Studies on chill coma recovery in the ladybird, Harmonia axyridis Ontogenetic profile, effect of repeated cold exposures, and capacity to predict winter survival

Michal Knapp, Philippe Vernon, D Renault

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

MICHAL KNAPP[1*], PHILIPPE VERNON[2], DAVID RENAULT[3,4]

[1] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Prague - Suchdol, 165 00, Czech Republic

[2] University of Rennes, CNRS, ECOBIO, UMR 6553, Station Biologique de Paimpont, 35380 Paimpont, France

[3] University of Rennes 1, UMR 6553 EcoBio CNRS, 263 Avenue du Gal Leclerc, 35000 Rennes Cedex, France

[4] Institut Universitaire de France, 1 Rue Descartes, 75231 Paris Cedex 05

* Correspondence:

Michal Knapp, Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Praha – Suchdol, 165 00, Czech Republic. Tel. +420 22438 3853 e-mail: knapp@fzp.czu.cz

Abstract

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

Highlights:

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

Key Words:

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

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2.1. Insect collection and rearing

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

2.2. Chill coma induction and chill coma recovery time measurements

For all beetles investigated in the following experiments (sections 2.3., 2.4. and 2.5.), chill coma was induced and chill coma recovery time (CCRt) was measured in a standardized way. Pools of six individuals were placed individually into clean Petri dishes and transferred to -3 °C for two hours. A freezing climatic chamber made on order by AVIKO-PRAHA a.s. company was employed for this purpose. The temperature inside the climatic chamber was monitored with a datalogger Tinytag PLUS2 (Supporting Information file Fig. A.1). After 2 hours at -3 °C, the Petri dishes were returned to room temperature (22 ± 1 °C) in an air-conditioned laboratory and the beetles were turned onto their dorsum. The duration necessary for observing the first movement of an appendage (most often a leg or a palpus) was 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

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

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

Five males and five females per parental pair were selected at random from 10 parental pairs (i.e., 100 ladybirds in total). Ladybird offspring were reared at 26 °C (photoperiod 16L:8D, 70 % humidity) and supplied with food (*Ephestia kuehniella* eggs) and water *ad libitum*. On October 7th, when the ladybirds were 15-25-day-old adults, the beetles were transferred to an 18 °C and 12L:12D photoperiod regime to initiate winter diapause. The insects were maintained individually in Petri dishes and supplied with water but without food (as they naturally stop feeding during winter diapause). On October 14th, the CCRt was measured (as described above) for each individual. One day later, the thermal regime was changed to fluctuating temperatures and a short photoperiod [8L(12 °C):16D(6 °C)] to mimic outdoor conditions in late autumn. Six days later, the overwintering phase started and the beetles, still maintained in the climatic chamber, were exposed to the mean temperature regime 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 17^{th,} 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

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

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

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

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

3. Results

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

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

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

4. Discussion

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

Changes in the degree of heat tolerance during ontogeny are widespread in insects, but adjustments to cold resistance during ontogeny have been less examined (e.g., Bowler and Terblanche, 2008; Klockmann et al., 2017; Knapp and Nědved, 2013; Zhao et al., 2017). Relevant experimental data that reported ontogenic variations of insect cold resistance exist mostly for insect model species (e.g., Arias et al., 2011; Colinet et al., 2013; Jensen et al., 2007; Pujol-Lereis et al., 2014; for the review of older studies see Bowler and Terblanche, 2008). In univoltine holometabolous species, differences in coping with cold exposures among stages could reflect adaptive responses, as the development stages can experience different thermal conditions, with the overwintering stage being most often the most cold-resistant one (Denlinger and Lee, 2010). Nevertheless, adjustments of insect cold hardiness are not only dictated by environmental temperatures, as differences have also been reported during the ontogeny of multivoltine species over the course of the growth season (e.g., Jensen et al., 2007). One of possible explanations is that levels of heat-shock proteins (HSPs), which 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 (LT_{50}) 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

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

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

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

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

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

5. Conclusions

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

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Appendices:

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

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

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

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

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

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

Tables

Table 1. Effect of repeated cold shocks on chill coma recovery time – model selection. The most parsimonious linear mixed effect model (the first line) was selected on the basis of the Akaike information criterion (AIC). The term "order" indicates the linear effects of the cold shock order on chill coma recovery time; the term "order^2" indicates the quadratic (non-linear) effect of the cold shock order on the chill coma recovery time**.** A difference in the AIC-value (Δ AIC) > 2 indicates a significant difference between models, i.e., the model with 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

