


# Cold tolerance of the invasive oak lace bug, *Corythucha arcuata*

Márton J. Paulin<sup>1</sup> | Csaba B. Eötvös<sup>1</sup> | Petr Zabransky<sup>2</sup> | György Csóka<sup>1</sup> |  
Martin Schebeck<sup>2</sup> 

<sup>1</sup>Forest Research Institute, Department of Forest Protection, University of Sopron, Mátrafüred, Hungary

<sup>2</sup>Institute of Forest Entomology, Forest Pathology and Forest Protection, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna, BOKU, Vienna, Austria

## Correspondence

Martin Schebeck, Institute of Forest Entomology, Forest Pathology and Forest Protection, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna, BOKU, Peter-Jordan-Straße 82/I, A-1190 Vienna, Austria.  
Email: [martin.schebeck@boku.ac.at](mailto:martin.schebeck@boku.ac.at)

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## Abstract

1. The North American oak lace bug (OLB), *Corythucha arcuata*, is an invasive species in Europe and a serious threat to oak-dominated forests.
2. Survival at low temperatures is one major factor determining the spread of invasive insects. Thus, we studied key traits, that is, cold-tolerance strategy, supercooling points (SCP) and chilling-related mortality, of overwintering adults to assess their potential to withstand harsh winters.
3. Samples for SCP measurements were collected once a month from November 2020 until March 2021 at three different locations in Hungary; specimens for chilling experiments were sampled in November 2020, January 2021 and March 2021.
4. SCPs of overwintering adults ranged from  $-29.68$  to  $-7.49$  °C, with only moderate variation among months; *C. arcuata* is a freeze-avoidant species.
5. Mortality rates of adults exposed to two sub-zero temperatures above the SCP ( $-3$  and  $-5$  °C) for 1, 2 and 3 weeks ranged between 0% and 69.1%, suggesting that OLB has a moderate risk to die from chilling injuries. Exposure time and sampling date affected mortality, with lowest survival rates after 3 weeks, collected in March 2021.

## KEYWORDS

chilling, cold hardiness, forest pest, invasive species, neobiota, *Quercus*, SCP, supercooling

## INTRODUCTION

Alien insect species can pose serious threats to various ecosystems worldwide (Csóka et al., 2010, 2012, 2017; Roques, 2010; Smith et al., 2018). While some of them do not have an evident impact on the newly-colonized ecosystems, others may become invasive, rapidly expanding their range and imposing severe pressures on the novel habitats (Blackburn et al., 2019; Haubrock et al., 2021; Levine & D'Antonio, 2003; Mollot et al., 2017; Shabani et al., 2020; Spatz et al., 2017; Vanbergen et al., 2013). Whether a non-native species can become invasive is influenced by several intrinsic, species-specific traits as well as by external biotic and abiotic factors. For example, a generalist

or specialist feeding behaviour, favourable or adverse thermal conditions (Paulin et al., 2020), availability of suitable host plants (Csóka et al., 2017, 2020), or regulatory effects of natural enemies (Csóka et al., 2009; Kos et al., 2021) can affect the success of a non-native species.

Temperature is a crucial environmental parameter, particularly for ectotherms such as insects, as it determines the developmental and reproductive performance of a species and regulates its cold- or heat-related survival (Bale et al., 2002; Battisti et al., 2005; Fomby et al., 2018; Lehmann et al., 2015; Lombardero et al., 2000; Lombardo & Elkinton, 2017; Ungerer et al., 1999). Therefore, temperature can either limit the spread of a non-native insect or facilitate its rapid range expansion, while also determining phenological and voltinism patterns (Ju et al., 2017; Kang et al., 2009; Reitz & Trumble, 2002; Ward et al., 2019). In particular, the ability to survive cold winter conditions in a newly

György Csóka and Martin Schebeck are equally contributing senior authors.

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colonized environment is of high importance to assess the likelihood a species will establish and increase its range (Leather et al., 1993; Marshall et al., 2020; Sobek-Swant et al., 2012; Véték et al., 2020). Therefore, information on the overwintering biology of a non-native insect is vital for both basic and applied fields.

