan and Sinclair,  
65 2011; Overgaard and MacMillan, 2017). Entering into chill coma has substantial ecological  
66 consequences for an individual, as immobile insects are not able to acquire food or escape  
67 from predators. While the measurement of the onset of chill coma can be challenging in  
68 immature insects, the recovery from chill coma, i.e., the ability of a specimen to exhibit a pre-  
69 determined behaviour, can be easily measured in both juveniles and adults (see Sinclair et al.,  
70 2015 for a discussion of this point).

71 Chill coma recovery time (CCRt) is expected to differ greatly among and within insect  
72 species and particularly among populations of different geographical origin (Andersen et al.,  
73 2015; Bowler and Terblanche, 2008; Chown, 2001). Within-population CCRt variations are

74 also frequently observed, as developmental plasticity (i.e., the environmental conditions  
75 experienced during preimaginal development partly determine adult stress tolerance) or  
76 individual acclimation (i.e., exposure to mild stress enhances individual stress tolerance;  
77 Colinet and Hoffmann, 2012; Terblanche and Chown, 2006) can affect this measure.  
78 Moreover, thermal tolerance can substantially vary over the course of an insect's life, but  
79 ontogenetic variations in the responses to low temperatures have been poorly examined  
80 compared to heat resistance (see Bowler and Terblanche, 2008). The existing studies have  
81 focused on preimaginal development or adult ageing (Colinet et al., 2013; David et al., 1998;  
82 Halle et al., 2015; Pujol-Lereis et al., 2014), while studies that included measures on both  
83 preimaginal and adult stages are commonly focused on a few developmental stages and age  
84 classes (e.g., Arias et al., 2011, but see Jensen et al., 2007 on *Drosophila melanogaster*).  
85 Differences between individuals in their cold tolerance are expected to have consequences for  
86 their overwintering success (Denlinger and Lee, 2010), but this prediction has only rarely  
87 been verified experimentally (e.g., Alford et al., 2014; Kostal et al., 2011).

88         The majority of studies investigating insect cold hardiness focus on the effects of a  
89 single exposure to low temperatures (Marshall and Sinclair, 2012). However, in the field,  
90 insects can be exposed to knockdown temperatures on multiple occasions over days, and these  
91 repeated exposures could affect insect performance in the following ways: 1) cold hardiness  
92 will progressively increase across successive exposures, depicting thermal acclimation; 2)  
93 cold hardiness will progressively decrease across successive exposures, revealing an  
94 accumulation of chill injuries; 3) cold hardiness will exhibit a complex pattern of a U-shaped  
95 cold hardiness response curve as a result of acclimation of the insect during the first sequence  
96 of the exposure to the cold conditions, followed by an accumulation phase of chill injuries  
97 (Colinet et al., 2016; Marshall and Sinclair, 2012, 2015).

98           In this study, we present the results of a pilot study on the thermal biology of the  
99 harlequin ladybird, *Harmonia axyridis* (Pallas, 1773). This species is currently considered one  
100 of the most invasive insects worldwide (Brown et al., 2011; Lombaert et al., 2011), with  
101 probable specific abilities to colonize cold environments (Barahona-Segovia et al. 2016). *H.*  
102 *axyridis* overwinters as an adult and creates large winter aggregations, frequently in human  
103 settlements (Berkvens et al., 2010). Previous studies measured supercooling points and lower  
104 lethal temperatures in adult *H. axyridis* originating from European and US invasive  
105 populations (Berkvens et al., 2010; Koch et al., 2004) and from Japanese native populations  
106 (Watanabe, 2002). To our knowledge, patterns of CCRt variation have not been investigated  
107 in this species thus far. We addressed three different questions using a single metric – CCRt:  
108 1) we examined the influence of ontogeny on CCRt and hypothesized that CCRt would  
109 decrease from the 1<sup>st</sup> to 4<sup>th</sup> larval instar because of the increasing probability of exposure to  
110 low temperatures in nature (late larval instars can experience colder autumn weather in  
111 Central Europe); 2) we investigated the effect of repeated cold exposures on CCRt in adult  
112 ladybirds and expected CCRt prolongation because of the accumulation of chill injuries  
113 during exposures to cold and insufficient damage repair during very short warm periods  
114 among subsequent cold exposures; 3) finally, we were interested in the predictive power of  
115 pre-overwintering CCRt for winter survival and expected that individuals achieving lower  
116 pre-overwintering CCRt would suffer from lower winter mortality. The ecophysiological  
117 meaning of CCRt clearly differs for summer active (questions 1 and 2) and winter diapausing  
118 insects (question 3). For summer active insects we employed CCRt as a measure that allowed  
119 us to investigate physiological changes during the ontogenetic / physiological responses to  
120 repeated cold shocks. For winter diapausing insects we tested the ecological relevance of  
121 CCRt as a proxy of insect cold tolerance.

