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# Cold tolerance of the harlequin ladybird *Harmonia axyridis* in Europe

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ABSTRACT

As an essential aspect of its invasive character in Europe, this study examined the cold hardiness of the harlequin ladybird *Harmonia axyridis*. This was done for field-collected populations in Belgium overwintering either in an unheated indoor or an outdoor hibernaculum. The supercooling point, lower lethal temperature and lower lethal time at 0 and −5 °C were determined. Possible seasonal changes were taken into account by monitoring the populations during each winter month. The supercooling point and lower lethal temperature remained relatively constant for the overwintering populations in the outdoor hibernaculum, ranging from −17.5 to −16.5 °C and −17.1 to −16.3 °C, respectively. In contrast, the supercooling point and lower lethal temperature of the population overwintering indoors clearly increased as the winter progressed, from −18.5 to −13.2 °C and −16.7 to −14.1 °C, respectively. A proportion of the individuals overwintering indoors could thus encounter problems surviving the winter due to premature activation at times when food is not available. The lower lethal time of field populations at 0 and −5 °C varied from 18 to 24 weeks and from 12 to 22 weeks, respectively. Morph type and sex had no influence on the cold hardiness of the overwintering adults. In addition, all cold tolerance parameters differed greatly between the laboratory population and field populations, implying that cold tolerance research based solely on laboratory populations may not be representative of field situations. We conclude from this study that the strong cold hardiness of *H. axyridis* in Europe may enable the species to establish in large parts of the continent.



## 1. Introduction

The Paleartic harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), native to a large part of Asia, was introduced on several occasions during the last century into North America and Europe as a biological control agent of aphid and coccid pests and has successfully established in several areas of both continents (Koch, 2003; Coutanceau, 2006; Brown et al., 2008a,b; Koch and Galvan, 2008). There is growing concern that, as in North America (Koch, 2003; Koch and Galvan, 2008), this invasive species will rapidly expand its distribution over the European continent and pose serious risks for native biodiversity, ecosystem services and plant and human health (Adriaens et al., 2008; Brown et al., 2008a,b). For a species to establish in a temperate or colder climatic zone, one of the crucial factors is that sufficient individuals of the species are able to survive through any cold winter period occurring in that area (Bale, 1995). Overwintering strategies and cold hardiness enable such invasive insect species in temperate areas to survive in sufficient numbers to allow rapid population build up and reinvasion of the local environment in high densities after each winter (Labrie et al., 2008).

A strategy used by *H. axyridis* to survive through cold winters is to move to concealed and sheltered locations where the individuals aggregate and create a protective microclimate in which the insects experience less extreme temperatures than in the surrounding area. In its natural range, adults of *H. axyridis* overwinter in cracks and crevices of rocks, concrete buildings, caves or sometimes in leaf litter (Obata, 1986; Sakurai et al., 1993). In invaded areas, the species frequently overwinters in houses or other artificial structures, where they can become a nuisance and may cause allergic reactions in inhabitants (Kidd et al., 1995; LaMana and Miller, 1996; Nalepa et al., 1996; Yarbourgh et al., 1999; Huelsman et al., 2002). In temperate areas of the northern hemisphere, migrations to such overwintering sites occur in autumn and are triggered by an increase in day time temperature above 18 °C following a cool period (Huelsman et al., 2002; personal observation). During the autumn migrations, adults swarm to overwintering sites driven by a hypsotactic orientation (i.e. movement toward prominent objects on the horizon) (Obata, 1986). Arriving at these sites, they are attracted to contrasting,

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shiny, light-coloured items (Obata, 1986; Hodek et al., 1993; Hodek and Honek, 1996; Koch, 2003). They gather on the sunlit south–south western side of these structures and subsequently slowly move to protected microhabitats at the location. In these microhabitats they aggregate, often *en masse*, and overwinter. It is unclear as to whether pheromones, thigmotatic stimuli, faeces or accumulated dead remains of *H. axyridis* play a role in locating these protected microhabitats, which appear to be frequented year after year (Hodek and Honek, 1996; Nalepa et al., 2000). In early spring, the adults leave their overwintering sites (Obata, 1986; Osawa, 2000).