Insects evolved various physiological and behavioural strategies to mitigate harmful effects of cold environmental conditions, especially during the winter season (Lee, 2010). Major adaptations are related to the freezing of body fluids as well as to physiological responses to avoid cold-related stress at temperatures above the freezing point of the hemolymph (Lee, 2010; Sinclair et al., 2015). There is general agreement on three basic types of insect cold tolerance strategies. First, freeze-avoidant (or freeze-intolerant) species die when ice is formed in their body fluids, but survive low temperatures when no internal ice formation has occurred yet. Second, freeze-tolerant insects can cope with ice formation in their bodies, as it is initially formed in the extracellular space or they can survive when certain parts of body tissues/fluids are frozen. Subsequently, they can survive even lower temperature conditions. Third, chill-susceptible species die because of cold-related effects without ice being formed within their bodies (Bale, 1993, 1996; Lee, 2010; Sinclair et al., 2015). Both freeze-intolerant and freeze-tolerant insects, however, have to cope with cold conditions at sub-zero temperatures without internal ice formation (even for long periods) and thus have to avoid harmful effects of chilling-related injuries (Lee, 2010).

To survive cold temperatures, many insects have evolved strategies to lower the freezing point of their hemolymph and remain in a supercooled state, which is facilitated by building up cryoprotectant substances, like sugars or polyols (Lee, 2010). The temperature when ice formation of body fluids finally occurs is the supercooling point (SCP), an important value in insect cold tolerance research, for example, to determine whether a species is freeze-avoidant or freeze-tolerant (Sinclair et al., 2015). Moreover, the SCP corresponds to the lower lethal temperature of freeze-avoidant species. However, these values do not necessarily explain the potential to survive harsh periods in the field, as the overwinter biology is determined by various physiological and behavioural strategies as well as by the environmental conditions which can strongly vary even on a small local scale (Lee, 2010; Sinclair et al., 2015). Studies on a species' performance at cold conditions, both above and below the freezing point of the hemolymph, help to understand an insect's overwinter performance and contribute to a better understanding of life histories.

The North American oak lace bug, OLB, *Corythucha arcuata* (Say, 1832) (Heteroptera: Tingidae) is a recent invader in Europe, both in urban and forest ecosystems (Csóka et al., 2020). It is native and widespread in the eastern part of the United States and across southern Canada (Barber, 2010), and was recently recorded as an invasive species in the Western US in Portland, Oregon (Rosetta, 2017). First European records were reported in Italy in 2000 (Bernardinelli, 2000). Since then, it was recorded in numerous countries: 2002 in Switzerland (Forster et al., 2005), 2003 in Turkey (Mutun, 2003), 2005 in Iran (Samin & Linnavuori, 2011), 2012 in Bulgaria (Dobrova et al., 2013), 2013 in Hungary (Csóka et al., 2013), Croatia (Hrašovec et al., 2013) and Serbia

(Pap et al., 2015; Poljaković-Pajnik et al., 2015), 2015 in Russia (Neimorovets et al., 2017), 2016 in Albania (E. Cota, personal communication), Ukraine (Shchurov et al., 2016), Slovenia (Jurc & Jurc, 2017) and Romania (Don et al., 2016), 2017 in Bosnia and Herzegovina (Dautbašić et al., 2018) and France (Streito et al., 2018), 2018 in Slovakia (Zúbrik et al., 2019), 2019 in Austria (Sallmannshofer et al., 2019), 2021 in Portugal (Gil & Grosso-Silva, 2021), and 2022 in Spain (Riba-Flinch, 2022).

Studies in its native range showed that *C. arcuata* completes two generations per year and might have a partial third generation (i.e., a small number of the third generation develops into the adult stage), with some adults from the second generation and those from the third overwintering (Connell & Beacher, 1947). In habitats with favourable, warm conditions *C. arcuata* can complete three generations per year and might even have a partial fourth generation (Bernardinelli, 2000).

In its native range, adults of OLB leave their overwintering habitats in spring, that is, early/mid-April, feed on the underside of flushing leaves, mate and subsequently lay eggs. Feeding causes discoloration of oak leaves, leaving a greyish-yellow spot (Connell & Beacher, 1947). Under natural conditions, embryonic development takes about 2–3 weeks and freshly hatched nymphs start sucking on leaves. Nymphal development is quite fast, going through five instars (intervals between moults of a few days) before reaching adulthood (Connell & Beacher, 1947).