122

## 123 2. Materials and Methods

### 124 2.1. Insect collection and rearing

125 The parental individuals used in study were collected August 2015 from shrubs and lime trees  
126 in the university campus of the Czech University of Life Sciences Prague, Czech Republic  
127 (GPS: 50°8' N, 14°21' E; 300 m a.s.l.). After transportation to the laboratory, the beetles were  
128 sexed, and parental pairs were formed. Each couple was placed in a separate Petri dish (9 cm  
129 in diameter) containing crumpled filter paper strips, which provided a suitable substrate for  
130 egg laying. New egg clutches were placed into Petri dishes and exposed to a standardized  
131 laboratory condition (16L:8D photoperiod, 70 % humidity, 26 °C). A temperature of 26 °C is  
132 the optimal rearing temperature for *H. axyridis* (Hodek et al., 2012; Knapp and Nėdved,  
133 2013). Newly hatched larvae were fed *ad libitum* with eggs of *Ephestia kuehniella* (Zeller,  
134 1879) (Lepidoptera: Pyralidae) and provided with water in cotton wool.

135

### 136 2.2. Chill coma induction and chill coma recovery time measurements

137 For all beetles investigated in the following experiments (sections 2.3., 2.4. and 2.5.), chill  
138 coma was induced and chill coma recovery time (CCRT) was measured in a standardized way.  
139 Pools of six individuals were placed individually into clean Petri dishes and transferred to -3  
140 °C for two hours. A freezing climatic chamber made on order by AVIKO-PRAHA a.s.  
141 company was employed for this purpose. The temperature inside the climatic chamber was  
142 monitored with a datalogger Tinytag PLUS2 (Supporting Information file Fig. A.1). After 2  
143 hours at -3 °C, the Petri dishes were returned to room temperature ( $22 \pm 1$  °C) in an air-  
144 conditioned laboratory and the beetles were turned onto their dorsum. The duration necessary  
145 for observing the first movement of an appendage (most often a leg or a palpus) was  
146 measured. As ladybirds can often “play dead” (remain motionless despite being already able



147 to move), individuals were stimulated by gentle teasing with an entomological pin every five  
148 seconds.

149

### 150 *2.3. Experiment 1: Effects of ontogeny on chill coma recovery time*

151 Three fecund parental pairs were selected at random, and their offspring were used in the  
152 laboratory experiment investigating the ontogenetic development of cold resistance in *H.*  
153 *axyridis*. Egg clutches, hatched larvae and 2<sup>nd</sup> generation adults were housed under the same  
154 rearing conditions as the parental pairs (16L:8D, 26 °C, 70 % humidity, food and water *ad*  
155 *libitum*). Following egg laying, egg clutches were randomly assigned to one of the following  
156 eight treatments to assess the effects of ontogenetic stages on CCRt: 1<sup>st</sup> larval instar, 2<sup>nd</sup> larval  
157 instar, 3<sup>rd</sup> larval instar, 4<sup>th</sup> larval instar, newly hatched adult (one-day old), four-day-old adult,  
158 16-day-old adult and 32-day-old adult. CCRt was measured only once for each individual (not  
159 repeatedly in the course of the experiment). Ladybirds are long-lived insects, with adult  
160 longevity exceeding a year; therefore, senescence is expected to take place far later than at the  
161 age of 32 days (Hodek et al., 2012). The first and second instar larvae were reared together (a  
162 complete clutch) in one Petri dish. When the larvae moulted to the third instar, they were  
163 assigned to new Petri dishes in groups of four to five individuals. The CCRt of six larvae per  
164 parental pair was measured per treatment, and the CCRt of five individuals per sex per  
165 parental pair per treatment was recorded for adult treatments.

166

### 167 *2.4. Experiment 2: Effects of repeated cold shock on the chill coma recovery time of adults*

168 Twelve males and twelve females aged 14 to 20 days obtained from the same parental pairs as  
169 those individuals from the previous experiment were reared at a constant 26 °C, photoperiod

170 16L:8D, 70 % humidity and with food and water *ad libitum*. One day before the CCRt  
171 measurements, the beetles were individually transferred to Petri dishes and were provided  
172 with water only (this short food deprivation period was used to reduce the possible confusing  
173 effects of variable stomach contents across repeated measurements). Repeated exposures to  
174 knockdown temperatures were achieved as follows: each ladybird was transferred to -3.0 °C  
175 for two hours, then returned to room temperature ( $22 \pm 1$  °C) for 30 minutes before being  
176 transferred again to -3.0 °C. This cyclic procedure was repeated four times (i.e., four  
177 exposures of two hours at -3.0 °C), and the CCRt was repeatedly measured for each  
178 individual after each exposure to the knockdown temperature (four CCRt measurements for  
179 each individual).

