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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 1st to 4th 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 ± 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 2nd 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: 1st larval instar, 2nd larval
157 instar, 3rd larval instar, 4th 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 ± 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 7th, 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 14th, 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 16th, December 15th, January 14th, February 17th and March 17th, 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, order²) was tested using F-tests (“anova.lme” function). To test
228 for the real necessity of both terms (order, order²) 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 1st to 2nd
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 2nd, 3rd and 4th 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 order²) 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 1st 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 (LT₅₀) in *Drosophila melanogaster*, which slightly
296 increased from the 2nd to the 3rd 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.**

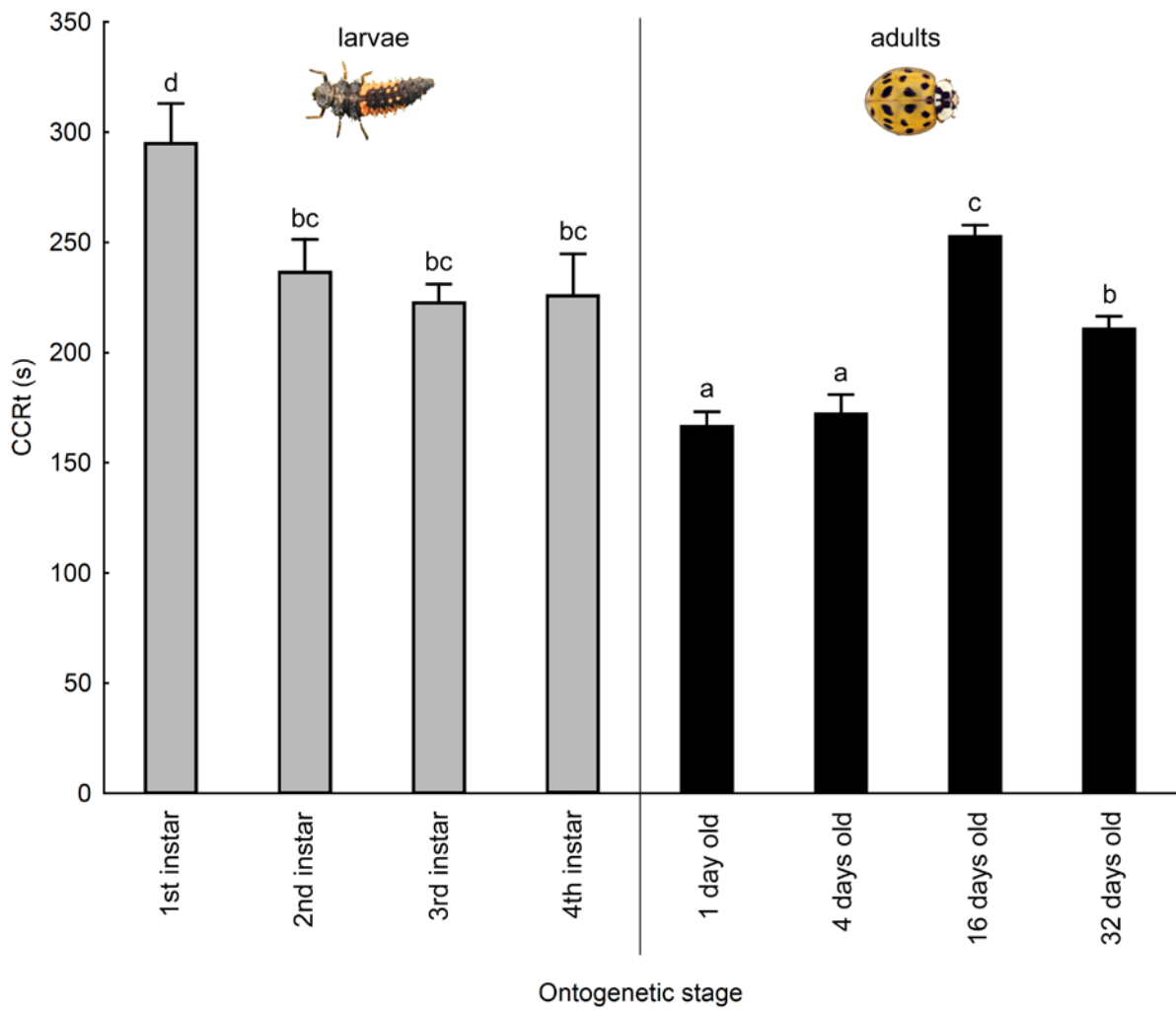
402 The most parsimonious linear mixed effect model (the first line) was selected on the basis of
 403 the Akaike information criterion (AIC). The term “order” indicates the linear effects of the
 404 cold shock order on chill coma recovery time; the term “order²” indicates the quadratic (non-
 405 linear) effect of the cold shock order on the chill coma recovery time. A difference in the
 406 AIC-value (Δ AIC) > 2 indicates a significant difference between models, i.e., the model with
 407 lower AIC-value is significantly more parsimonious.