OLB utilizes various oak species (*Quercus* spp.) as hosts. In its native range, it mostly feeds on white oaks, such as eastern white oak *Quercus alba* L., 1753, the swamp white oak *Quercus bicolor* Willd., 1801, bur oak *Quercus macrocarpa* Michx., 1801, or post oak *Quercus stellata* Wangenh., 1787 (Csóka et al., 2020). In the Western US it was recently found on Oregon white oak *Quercus garryana* Douglas ex Hook., 1839 (Rosetta, 2017).

In the invaded European range, 27 oak species were described as suitable hosts (Csóka et al., 2020). In the last decade, OLB showed an incredibly fast range expansion and severe outbreaks have been reported from 20 European countries (Paulin et al., 2020). As almost all native European oak species are suitable hosts, at least 30 million hectares of oak-dominated forests are a potential habitat (Csóka et al., 2020). Although there are major gaps of knowledge concerning the further spread and damage of OLB, it is expected to become a serious oak pest in Europe (Csóka et al., 2020; Paulin et al., 2020). For example, a study on *Quercus robur* revealed that severe infestations can substantially reduce the rate of photosynthesis and transpiration (Nikolić et al., 2019). In addition, remote sensing tools were successfully applied to evaluate the effects of OLB on the photosynthetic activity of trees and thus on the progress of damages to oak forest in time and space (Kern et al., 2021). Although detailed studies are currently lacking, oak stands with heavy OLB infestations over several years had an earlier acorn abscission and smaller acorn size (Csóka et al., 2020; Paulin et al., 2020).

Knowledge of the overwintering biology of *C. arcuata* is scarce, but it might be well-adapted to cold winters in its native range which covers major parts of North America (Drake & Ruhoff, 1965). Field observations showed that OLB adults stop feeding in late autumn

(October/November) and migrate to their overwintering microhabitats, which are under raised bark, in bark crevices, in branch forks covered by leaf litter or in moss layers on tree trunks (Connell & Beacher, 1947; Csepelényi et al., 2017; Kovač et al., 2021; Paulin et al., 2021). No overwintering nymphs have been found in Central Europe so far (Csepelényi et al., 2017; Paulin et al., 2021). Other data on its performance during winter and responses to cold are currently lacking.

To fill these major gaps of knowledge, we performed a set of experiments on the cold tolerance of OLB. For example, data on the cold tolerance strategy or on the potential to cope with low temperatures for various periods of time can be used to evaluate the effects of cold on survival, and thus on population dynamics. Moreover, these data are useful to assess the habitat suitability and future spread of *C. arcuata* in Europe. We conducted various laboratory experiments with field-collected adult *C. arcuata* over one winter season from three Central European, that is, Hungarian, locations. Our main goals were (i) to conduct SCP measurements and assess the lower limits of survival at short-time cold exposure under laboratory conditions, (ii) to identify the species' cold tolerance strategy (i.e., freeze-tolerant or freeze-intolerant) by recording survival after individual SCPs and (iii) to evaluate chilling-related mortality by studying OLB survival after long-time exposure to sub-zero temperatures above the SCP (i.e., before the freezing of body fluids).

## METHODS

### Insect collection and site information

During winter 2020/21 (from November until March), adult individuals were sampled from three Hungarian locations once per month: Gyula (46°41'37.4" N, 21°20'07.0" E; elevation: 87 m), Szolnok (47°12'13.0" N, 20°10'56.7" E; elevation: 86 m) and Mátrafüred (47°49'48.4" N, 19°57'50.2" E; elevation: 359 m). The forests in the locations Szolnok and Gyula showed a similar stand structure. The site in Szolnok was a pure *Q. robur* stand (personal communication: National Forestry Company 'KEFAG Inc.'). In Gyula it consisted mostly of *Q. robur* (92%) and to a minor extent of *Fraxinus excelsior* (8%) (personal communication: National Forestry Company 'DALERD Inc.'). The average age of trees in Szolnok was 44 years, in Gyula about 60 years. The stands at Mátrafüred comprised *Q. petraea* (36%) and *Q. cerris* (64%), the average age of trees was 75 years (personal communication: National Forestry Company 'Egererdő Inc.').

Air temperature was recorded 2 m above ground in each location during the entire sampling period. In Gyula and Mátrafüred we had access to data recorded by weather monitoring stations, in Szolnok a temperature data logger (model: Voltcraft DL-141TH) was installed. All devices recorded the data in 10-min intervals, from 1 November 2020 to 29 March 2021. Subsequently, we calculated daily mean temperatures, daily maxima and daily minima.