180

### 181 2.5. Experiment 3: Chill coma recovery time and winter survival of adults

182 Five males and five females per parental pair were selected at random from 10 parental pairs  
183 (i.e., 100 ladybirds in total). Ladybird offspring were reared at 26 °C (photoperiod 16L:8D, 70  
184 % humidity) and supplied with food (*Ephestia kuehniella* eggs) and water *ad libitum*. On  
185 October 7<sup>th</sup>, when the ladybirds were 15-25-day-old adults, the beetles were transferred to an  
186 18 °C and 12L:12D photoperiod regime to initiate winter diapause. The insects were  
187 maintained individually in Petri dishes and supplied with water but without food (as they  
188 naturally stop feeding during winter diapause). On October 14<sup>th</sup>, the CCRt was measured (as  
189 described above) for each individual. One day later, the thermal regime was changed to  
190 fluctuating temperatures and a short photoperiod [8L(12 °C):16D(6 °C)] to mimic outdoor  
191 conditions in late autumn. Six days later, the overwintering phase started and the beetles, still  
192 maintained in the climatic chamber, were exposed to the mean temperature regime  
193 experienced in outdoor shelters in Prague (data were computed as the long-term mean

194 temperature 1 cm below the soil surface; hourly means from the meteorological station  
195 situated in the Crop Research Institute, Praha-Ruzyně were used). The beetles were  
196 maintained in clear Petri dishes containing a small watered piece of cotton wool to limit  
197 desiccation and placed into the climatic chamber set to the overwintering regime. The climatic  
198 chamber was adjusted each hour with a new temperature value (the temperatures applied are  
199 attached as Supporting Information file Table A.1). The mean temperature during the  
200 overwintering phase was 0.7 °C, and the minimum temperature reached was -4.1 °C.

201 In the course of the overwintering experiment, the beetles were checked at monthly  
202 intervals (November 16<sup>th</sup>, December 15<sup>th</sup>, January 14<sup>th</sup>, February 17<sup>th</sup> and March 17<sup>th</sup>, 2016 –  
203 when the overwintering experiment was terminated). During all inspections, the Petri dishes  
204 with beetles were moved to outdoor conditions to minimize unwanted warming of the  
205 experimental beetles, the survival of each individual was recorded, and a new piece of  
206 watered cotton wool was inserted into each Petri dish (the old one was removed). Live  
207 diapausing ladybirds are commonly tightly attached to the substrate, whereas dead individuals  
208 have dropped off and are lying freely on their dorsum. Petri dishes with dead individuals were  
209 not removed from the experiment as there was a little risk of false death records (an animal  
210 seeming to be dead but is still alive); however, no false records were revealed.

211

## 212 *2.6. Statistical analyses*

213 To analyse ontogenetic changes in the CCRt, a linear mixed-effects model (LME) was run  
214 using the “lme” function implemented in the “nlme” package (Pinheiro et al., 2013) for R (R  
215 Development Core Team, 2016). Parental pair identity was used as a random effect in our  
216 analysis. Ontogenetic stage was used as a fixed effect in our model. Tukey’s HSD test was  
217 employed to test for significant differences between particular ontogenetic stages using the

218 “glht” function as implemented in the “multcomp” package (Hothorn et al., 2008). Raw data  
219 serving as inputs for the abovementioned analysis are available in the Supporting Information  
220 file (Table A.2).

221 To analyse the effects of repeated cold exposures on the CCRt, a linear mixed-effects  
222 model (LME) was conducted using the “lme” function implemented in the “nlme” package  
223 (Pinheiro et al., 2013) for R (R Development Core Team, 2016). Beetle identity was used as a  
224 random effect in our analysis. The order of cold exposure (from 1 to 4; a continuous variable)  
225 was used as a fixed effect in our model, while a quadratic term was also included in the model  
226 for the case where the response would not be purely linear. The significance of the  
227 independent variables (order, order<sup>2</sup>) was tested using F-tests (“anova.lme” function). To test  
228 for the real necessity of both terms (order, order<sup>2</sup>) in the final model, the most parsimonious  
229 model based on AIC-values was also selected (Akaike, 1974). Raw data are available in the  
230 Supporting Information file (Table A.3).

231 To analyse the effects of CCRt on *H. axyridis* survival during winter, the Cox  
232 proportional-hazards model (Cox-PH) was run using the “coxph” function implemented in the  
233 “survival” package (Therneau, 2015) for R (R Development Core Team, 2016). Winter  
234 survival in months (one to five months) was used as the response variable, and CCRt was  
235 used as an independent variable in the model. Beetles that survived until the end of the  
236 experiment (March) were excluded in the model, as their future longevity was unknown. Raw  
237 data serving as inputs for the above analysis are available in the Supporting Information file  
238 (Table A.4).