Figures

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

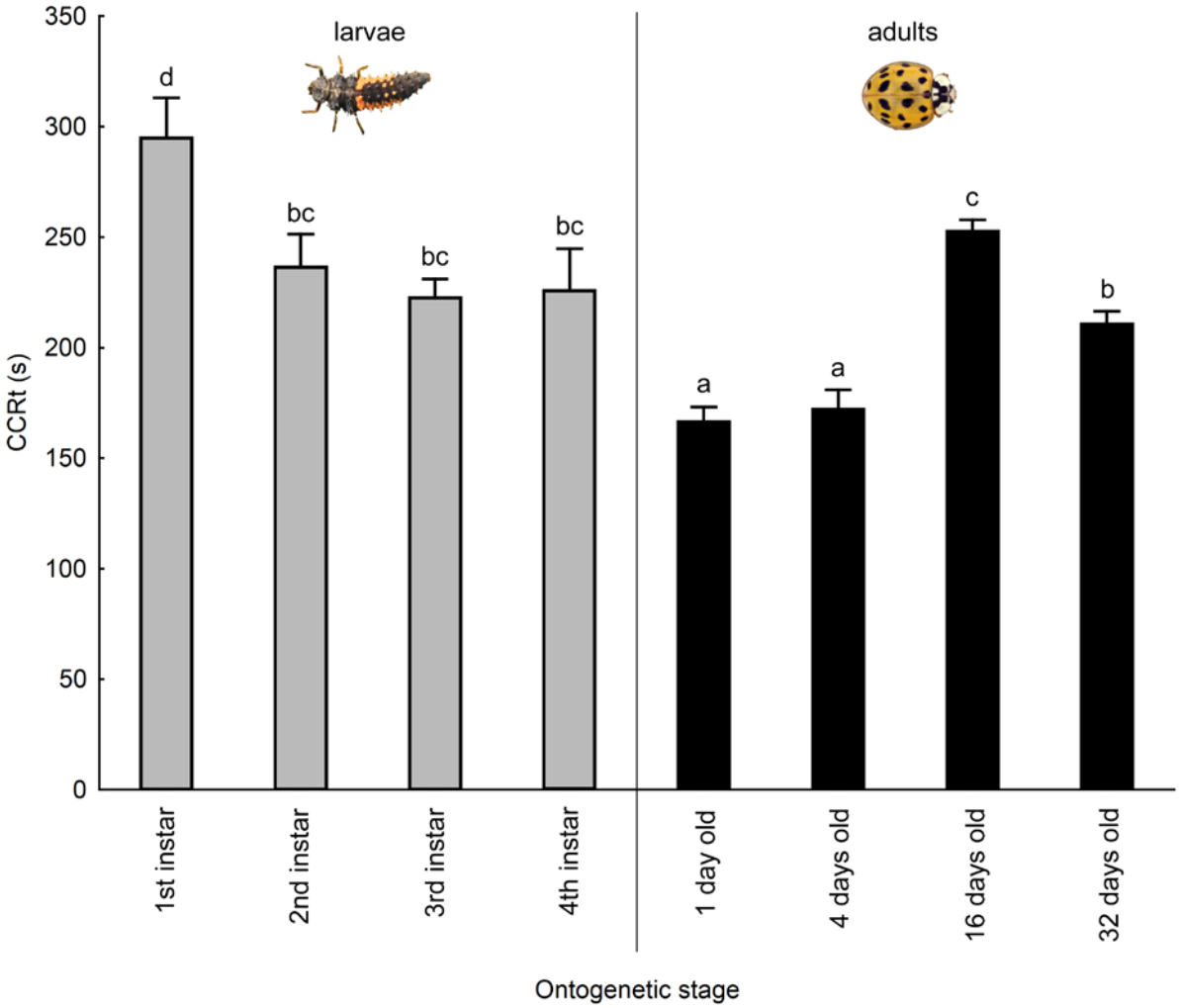


Fig. 2. Effect of repeated cold shocks on chill coma recovery time in adult *Harmonia axyridis*. Mean chill coma recovery time (CCRt) values + SEM are shown. Each ladybird (24 in total) was exposed to four successive cold shocks (two hours at -3 °C) interrupted by short warm periods (30 minutes at 22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