Hibernating adults were collected from their overwintering habitats, that is, under raised bark and in bark crevices. Live bugs were

express-mailed to the laboratory in Vienna (with cooling packages). Subsequently, SCP measurements and chilling experiments were conducted immediately upon arrival of samples. Before the trials were performed survival of individual samples was controlled to ensure that only alive specimens were tested.

### Short-time exposure to cold temperatures and supercooling points

SCPs were determined using a standard procedure as, for example, described in Véték et al. (2020). In brief, living adults of *C. arcuata* were attached to a type-T (copper-constantan) thermocouple and connected with a recording potentiometer (details on collection date and sample size per location see Table S1). Specimens were attached to the tip of the thermocouple using petroleum jelly and subsequently transferred into a 1.6 mL 'Eppendorf' tube which was again put in a larger 'Falcon' tube, to ensure a constant cooling rate, that is, 1 °C/min (as described in Véték et al. (2020)). Afterwards, samples were cooled down in a freezer until individual SCPs were recorded, as indicated by an exotherm, because of the release of latent heat of crystallization (Sinclair et al., 2015). Temperatures of the thermocouples were recorded once per second using the program 'Personal DaqVieq' (version 2.0.4, Measurement Computing GmbH). After individual SCPs were reached, samples were immediately removed from the freezing device. To assess the cold tolerance strategy of *C. arcuata* (i.e., freeze-tolerant or freeze-intolerant), specimens were subsequently transferred to room temperature and survival was observed 12 and 24 h after cold exposure by controlling for coordinated movements.

### Long-time exposure to sub-zero temperatures and chilling mortality

To assess the overwinter survival of *C. arcuata* at sub-zero temperatures above the SCP, even for longer periods, specimens were exposed to -3 and -5 °C in incubators (Sanyo MIR-553) with constant darkness for 1, 2 and 3 weeks, respectively (experimental conditions were selected based on the relatively mild winter conditions at the study sites where periods with a daily average below -5 °C for more than 1 week are rare; unpublished data and Figure 3). Batches between 10 and 29 living individuals (sampled in November 2020, January 2021 and March 2021; details see Table S2) were transferred to small plastic boxes (9 × 6 × 5 cm, lid with aeration holes) containing a piece of paper tissue (i.e., a separate plastic box for each temperature condition and each exposure time). After each exposure time, randomly chosen batches were removed from the incubators, transferred to room temperature and survival of each individual was assessed by the presence of coordinated movements within a period of 3 h. Subsequently, mortality rates per batch were inferred from the numbers of dead and living individuals.

To ensure that mortality at sub-zero conditions was caused by low temperatures (and not by desiccation during periods of up to

3 weeks), OLB were provided with dry and slightly moistened paper tissue. As the mortality rates between dry and moist conditions ( $z$ -value = 1.808,  $df = 1$ ,  $p = 0.0706$ ) and between  $-3$  and  $-5$  °C ( $z$ -value = 0.641,  $df = 1$ ,  $p = 0.5220$ ) did not differ significantly among each other, these variables were not included individually in the subsequent statistical analyses.

## Data analysis

We used Generalized Linear Mixed Models (GLMM) to assess the effects of low temperatures on the survival of *C. arcuata* (Bates et al., 2015; Bolker et al., 2009). The SCP data fitted best to a gamma distribution, while the data on the chilling experiments fitted best to a lognormal distribution on the quantile to quantile (QQ) plot. Both allowed us to use the Penalized Quasi Likelihood (PQL) method with the appropriate GLM family to obtain estimates from the GLMM (Breslow & Clayton, 1993). After the PQL tests we used ANOVA and Tukey's post-hoc tests to calculate significance levels.

In the analysis of SCP values, we included the site and the month of collection as fixed effect and the ID of the individuals as random factor. When analysing the data of chilling experiments our model included the month, the location and the exposure times as fixed effect and the samples as random factor.

Analyses and data visualization were performed with RStudio version 4.2.2 (RCoreTeam, 2022), using the packages 'MASS' (Venables & Ripley, 2002), 'readr' (Wickham & Hester, 2018), 'ggplot2' (Wickham, 2016), 'car' (Fox & Weisberg, 2019), 'nlme' (Pinheiro et al., 2022), 'multcomp' (Hothorn et al., 2008) and 'tidyverse' (Wickham et al., 2019).