239 The effects of sex on the CCRt were analysed in the preliminary analyses performed  
240 for all experiments. As there were no significant effects of sex in any model, this term was not  
241 included in the final models.

242

### 243 3. Results

244 There were significant changes in chill coma recovery time (CCRT) during the ontogenetic  
245 development of the harlequin ladybird, *Harmonia axyridis* (LME:  $F = 19.92$ ,  $P < 0.001$ ). The  
246 CCRT decreased by ca. 22 % in the course of early larval development from the 1<sup>st</sup> to 2<sup>nd</sup>  
247 larval instars and reached the lowest value in teneral adults (between one and four days old  
248 adults). The CCRT of older adults (16 or 32 days old adults) was increased and did not differ  
249 from the CCRT of the 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> larval instars (Fig. 1).

250 Repeated two-hour exposures to sub-zero temperatures interrupted by 30-minute  
251 recovery periods at 22 °C resulted in increasing the CCRT from the second cold exposure  
252 onwards in *H. axyridis*. However, the CCRT decreased from the first to the second cold  
253 exposure; thus, alongside the significant linear term (LME:  $F = 23.92$ ,  $P < 0.001$ ), there was  
254 also a significant quadratic term in the final statistical model ( $F = 11.24$ ,  $P = 0.001$ ). The most  
255 parsimonious model included both terms (order and order<sup>2</sup>) according to the AIC (Table 1),  
256 demonstrating that a U-shaped response to repeated cold exposures was measured in *H.*  
257 *axyridis* (Fig. 2).

258 No significant relationship was observed between the CCRT of the pre-overwintering  
259 individuals and their ability to survive during the winter (Cox-PH:  $z = -1.50$ ,  $P = 0.13$ ). The  
260 observed trend was even opposite that of the hypothesized one: the four individuals who had  
261 the lowest CCRT died during the first months of the winter, whereas the three individuals with  
262 the highest CCRT survived until the last month of the winter (two of them even survived the  
263 whole winter; Fig. 3).

264

#### 265 4. Discussion

266 In this work, we used CCRt to examine different aspects of ladybird thermal physiology: we  
267 1) investigated the physiological changes during ontogeny, 2) examined the physiological  
268 responses of summer active ladybirds to repeated cold shocks, and 3) investigated the  
269 relevancy of the CCRt as a proxy for winter cold hardiness. Significant variations of the CCRt  
270 were measured across the ontogeny of *Harmonia axyridis*, with the lowest CCRt values  
271 observed in teneral adults. Repeated cold shocks altered the CCRt of *H. axyridis*, and a U-  
272 shaped response was measured, with reduced CCRt during the first phase, followed by a  
273 CCRt increase when the number of cold shocks was increased. Finally, we found no  
274 relationship between the CCRt of pre-overwintering individuals and their subsequent ability  
275 to survive during the winter months.

276 Changes in the degree of heat tolerance during ontogeny are widespread in insects, but  
277 adjustments to cold resistance during ontogeny have been less examined (e.g., Bowler and  
278 Terblanche, 2008; Klockmann et al., 2017; Knapp and Nēdved, 2013; Zhao et al., 2017).  
279 Relevant experimental data that reported ontogenic variations of insect cold resistance exist  
280 mostly for insect model species (e.g., Arias et al., 2011; Colinet et al., 2013; Jensen et al.,  
281 2007; Pujol-Lereis et al., 2014; for the review of older studies see Bowler and Terblanche,  
282 2008). In univoltine holometabolous species, differences in coping with cold exposures  
283 among stages could reflect adaptive responses, as the development stages can experience  
284 different thermal conditions, with the overwintering stage being most often the most cold-  
285 resistant one (Denlinger and Lee, 2010). Nevertheless, adjustments of insect cold hardiness  
286 are not only dictated by environmental temperatures, as differences have also been reported  
287 during the ontogeny of multivoltine species over the course of the growth season (e.g., Jensen  
288 et al., 2007). One of possible explanations is that levels of heat-shock proteins (HSPs), which  
289 are partially responsible for the actual cold resistance and are also involved in various stress

290 responses unrelated to cold challenge, vary substantially in the course of insect ontogeny  
291 (Colinet et al., 2013), including in the harlequin ladybird (Wang et al., 2017).