Adults of *H. axyridis* undergo several physiological changes during their overwintering. They start in a state of reproductive diapause and have a greatly enlarged fat body, a decreased respiration rate and an atrophied corpus allatum. Additionally, females have reduced ovaria (oocytes are resorbed and not vitellinized) (Sakurai et al., 1992; Hodek and Honek, 1996; Iperti and Bertand, 2001). As the diapause continues, the fat body is slowly utilised for energy (body weight subsequently also decreases), the digestive tract is emptied and cold hardiness increases. During the course of the winter the beetles switch from diapause to a state of quiescence (Iperti and Bertand, 2001).

Cold tolerance studies are considered a fundamental step in the assessment of establishment potential as part of an environmental risk analysis for non-native biological control agents (Hart et al., 2002a,b; Van Lenteren et al., 2003; Bazzocchi et al., 2004; Tullett et al., 2004; Hatherly et al., 2005a,b, 2008; van Lenteren et al., 2008). In this study we have examined the cold hardiness of a long-term laboratory strain of *H. axyridis* and two European field populations overwintering in an outdoor and indoor hibernaculum. Cold tolerance of *H. axyridis* has been studied by Watanabe (2002) and Koch et al. (2004) for overwintering populations in Japan and the USA, respectively. The cold tolerance of the invading populations in Europe has, however, never been the subject of detailed investigation. Furthermore, other studies have not assessed the overwintering ability of *H. axyridis* as a function of hibernaculum type. In this study, the cold hardiness of *H. axyridis* was evaluated by determining their supercooling point, lower lethal time and lower lethal temperature (e.g. Hart et al., 2002a,b; Watanabe, 2002; Hatherly et al., 2004, 2005a,b, 2008; Koch et al., 2004; Tullett et al., 2004).

## 2. Materials and methods

### 2.1. Insect populations

Four populations of *H. axyridis* were used in this study.

Field population (A) consisted of individuals collected from an outdoor hibernaculum in Wilrijk (51°30′N to 4°46′E), Belgium. These individuals were collected in spaces along the doorframes of an old deserted bunker located in the middle of the nature reserve "Fort 7". Field population (B) consisted of individuals overwintering in an indoor hibernaculum in Gaasbeek (50°47′N to 4°11′E), Belgium. These individuals were collected in spaces along window frames of an unheated stairwell at the southwest wing of a large house, situated in the middle of the park "domein Groenenbeek". Individuals of both populations were collected once a month in early November and December 2007 and January and February 2008 and used immediately in experiments. Additionally, two populations (C and D) were established in the laboratory using insects collected in a park in Ghent, Belgium. The latter populations were reared following the methods described in Berkvens et al. (2008a,b). A non-melanic population (C) and melanic population (D) were set up with individuals collected in October 2007 and maintained at 23 °C, 70% RH and a 16:8 h (L:D) photoperiod. The insects were fed frozen eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Phycitidae) as their diet. The cold tolerance of populations C and D was measured using 2-week-old adults of the 3rd laboratory generation.

### 2.2. Indices of cold tolerance

The supercooling point (SCP), median lower lethal temperature (LTemp50) and median lower lethal time (LTime50) at 0 and −5 °C were measured for adults of all four populations. Morph type, fresh body weight and sex were determined for individuals used in the SCP and LTemp50 experiments, to evaluate the impact of these factors on each cold tolerance parameter. Body weight was determined before the adults were subjected to the experimental exposures; the insects were weighed using a semi-microbalance Sartorius Genius ME215P (Sartorius AG, Goettingen, Germany) (±0.01 mg). Sex was determined after the experiments were terminated using the method described by McCornack et al. (2007). Tested adults of the laboratory-reared populations were ca. 2 weeks old, but the age of field-collected individuals was not known.