## RESULTS

### Short-time exposure to cold temperatures and supercooling points

SCP experiments were used to infer whether OLB is freeze-tolerant or freeze-intolerant, and to assess lower limits of survival under laboratory conditions over one winter season. Adults of *C. arcuata* have a high potential to withstand short-time exposure to low sub-zero temperatures and the supercooling capacity remained at quite high levels from November 2020 to March 2021 in all three locations (Figure 1).

Our models showed no significant differences among SCP values from month to month ( $F$ -value = 1,  $df = 4$ ,  $p = 0.3908$ ). However, we found significant differences between the locations ( $F$ -value = 34,  $df = 2$ ,  $p < 0.0001$ ). After Tukey's post-hoc tests, we found significantly higher mean SCP values at Mátrafüred as compared to the other two locations, while the interaction of months and locations showed that the mean SCP values at Szolnok were significantly lower in November, December and February than the respective values in Mátrafüred (Figure 1, Table 1).

In the southernmost location Gyula, a mean SCP over all months of  $-21.47$  °C ( $\pm 2.82$  °C; SD) was observed. The lowest SCP in this

site was  $-26.78$  °C measured in December 2020, the highest was  $-11.54$  °C measured in March. Monthly average SCP values ranged between  $-20.93$  and  $-22.66$  °C, with the lowest mean in February 2021.

A similar pattern of mean SCPs was observed in the central location Szolnok. An overall average SCP of  $-22.80$  °C ( $\pm 3.42$  °C) was found, with the lowest SCP of  $-28.20$  °C measured in December 2020 and the highest SCP of  $-11.76$  °C in February 2021. Average monthly SCP values ranged from  $-22.10$  to  $-23.15$  °C, meaning OLB from this location showed the highest resistance to short-time cold exposure.

At the northernmost site Mátrafüred an overall mean SCP of  $-19.29$  °C ( $\pm 4.52$  °C) was observed. The lowest SCP in this site was  $-29.68$  °C (in February 2021), which was the lowest value among all individuals studied here, while the highest was  $-7.49$  °C (in March 2021). Average monthly SCP values ranged from  $-18.11$  to  $-20.46$  °C.

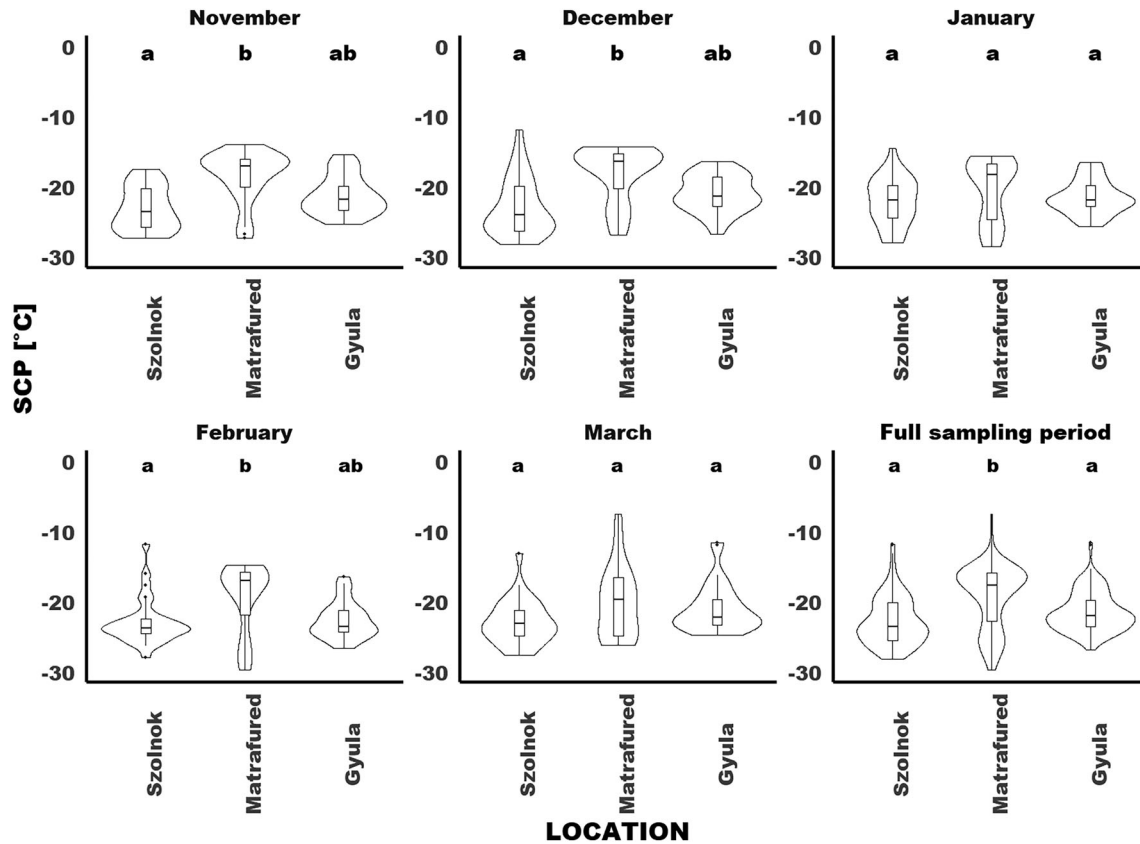
To determine whether *C. arcuata* is freeze-tolerant or freeze-intolerant (i.e., information on the mode of ice formation at the SCP), we tested whether individuals were alive (by controlling for coordinated movements) after they had reached their individual SCPs. As none of the individuals showed any coordinated movements (even not after 24 h), we concluded that adult *C. arcuata* are freeze-avoidant.