292           There is no unified ontogenetic profile of cold resistance observed for a wide range of  
293 insect species. In our study, CCRt was significantly higher in the 1<sup>st</sup> larval instar compared  
294 with later larval instars. Interestingly, Jensen et al. (2007) observed a distinct pattern of lower  
295 lethal temperature causing 50 % mortality (LT<sub>50</sub>) in *Drosophila melanogaster*, which slightly  
296 increased from the 2<sup>nd</sup> to the 3<sup>rd</sup> larval instar. We measured the lowest CCRt value in teneral  
297 ladybird adults, which is consistent with the finding of Jensen et al., (2007) for *D.*  
298 *melanogaster*, but opposite the pattern reported for *Tenebrio molitor* (Arias et al., 2011).  
299 Differences in the changes of ontogenetic patterns of CCRts across species may result from  
300 the distinct life histories of the studied species, particularly in terms of overwintering  
301 strategies. Importantly, the contrasting cold resistance patterns among studies can also be  
302 caused by the various proxies that are employed, as these measures are not necessarily  
303 correlated (Andersen et al., 2015).

304           The higher cold resistance of teneral adults (few hours/days old) of *D. melanogaster*  
305 reported by Colinet et al. (2013) was also found in our study for *H. axyridis*. The cold  
306 resistance of adult ladybirds subsequently decreased with increasing age, a pattern also  
307 observed for *D. melanogaster* and *Ceratitis capitata* (David et al., 1998; Pujol-Lereis et al.,  
308 2014). Decreased cold hardiness with age in adult insects was thought to be related to  
309 senescence-associated processes (Bowler and Terblanche, 2008; Halle et al., 2015). However,  
310 this hypothesis is unlikely for our 32-day-old *H. axyridis* whose longevity can easily reach  
311 one year; senescence is thus expected to take place later in their lives (Hodek et al., 2012; note  
312 also that the CCRt slightly decreased from the age of 16 days to 32 days in our study).  
313 Moreover, increasing cold resistance in the course of senescence was observed for the sub-  
314 Antarctic fly *Anatalanta aptera* (Lalouette et al., 2010). Altogether, our results have important

315 methodological implications for future studies on insect cold hardiness, as the exact age of  
316 experimental animals should be precisely stated, and animals of the same stage/age should be  
317 compared with each other in studies investigating issues other than the effects of ontogeny.

318         Low temperatures cause chill injuries to chill-susceptible insects, and the extent of the  
319 injuries depends on the severity of the treatment, i.e., the severity of the temperatures  
320 experienced and the duration of exposure (Marshall and Sinclair, 2015; Overgaard and  
321 MacMillan, 2017). Despite the fact that thermal fluctuations are ubiquitous in nature, the  
322 majority of ecophysiological studies are focused on exposures of insects to a single period at  
323 low temperature (Marshall and Sinclair, 2012; see the literature focusing on fluctuating  
324 thermal regimes and repeated cold exposures, e.g., Colinet et al., 2016; Marshall and Sinclair,  
325 2015). In this study, we observed the changes in CCRt values in *H. axyridis* subjected to  
326 repeated cold shocks and found that values decreased in the first stage followed by a  
327 continuous increase from the second exposure onwards. The observed U-shaped response  
328 curve depicted the interplay between acclimation (rapid cold-hardening response; Lee et al.,  
329 1987) elicited by the first cold exposure and the accumulation of chill injuries (e.g., the  
330 creation of reactive oxygen species; Lalouette et al., 2011) inflicted by each successive cold  
331 shock. The short warm periods may not have been sufficient for restoring the aerobic  
332 metabolism of the insects and completely repairing the physiological injuries caused by  
333 exposures to low temperatures (Colinet et al., 2016). A few hours may have been needed for a  
334 complete recovery of the injuries accumulated by cold-shocked ladybirds. For example, five  
335 cold exposures separated by recovery periods of 24 hours lowered CCRt values in the red  
336 flour beetle (Scharf et al., 2018). The physiological responses of insects to repeated cold stress  
337 are still not known in detail and it is possible that rapid switching between stress and injury  
338 repair states itself poses some physiological costs (Marshall and Sinclair, 2015).



339           We failed to report any significant relationship between the CCRt of pre-  
340 overwintering specimens of *H. axyridis* and their subsequent overwintering success. This  
341 finding is surprising as between- and within-species variations in CCRt commonly exist in  
342 insects (Chown, 2001). This variation seems to be adaptive as species' CCRt generally  
343 correlates to their distribution ranges (winter conditions experienced; Andersen et al., 2015;  
344 Chown, 2001), and overwintering individuals often have a significantly lower CCRt  
345 compared with their relatives sampled during the breeding season (Chown, 2001). Our data  
346 even showed slight support for the opposite pattern, i.e., individuals with the lowest CCRt  
347 died in early winter while those with the highest CCRt survived. A possible explanation is a  
348 higher metabolic rate in animals with lower CCRt (Williams et al., 2016). Animals with  
349 higher energy demands can die during overwintering due to the depletion of their energy  
350 reserves.