### 2.3. Supercooling point

The SCP is the absolute lowest limit of cold tolerance of a freeze avoiding insect, being the temperature at which the body tissues and fluids freeze (Bale, 1987; Koch et al., 2004). The SCPs of *H. axyridis* individuals were measured using a Picotech TC-08 thermocouple data logger and a low temperature programmable Haake Phoenix II CP30 alcohol bath. An adult *H. axyridis* was placed individually in a 1.5 ml Eppendorf tube. A thermocouple was led through a small opening in the Eppendorf lid and placed near the coccinellid's body. The opening in the Eppendorf lid was sealed afterwards with Pritt Poster Buddy. The Eppendorf tube was then placed individually in a glass test tube which was suspended in the alcohol bath. The starting temperature was set at 23 °C. The test individuals were then gradually cooled at a rate of 0.5 °C $min^{-1}$ to their SCP. The SCP of each individual was detected by the release of heat (exotherm) when the beetle froze; 32 individuals per population and per month were used to determine the mean SCP.

### 2.4. Lower lethal temperature and lower lethal time

In addition to the mortality caused by freezing at the SCP, an insect can also die at temperatures above the SCP. This is caused by the accumulation of chill injury. If the injury results from rapid cooling in absence of freezing, this is categorised as direct chilling injury; indirect chilling injury occurs as a consequence of a long-term exposure to low temperatures (Chown and Terblanche, 2006). Chill injury is characterized in this study by the LTemp50 (direct chilling injury) and LTime50 (indirect chilling injury) (i.e. the temperature at which 50% of the test individuals die and the time required to kill 50% of the population at a certain temperature) respectively.

The same experimental design as described for the SCP studies was used to determine the LTemp50. Adults were suspended in an alcohol bath at 23 °C and were cooled to one of seven temperatures (−5, −7.5, −10, −12.5, −15, −17.5, and −20 °C) at a rate of 0.5 °C $min^{-1}$. They were kept at the minimum low temperature for 1 min after which they were warmed back up to 23 °C, again at a rate of 0.5 °C $min^{-1}$. The adults were then transferred individually to a Petri dish (diameter: 9 cm and height: 1.3 cm) and kept in an incubator set at 23 °C, 70% RH and a 16 h photoperiod. The insects were not provided with food or water. After 24 h, their survival was recorded. A group of 32 adults was exposed at each temperature and the data was analysed by logit regression to estimate the LTemp50 of each population.

To determine the LTime50, 72 Petri dishes (diameter: 9 cm and height: 1.3 cm) each containing 10 adult *H. axyridis*, were set up at room temperature with no food or water provided. The Petri dishes were then transferred successively to incubators set at 15 and 5 °C, and held at each temperature for 30 min to avoid possible mortality due to cold shock. The Petri dishes were then equally divided between 2 incubators set at 0 or −5 °C. The internal environment in both incubators was kept in total darkness at all times and relative humidity was not controlled. At different time intervals over the following 6 months, 3 Petri dishes (in total 30 individuals) were taken from the 0 and −5 °C incubators, transferred successively to incubators set at 5 and 15 °C, and again held for 30 min at each temperature. The insects were finally transferred to an incubator set at 23 °C and maintained for 24 h without food or water, after which survival was recorded. The LTime50 at 0 and −5 °C was estimated for each population using Cox-proportional hazards models.

### 2.5. Climatic data

Outdoor climatic data of October, November and December 2007 and January and February 2008 were obtained from the Belgian Royal Meteorological Institute (RMI). A HOBO H8 Family data logger was placed in the unheated indoor hibernaculum to monitor temperature.

### 2.6. Statistics

Data analysis was carried out using Stata/SE 10.1 (Statacorp, 2007). The SCP was determined by applying a bootstrapped median regression. The LTemp50 was calculated by means of a logistic regression. Analysis started each time with a saturated model and interactions and non-significant main factors were dropped at a significance level of 0.05. The LTime50 for 0 and −5 °C was determined using Cox-proportional hazards models. Proportionality of hazards was tested by means of Schoenfeld residuals.

## 3. Results

### 3.1. Supercooling point

The lowest SCP was recorded for the indoor overwintering field population (B) collected in November (−18.2 °C) and the highest for the non-melanic laboratory population (C) (−13.2 °C) (Fig. 1). A bootstrap regression indicated that neither sex nor body weight influenced the SCP. Additionally, morph type did not affect the SCP of the field populations (A) and (B). The melanic adults of population (D), however, had a lower SCP than the non-melanic adults of population (C) ($p = 0.034$).