### Long-time exposure to sub-zero temperatures and chilling mortality

To understand the effects of sub-zero temperatures above the SCP on the survival of overwintering adult *C. arcuata*, even for long periods (i.e., chilling injuries without internal ice formation), we assessed the mortality after 1, 2 and 3 weeks. Over all sampling dates and exposure times, the average mortality rates of adult OLBs were 15.11% ( $\pm 14.37\%$ ; SD) in Gyula, 14.03% ( $\pm 15.36\%$ ) in Mátrafüred and 12.81% ( $\pm 16.77\%$ ) in Szolnok, respectively, showing no significant differences among each other (Figure 2, Table S3). However, when using the PQL method we found significant differences in mean mortality rates when testing for the length of cold exposure ( $F$ -value = 12.3156,  $df = 2$ ,  $p < 0.0001$ ).

In addition, sampling time significantly affected mean mortality rates ( $F$ -value = 53.2668,  $df = 2$ ,  $p < 0.0001$ ). Tukey's post-hoc test indicated that mean mortality rates of specimens collected in March (28.9%) were significantly different from those samples collected in November (6.78%;  $z$ -value = 3.558,  $df = 2$ ,  $p = 0.0009$ ) or January (6.32%;  $z$ -value = 3.411,  $df = 2$ ,  $p = 0.0015$ ). Mean mortality rates of November and January did not differ significantly from each other ( $z$ -value =  $-1.153$ ,  $df = 2$ ,  $p = 0.467$ ). Looking at the interaction between the month of collection and exposure time we found that the significant differences resulted from the significantly higher mortality in March with 21-day exposure time (Figure 2, Table 2).

On the population level, mortality rates were similar in the various sites. In Gyula the average mortality rate of samples collected in