351           A possible shortcoming of our study is that overwintering success was investigated for  
352 laboratory-reared insects that experienced standardized environmental conditions. Moreover,  
353 their genetic variability may have been limited (all were offspring of 10 parental pairs). This  
354 experimental setting may have reduced the differences in CCRt values among individuals  
355 compared with the variation existing for individuals in the field. As laboratory rearing could  
356 affect ladybird overwintering performance (Berkvens et al., 2010), we encourage a repetition  
357 of this experiment using pre-overwintering individuals collected directly in the field. As  
358 winter survival in insects is a result of complex interactions between individual physiological  
359 traits and environmental conditions, future research investigating the effects of individual cold  
360 resistance (represented by various measures/proxies) on winter survival under various  
361 overwintering conditions for various insect species is needed to disentangle the effects of  
362 individual traits on winter survival in insects. If additional studies confirm that CCRt is not  
363 the most reliable predictor of insect overwintering success, chill coma temperatures or lower

364 lethal temperatures, both good predictors of the latitudinal distribution of *Drosophila* species,  
365 (Andersen et al., 2015) might be used as better indicators of overwintering success.

366

## 367 **5. Conclusions**

368 This study provides the first measurements of chill coma recovery time (CCRt) in the invasive  
369 harlequin ladybird, *Harmonia axyridis*. We showed substantial changes in CCRt in the course  
370 of the ontogenetic development of *H. axyridis*. The significant ontogenetic signal in *H.*  
371 *axyridis* cold hardiness poses important methodological implications for future studies, as  
372 animals of the same stage/age should be compared with each other. We also showed that  
373 repeated exposures to cold shocks interrupted by short warm periods caused a U-shaped cold  
374 resistance response. Individual CCRt was a bad predictor of *H. axyridis* overwintering  
375 success. Together with the limited evidence in the existing literature, our results indicated that  
376 CCRt is not a reliable measure of insect overwinter success. Future studies investigating the  
377 relationship between prewintering CCRt and following winter survival for various insect  
378 species are needed to extend the generality of this pattern.

379

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386

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389

390 **Appendices:**

391 The following additional supporting information may be found in the online version of this  
392 article.

393 **Table A.1 – Temperature profile experienced by ladybirds during overwintering in**  
394 **computer-instructed freezing climatic chambers.**

395 **Table A.2 – Raw data analysed in experiment 1 (Ontogeny).**

396 **Table A.3 – Raw data analysed in experiment 2 (Repeated cold shock).**

397 **Table A.4 – Raw data analysed in experiment 3 (Winter survival).**

398 **Fig. A.1 – Temperature record from a datalogger placed between the Petri dishes with**  
399 **ladybirds during chill coma induction.**

400 **Tables**

401 **Table 1. Effect of repeated cold shocks on chill coma recovery time – model selection.**