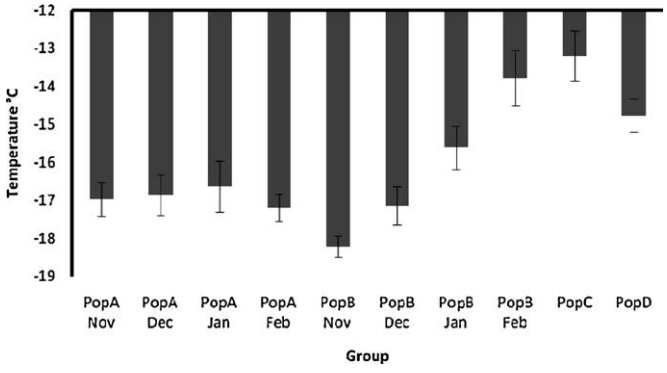


**Fig. 1.** SCP (mean ± SE) of field populations (A) and (B) collected in the months November, December, January and February and a non-melanic (C) and melanic laboratory-reared population (D).

The SCP of the outdoor overwintering field population (A) remained similar during the 4-month monitoring period, but the SCP of the indoor overwintering field population (B) increased as winter progressed. The SCP of the indoor overwintering population was marginally lower in November than in December ($p = 0.093$) and significantly lower in November than in January ($p = 0.016$) and February ($p = 0.001$). Only in February was the SCP of the outdoor overwintering field population (A) significantly lower than that of the indoor overwintering field population (B) ($p = 0.011$). All adults of the field populations (A) and (B) collected in November and December had lower SCPs than the adults of populations (C) and (D) (all $p$-values $\leq 0.035$). The adults of the indoor overwintering field population (B) collected in January had a lower SCP than the adults of the non-melanic population (C) ($p = 0.008$), but had a similar SCP to those of the melanic population (D).

### 3.2. Lower lethal temperature

Fig. 2 shows the relationship between exposure to lower temperatures and mortality in four populations, at four sampling times. Using logistic regression models the LTemp50 of each population and (for population (A) and (B)) sampling time was estimated (Table 1). A Pearson correlation coefficient $r$ of 0.923 ($p < 0.001$, $n = 9$) was found between the SCP and LTemp50. Sex and fresh body weight again had no influence on the LTemp50. As for the SCP, there were no significant differences in the survival of the outdoor overwintering field population (A) over the four winter months. Likewise, the indoor overwintering field population B had a higher tolerance to subzero temperatures in the first winter months (November and December) than later (January and February) (all $p$-values $\leq 0.011$). Furthermore, adults of the indoor overwintering population (B) survived exposure to the low temperatures less successfully than those of the outdoor overwintering population (A) during January and February ($p < 0.001$). There was no difference in survival between the melanic and non-melanic populations (C) and (D). All adults of the field populations (A) and (B) collected in November and December tolerated low temperatures better than the adults of the populations (C) and (D) (all $p$-values $\leq 0.022$), reflecting the pattern observed for the SCPs.

### 3.3. Lower lethal time

Figs. 3 and 4 show the percentage mortality of adults when continuously exposed to 0 and −5 °C, respectively for up to 6 months. Only a small number of adults of the outdoor overwintering population (A) could be collected in November, hence lower lethal times could not be assessed after 15 and 9 weeks for 0 and −5 °C, respectively. Likewise, the indoor overwintering population (B) was not large enough to continue with the lower lethal time experiments after December. Using the Cox-proportional hazards model LTime50 was calculated for the different populations kept at 0 °C (Table 2). Contrary to the SCP and LTemp50, there was a decline in cold tolerance of the outdoor overwintering population (A) in these experiments. Adults of this population survived better at 0 °C when collected in December or January than in February ($p \leq 0.001$ and $p \leq 0.027$ respectively) and marginally better in December than in January ($p = 0.067$). There was a higher survival of adults of the indoor overwintering population (B) at 0 °C in December than in November ($p \leq 0.001$). There were no differences in the survival of field populations (A) and (B), or between both laboratory-reared populations (C) and (D). Adults of the overwintering populations (A) and (B), however,