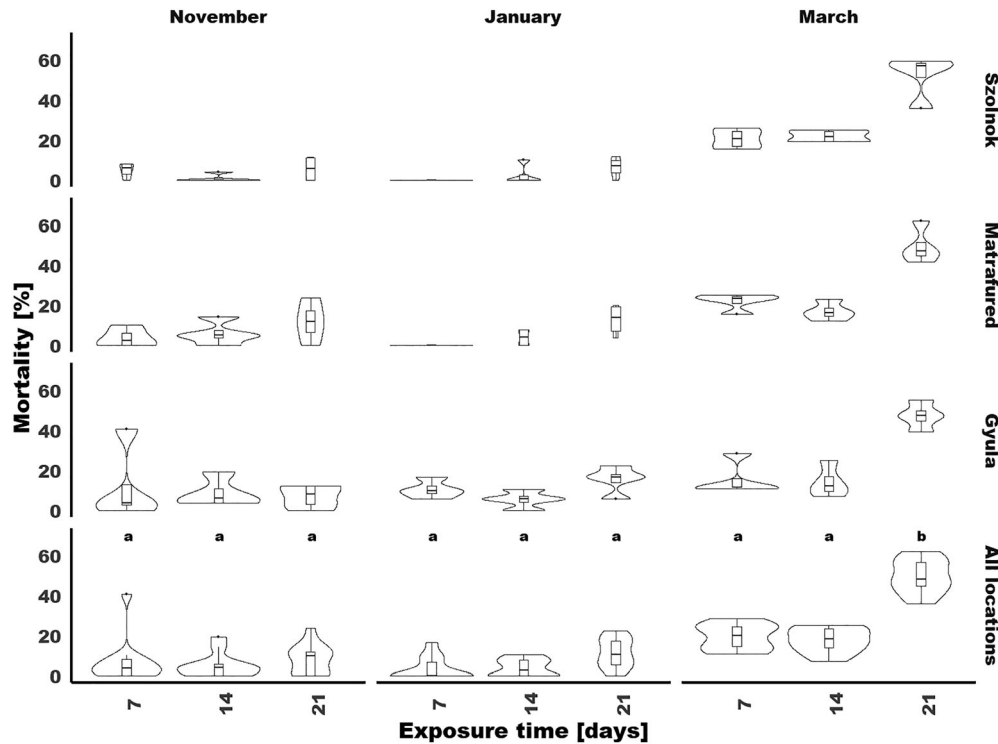


**FIGURE 1** Violin plots of supercooling point (SCP) values of adult *Corythucha arcuata* from three sites (Gyula, Mátrafüred and Szolnok) measured from November 2020 to March 2021. Each violin plot includes a standard box plot, which indicates the summary statistics (median, interquartile range and outliers), supplemented by a rotated, smoothed probability density distribution that describes the underlying shape of the data. Different lower-case letters refer to significant differences.

**TABLE 1** Results of Tukey's post-hoc tests comparing mean supercooling point (SCP) values among three study sites (Gyula, Mátrafüred and Szolnok) from November 2020 to March 2021. Significant differences are in bold.

|          |                           | Estimate       | Standard error | df         | t ratio       | p value       |
|----------|---------------------------|----------------|----------------|------------|---------------|---------------|
| November | <b>Szolnok-Mátrafüred</b> | <b>-0.0188</b> | <b>0.00388</b> | <b>423</b> | <b>-4.829</b> | <b>0.0002</b> |
|          | Szolnok-Gyula             | -0.0073        | 0.00385        | 423        | -1.903        | 0.8509        |
|          | Mátrafüred-Gyula          | 0.0114         | 0.00374        | 423        | 3.051         | 0.1388        |
| December | <b>Szolnok-Mátrafüred</b> | <b>-0.0191</b> | <b>0.00374</b> | <b>423</b> | <b>-5.091</b> | <b>0.0001</b> |
|          | Szolnok-Gyula             | -0.0080        | 0.00374        | 423        | -2.123        | 0.7190        |
|          | Matrafured-Gyula          | 0.0111         | 0.00371        | 423        | 2.993         | 0.1603        |
| January  | Szolnok-Mátrafüred        | -0.0064        | 0.00378        | 423        | -1.695        | 0.9338        |
|          | Szolnok-Gyula             | -0.0024        | 0.00378        | 423        | -0.635        | 1.0000        |
|          | Mátrafüred-Gyula          | 0.0040         | 0.00378        | 423        | 1.060         | 0.9993        |
| February | <b>Szolnok-Mátrafüred</b> | <b>-0.0136</b> | <b>0.00371</b> | <b>423</b> | <b>-3.662</b> | <b>0.0222</b> |
|          | Szolnok-Gyula             | -0.0010        | 0.00371        | 423        | -0.274        | 1.0000        |
|          | Mátrafüred-Gyula          | 0.0126         | 0.00371        | 423        | 3.388         | 0.0538        |
| March    | Szolnok-Mátrafüred        | -0.0112        | 0.00378        | 423        | -2.954        | 0.1764        |
|          | Szolnok-Gyula             | -0.0079        | 0.00378        | 423        | -2.086        | 0.7437        |
|          | Mátrafüred-Gyula          | 0.0033         | 0.00378        | 423        | 0.868         | 0.9999        |





**FIGURE 2** Violin plots of chilling mortality rates of adult *Corythucha arcuata* from three sites (Gyula, Mátrafüred and Szolnok) observed from November 2020 to March 2021 (as there was no significant difference according to site, statistical analysis for differences in exposure length were applied only for all locations). Each violin plot includes a standard box plot, which indicates the summary statistics (median, interquartile range and outliers), supplemented by a rotated, smoothed probability density distribution that describes the underlying shape of the data. Different lower-case letters refer to significant differences.