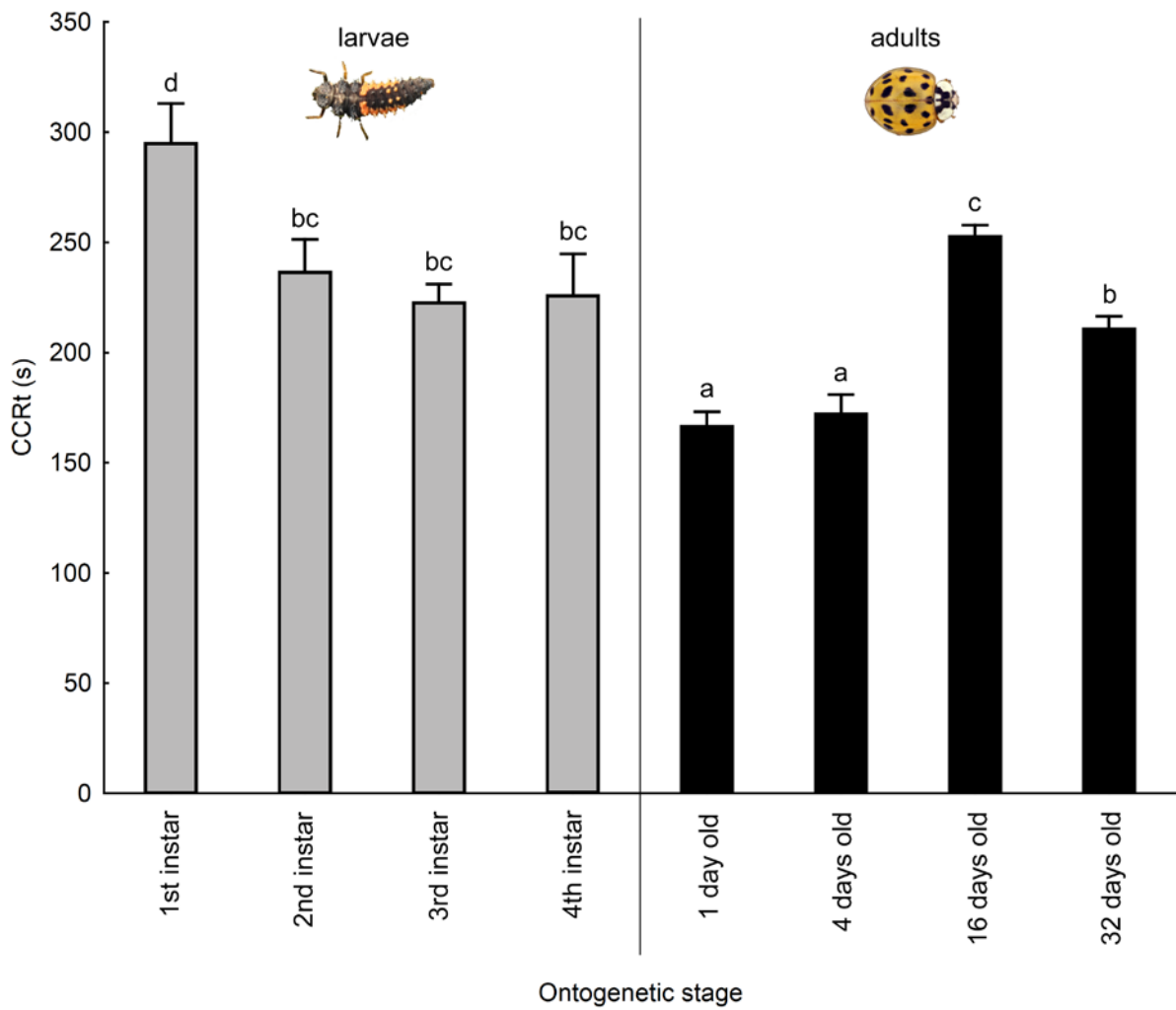
402 The most parsimonious linear mixed effect model (the first line) was selected on the basis of  
 403 the Akaike information criterion (AIC). The term “order” indicates the linear effects of the  
 404 cold shock order on chill coma recovery time; the term “order<sup>2</sup>” indicates the quadratic (non-  
 405 linear) effect of the cold shock order on the chill coma recovery time. A difference in the  
 406 AIC-value ( $\Delta$  AIC) > 2 indicates a significant difference between models, i.e., the model with  
 407 lower AIC-value is significantly more parsimonious.

<b>Model structure</b>			
<b>Independent variable</b>	<b>Random effect</b>	<b>AIC-value</b>	<b><math>\Delta</math> AIC</b>
~ order + order <sup>2</sup>	individual beetle	826.303	0.000
~ order <sup>2</sup>	individual beetle	836.124	9.821
~ order	individual beetle	837.654	11.352

408

409 **Figures**

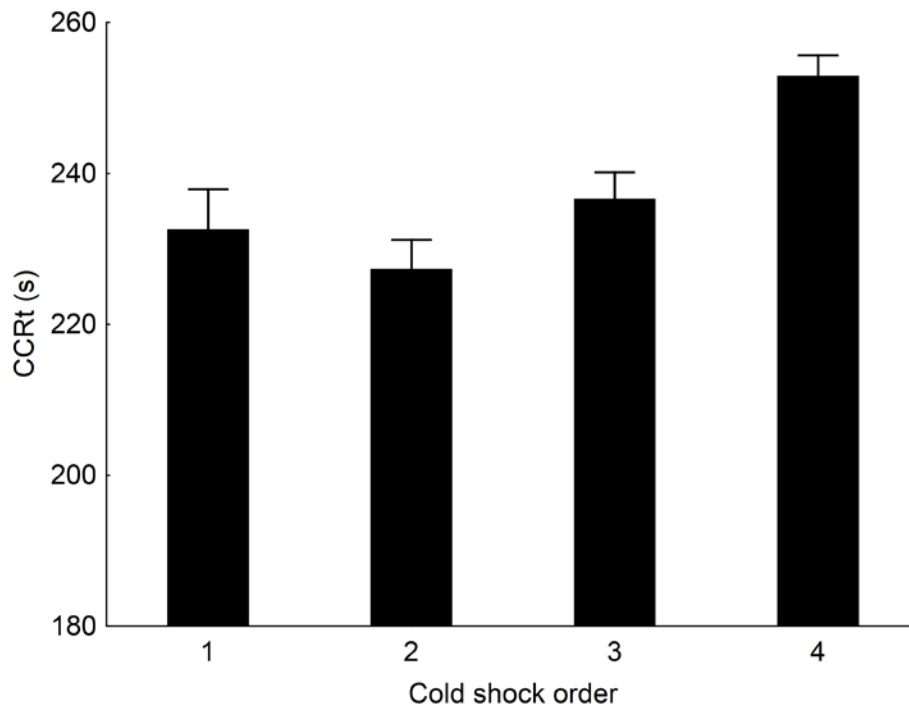
410 **Fig. 1. Effect of ontogenetic stage on cold resistance of *Harmonia axyridis*.** The mean chill  
411 coma recovery time (CCRT) values + SEM based on 18 larvae measured per instar (grey bars)  
412 and 30 adults (15 males and 15 females) measured per adult age class (black bars) are shown.  
413 Significant differences (Tukey post hoc test:  $P < 0.05$ ) in CCRT among particular ontogenetic  
414 stages are indicated by unshared letters above the bars.