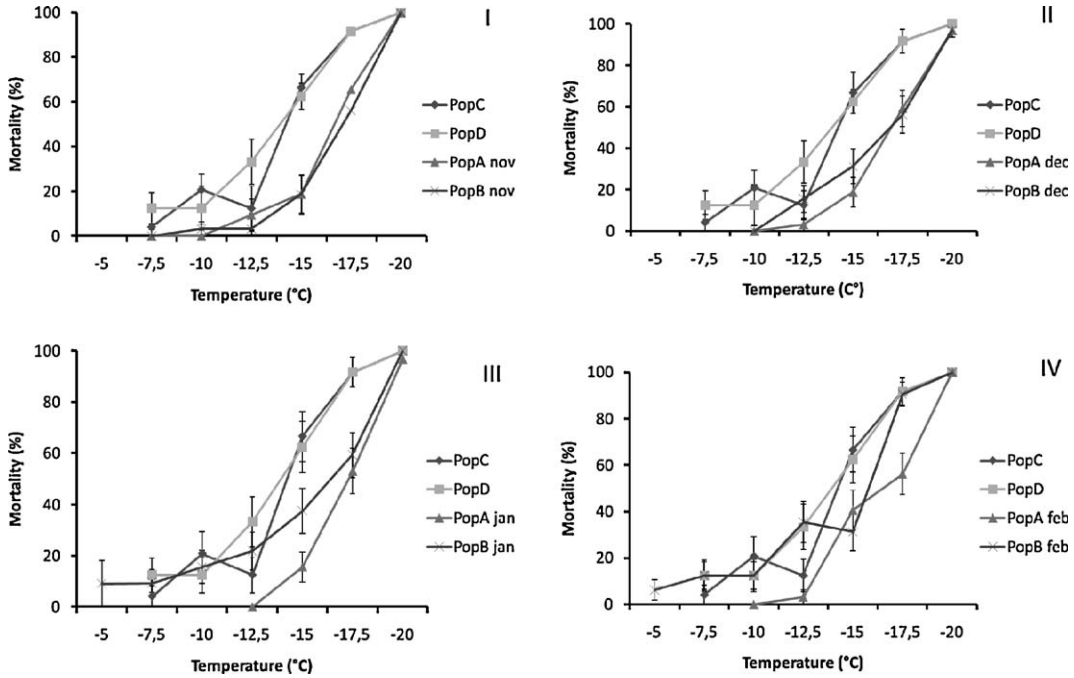


**Fig. 2.** Mortality rates (mean ± SE) of field populations (A) and (B) collected in the months November (I), December (II), January (III) and February (IV); for all months mortality rates of a non-melanic (C) and melanic laboratory-reared population (D) are shown for comparison.

**Table 1**
Mean LTemp50 (°C) and lower and upper confidence intervals (CL, °C) of field populations (A) and (B) collected in the months November, December, January and February and a non-melanic (C) and melanic laboratory-reared population (D).

| Group | PopA Nov | PopA Dec | PopA Jan | PopA Feb | PopB Nov | PopB Dec | PopB Jan | PopB Feb | PopC | PopD |
|---|---|---|---|---|---|---|---|---|---|---|
| LTemp50 | −16.4 | −16.8 | −17.1 | −16.3 | −16.7 | −16.3 | −15.3 | −14.1 | −13.4 | −13.4 |
| Lower CL | −17.0 | −17.4 | −17.6 | −17.0 | −17.4 | −17.0 | −16.3 | −15.1 | −14.7 | −14.4 |
| Upper CL | −15.8 | −16.2 | −16.6 | −15.7 | −16.1 | −15.6 | −14.3 | −13.3 | −13.0 | −12.5 |

showed higher survival when exposed to 0 °C than those of populations (C) and (D) (all $p \leq 0.001$).