Model structure			
Independent variable	Random effect	AIC-value	Δ AIC
~ order + order ²	individual beetle	826.303	0.000
~ order ²	individual beetle	836.124	9.821
~ order	individual beetle	837.654	11.352

408

409 **Figures**

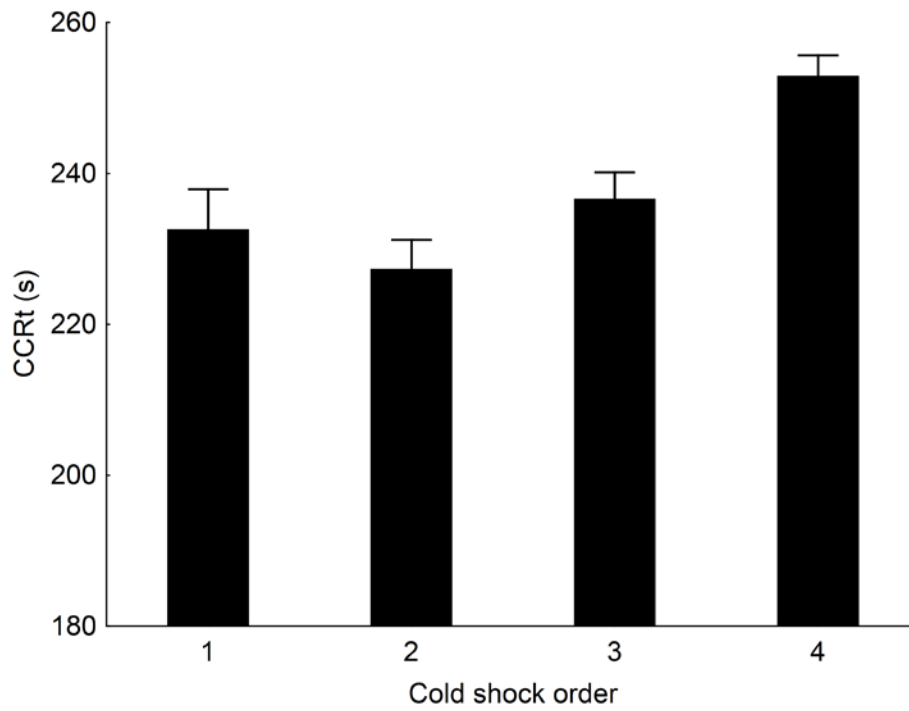
410 **Fig. 1. Effect of ontogenetic stage on cold resistance of *Harmonia axyridis*.** The mean chill
411 coma recovery time (CCRT) values + SEM based on 18 larvae measured per instar (grey bars)
412 and 30 adults (15 males and 15 females) measured per adult age class (black bars) are shown.
413 Significant 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

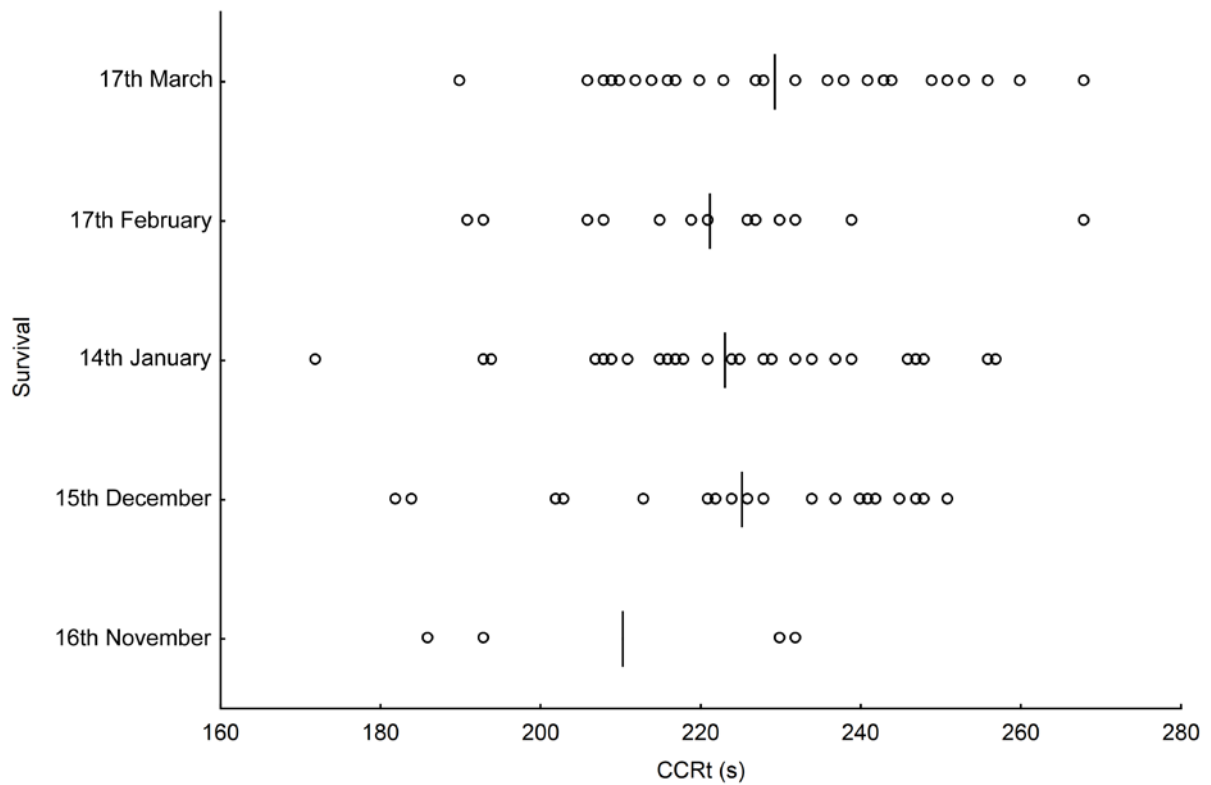
417 **Fig. 2. Effect of repeated cold shocks on chill coma recovery time in adult *Harmonia***
418 ***axyridis*.** Mean chill coma recovery time (CCRT) values + SEM are shown. Each ladybird (24
419 in total) was exposed to four successive cold shocks (two hours at -3 °C) interrupted by short
420 warm 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 17th 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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