415

416

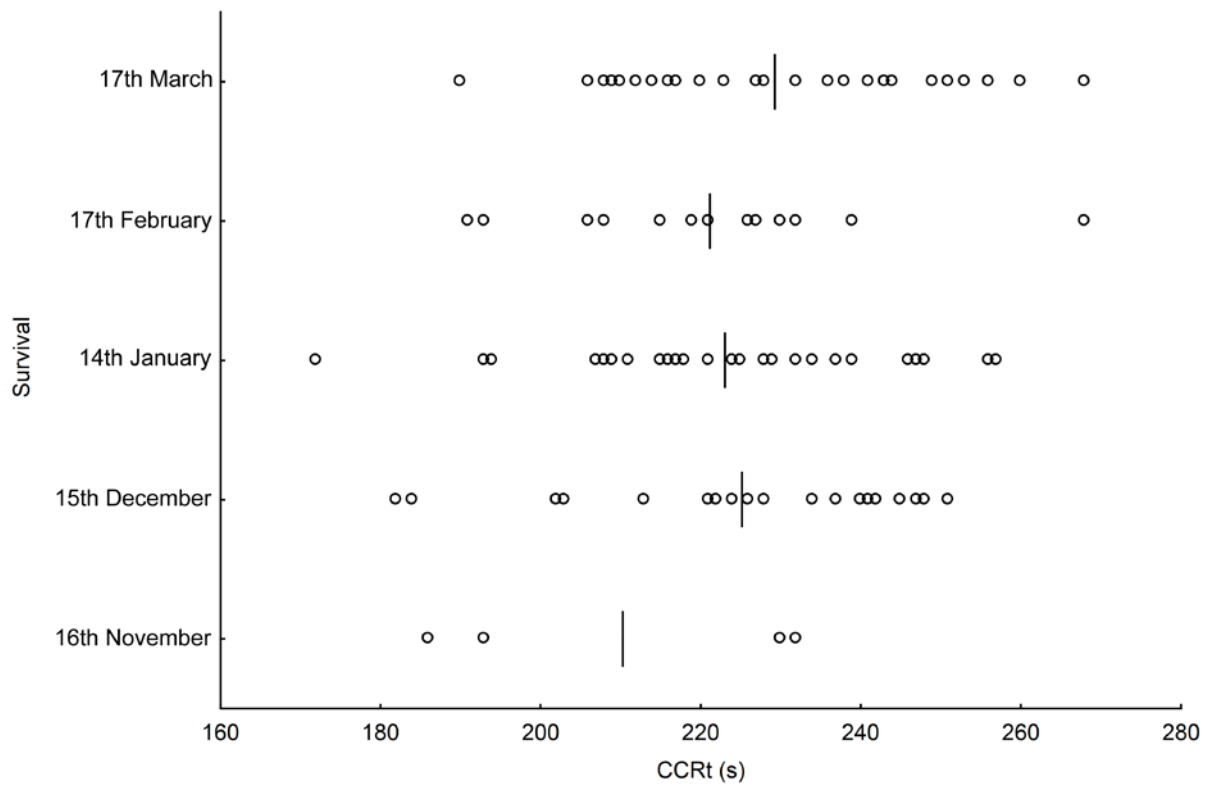
417 **Fig. 2. Effect of repeated cold shocks on chill coma recovery time in adult *Harmonia***  
418 ***axyridis*.** Mean chill coma recovery time (CCRT) values + SEM are shown. Each ladybird (24  
419 in total) was exposed to four successive cold shocks (two hours at -3 °C) interrupted by short  
420 warm periods (30 minutes at 22 °C). The CCRT was measured for each individual after each  
421 cold shock.



422

423

424 **Fig. 3. Relationships between individual pre-overwintering chill coma recovery time and**  
 425 **subsequent winter survival.** Dates on the vertical axis indicate control dates, i.e., the dates  
 426 on which a given beetle was seen alive for the last time. Survival until March 17<sup>th</sup> indicates  
 427 successfully completed overwintering. Short vertical lines indicate the mean pre-  
 428 overwintering chill coma recovery time (CCRt) achieved by individuals who were seen alive  
 429 for the last time during a given control.



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