For the adults of the outdoor overwintering population (A) at −5 °C, there was a higher survival in December than in November ($p = 0.039$), January ($p = 0.048$) or February ($p \leq 0.001$). Also, survival was higher in January than in February ($p \leq 0.001$) (Table 2). Survival of the indoor overwintering population (B) at −5 °C was also higher in December than in November ($p = 0.025$). In December, survival of the outdoor population (A) at −5 °C was higher than that of the indoor population (B) ($p \leq 0.001$). The non-melanic population (C) survived in higher numbers at −5 °C than the melanic population (D) ($p = 0.007$), but neither populations survived as well as the overwintering field populations (A) and (B) (all $p \leq 0.001$). Adults of the outdoor overwintering population (A) tolerated 0 and −5 °C equally well, while the indoor overwintering population (B) and both laboratory-reared populations (C) and (D) had higher survival rates at 0° than at −5 °C.

## 4. Discussion

The SCP and LTemp50 values were broadly similar (differing by a maximum of 1.5 °C), indicating that the mortality of coccinellids exposed to brief acute subzero temperatures was generally caused by a freezing of the body liquids. When the ladybirds were exposed to less severe subzero temperatures for longer periods of time, mortality occurred at higher temperatures. This is a typical response for a 'chill intolerant' insect (Bale, 1993, 1995).

The SCP and LTemp50 values of the outdoor overwintering field population of *H. axyridis* in Belgium are similar to those observed by Watanabe (2002) and Koch et al. (2004) for overwintering populations in Japan and the USA, respectively. Our results indicate that *H. axyridis* can survive at the average winter temperatures occurring in temperate regions of Europe. The highest SCP and LTemp50 of the outdoor overwintering field population were both about −16.5 °C, and more than 50% of these individuals survived exposure to 0 and −5 °C for more than 4.5 and 4 months, respectively. Furthermore, given that the beetles usually create a protective microclimate within their aggregation when overwintering (Koch et al., 2004), they can possibly survive in environments with even lower subzero temperatures. The SCP and LTemp50 of individuals overwintering in an unheated indoor hibernaculum were consistently below −14 °C, implying that they would also be able to withstand subzero temperatures if they were suddenly exposed to outdoor winter conditions. The current study only focussed on survival of *H. axyridis* through winter and the fitness of surviving adults was not assessed. Further research should therefore investigate the effect of winter temperatures on the reproductive capacity of adults emerging from hibernation.

As reported in Watanabe (2002), the SCP and lower lethal temperature of the outdoor overwintering field individuals remained stable during the winter months, while their lower lethal time showed a significant decrease as the winter progressed. Both the SCP and lower lethal temperature are 'instantaneous' measures of cold tolerance, which appear to change little as long as the cryoprotectant profile and concentration remain reasonably consistent through winter. The causes of mortality during longer exposures to less extreme subzero temperatures, however, appear to be of a different nature. Hence the lethal time, reflecting