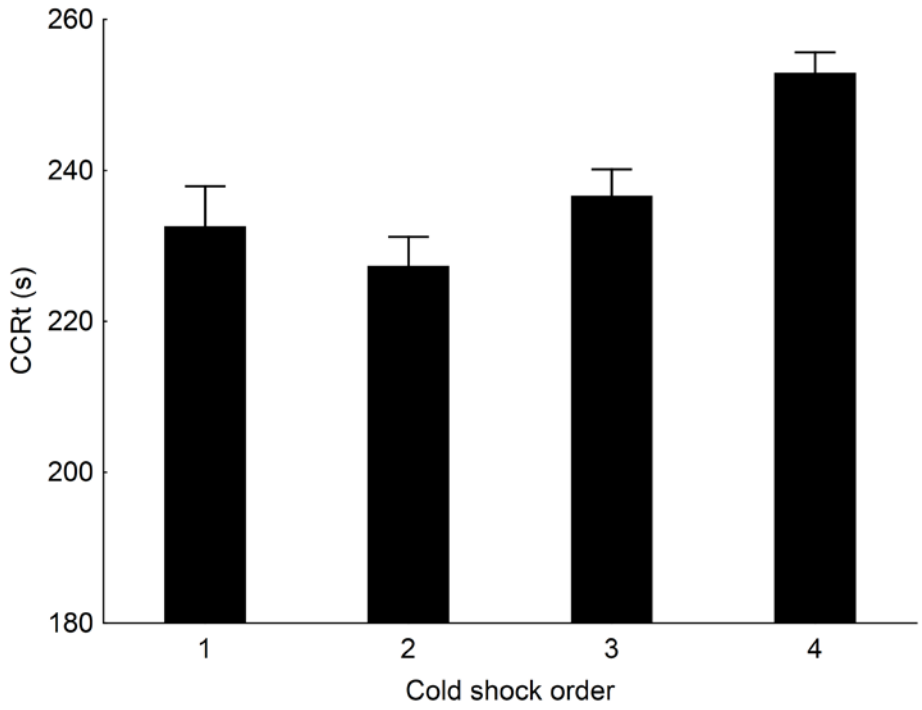
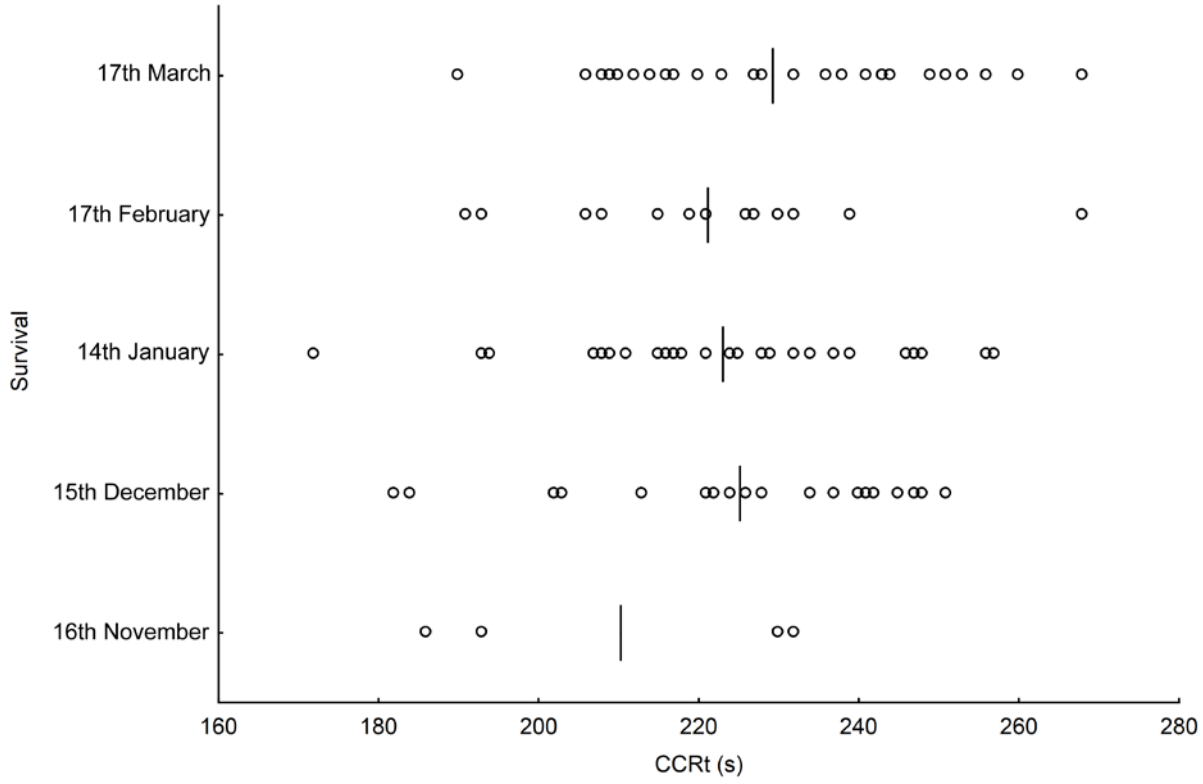


Fig. 3. Relationships between individual pre-overwintering chill coma recovery time and subsequent winter survival. Dates on the vertical axis indicate control dates, i.e., the dates on which a given beetle was seen alive for the last time. Survival until March 17th indicates successfully completed overwintering.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



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