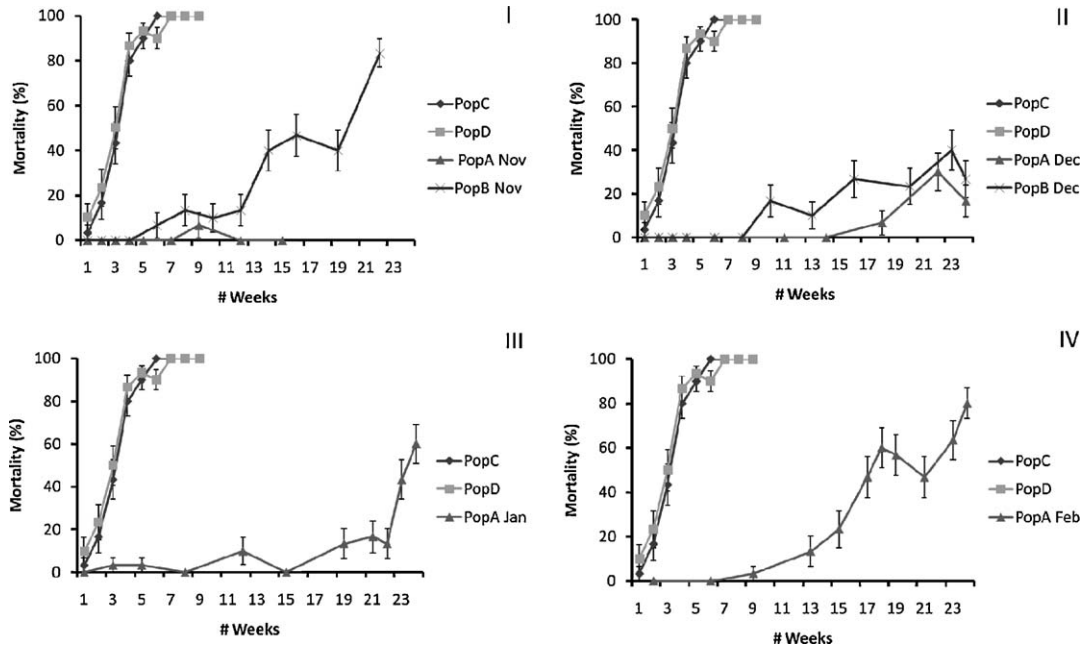


**Fig. 3.** Mortality rates (mean ± SE) at 0 °C of field populations (A) and (B) collected in the months November (I), December (II), January (III) and February (IV); for all months mortality rates of a non-melanic (C) and melanic laboratory-reared population (D) are shown for comparison.

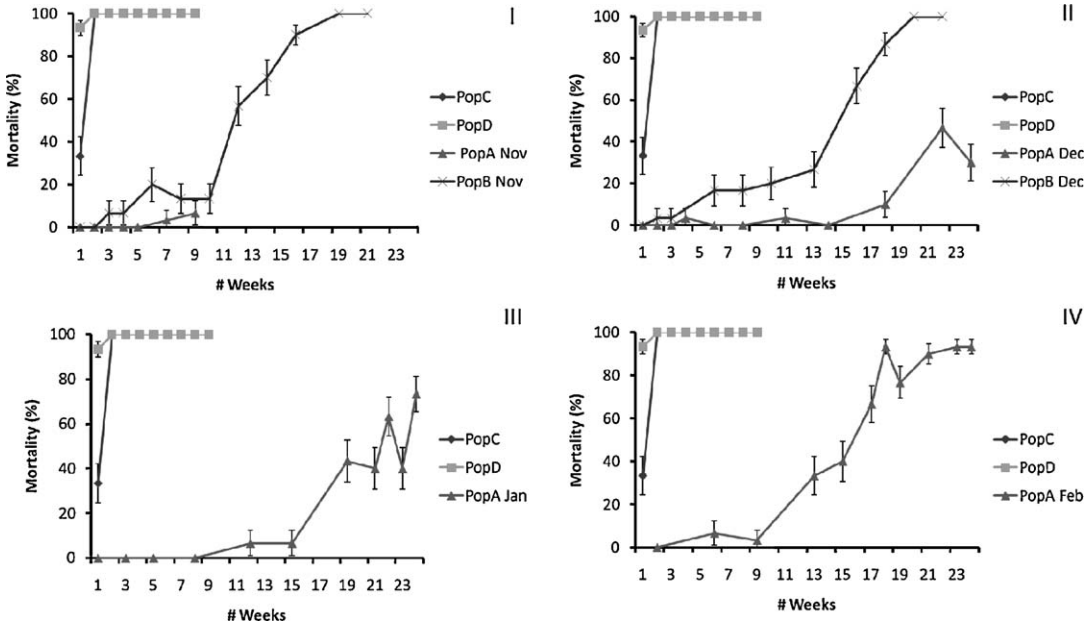


**Fig. 4.** Mortality rates (mean ± SE) at −5 °C of field populations (A) and (B) collected in the months November (I), December (II), January (III) and February (IV); for all months mortality rates of a non-melanic (C) and melanic laboratory-reared population (D) are shown for comparison.

**Table 2**
LTime50 (mean ± SE; weeks) at 0° and −5 °C of field populations (A) and (B) collected in the months November, December, January and February and a non-melanic (C) and melanic laboratory-reared population (D).

| Temperature (°C) | | PopA Nov | PopA Dec | PopA Jan | PopA Feb | PopB Nov | PopB Dec | PopC | PopD |
|---|---|---|---|---|---|---|---|---|---|
| 0 | LTemp50 | [a] | [a] | 24 | 18 | 22 | 23 | 4 | 3 |
| | ±SE | [a] | [a] | 0.2 | 0.3 | 0.6 | [a] | 0.1 | 0.1 |
| −5 | LTemp50 | [a] | [a] | 22 | 17 | 12 | 16 | 2 | 1 |
| | ±SE | [a] | [a] | 0.4 | 0.3 | 0.5 | 0.3 | 0.1 | [a] |

[a] No values could be calculated due to too few data points.

cumulative effects of cold stress, is a more reliable index of cold tolerance being more representative of the conditions experienced by insects during temperate winters.

During the first months of winter the indoor and outdoor overwintering field population had a comparable level of cold tolerance, presumably because they were exposed to similar outdoor environmental conditions during the preceding months and subsequently underwent similar cold hardening processes. However, in contrast to the outdoor overwintering population which had a consistent SCP and LTemp50 throughout winter, both the SCP and LTemp50 of the indoor overwintering population rose as the winter progressed, i.e. cold tolerance was reduced. Most insect species require low ambient temperatures and/or a state of diapause to induce the production of cryoprotectant compounds and increase their cold tolerance (Sømme, 1982; Bale, 1987; Slachta et al., 2002; Wang et al., 2006). The higher average temperatures in the indoor hibernaculum (temperatures were always higher than +5 °C but decreased to a minimum of −8.4 °C in the outdoor hibernaculum), may have led to a lower production of cryoprotectant compounds such as myo-inositol (Watanabe, 2002), or reduced the concentration of some compounds that had previously accumulated to a higher level. Additionally, higher ambient temperatures can also weaken the diapause intensity of overwintering insects (Hodek and Honek, 1996). The relationships between diapause and cold tolerance in insects is, however, not yet clear, Some species such as certain tropical and Antarctic insects, show no relationship, whereas several temperate insects with a facultative winter diapause, for example *H. axyridis* (Koch, 2003), do not enter their protective state of cold tolerance unless they are in the diapause stage (Slachta et al., 2002; Danks, 2005). Future research is needed to determine whether the lower level of cold tolerance of indoor overwintering individuals of *H. axyridis* triggers an earlier activation of these adults compared with the outdoor overwintering individuals. If so, this earlier activation could have an impact on adult survival in relation to the availability of food resources in early spring. In their study in Canada, Labrie et al. (2008) collected *H. axyridis* adults from the field in November and placed them in controlled chambers set at −5 and +10 °C. About 45% of the adults kept at −5 °C up to the end of the winter in April were still alive, whereas all adults kept at 10 °C had died; in addition, adults kept at the higher temperature had depleted their energy reserves.

Sex and morph type did not influence the SCP and LTemp50 of the field individuals in our study, which is in accordance with the observations of Koch et al. (2004).

When assessing the cold tolerance of a potential biocontrol agent as part of an environmental risk assessment (Hart et al., 2002a,b; Van Lenteren et al., 2003; Bazzocchi et al., 2004; Tullett et al., 2004; Hatherly et al., 2005a,b, 2008; van Lenteren et al., 2008), it would be more practical to use individuals from a laboratory strain than to try to collect individuals from the field in winter (in its area of origin). In our study however, the cold tolerance differed greatly between the laboratory-reared and field-collected individuals of *H. axyridis*. Unlike those overwintering in the field, the laboratory individuals were not in a state of diapause at the time of testing. In the course of our study three attempts were made to induce diapause in the non-melanic and melanic populations, which had been established from individuals collected in the field in June 2008. In contrast to our earlier experience (Berkvens et al., 2008a,b), we did not succeed in inducing reproductive diapause in the majority of individuals exposed to short day conditions.

In conclusion, this study indicates that *H. axyridis* is sufficiently cold tolerant to withstand winters generally occurring in temperate regions of Europe, and that further establishment of the species in these areas may not be prevented by subzero winter temperatures. In areas where low subzero temperatures occur in winter (e.g. Northern Europe and Scandinavia) and/or less extreme subzero temperatures persist for long periods of time, human habitation could provide a "cold-free space" for the species to survive (Koch et al., 2004; Labrie et al., 2008), but more research is needed to determine the success of indoor overwintering in *H. axyridis*.

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