**TABLE 2** Results of Tukey's post-hoc tests comparing mean mortality rates under chilling conditions ( $-3$  and  $-5$  °C) after 7, 14 and 21 days among three study sites (Gyula, Mátrafüred and Szolnok) from November 2020 to March 2021. Significant differences are in bold.

|          | Contrast                        | Estimate | Standard error | df | t ratio | p value |
|----------|---------------------------------|----------|----------------|----|---------|---------|
| November | 7 day exposure–14 day exposure  | 0.2447   | 0.413          | 99 | 0.593   | 0.9996  |
|          | 7 day exposure–21 day exposure  | −0.1422  | 0.338          | 99 | −0.421  | 1.0000  |
|          | 14 day exposure–21 day exposure | −0.3869  | 0.393          | 99 | −0.984  | 0.9865  |
| January  | 7 day exposure–14 day exposure  | −0.0897  | 0.609          | 99 | −0.147  | 1.0000  |
|          | 7 day exposure–21 day exposure  | −1.0206  | 0.478          | 99 | −2.134  | 0.4568  |
|          | 14 day exposure–21 day exposure | −0.9308  | 0.442          | 99 | −2.104  | 0.4764  |
| March    | 7 day exposure–14 day exposure  | 0.0878   | 0.151          | 99 | 0.582   | 0.9997  |
|          | 7 day exposure–21 day exposure  | −0.9116  | 0.112          | 99 | −8.130  | <0.0001 |
|          | 14 day exposure–21 day exposure | −0.9994  | 0.120          | 99 | −8.306  | <0.0001 |

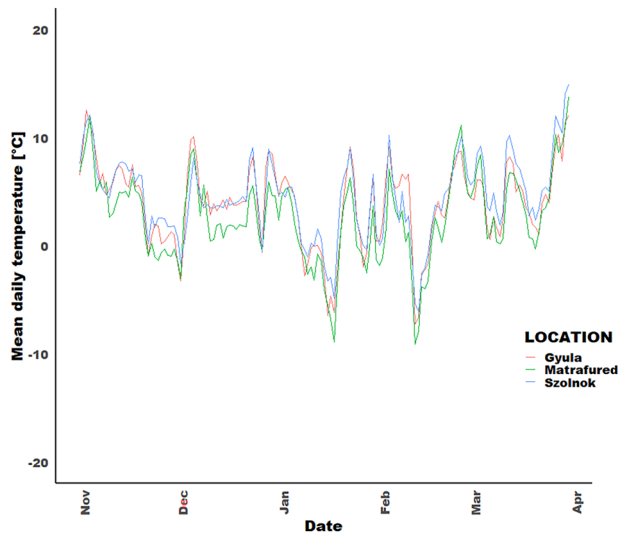
March was 25.65% ( $\pm 17.43\%$ ; SD), in November 9.25% ( $\pm 11.4\%$ ) and in January 10.44% ( $\pm 6.43\%$ ). In Szolnok, the highest average mortality rate of OLB was also recorded in March with 31.58% ( $\pm 16.59\%$ ), in November it was 3.89% ( $\pm 4.62\%$ ) and in January 2.97% ( $\pm 4.65\%$ ). In Mátrafüred, the mean mortality rate in March was 29.35% ( $\pm 15.95\%$ ), in November 7.19% ( $\pm 7.51\%$ ) and in January 5.55% ( $\pm 7.39\%$ ).

Taken together, the mean mortality rates under mild sub-zero conditions at the beginning of the winter season and in mid-winter

were quite low and clearly increased with the onset of spring. Moreover, longer cold exposure clearly resulted in higher OLB mortality rates at the end of winter.

### Air temperatures in the field

In Gyula (southern location) the lowest daily average temperature was  $-6.25$  °C ( $\pm 1.30$  °C; SD) measured on 12 February 2021. The lowest



**FIGURE 3** Daily average temperatures from three Hungarian locations (Gyula, Mátrafüred and Szolnok) from November 2020 to March 2021.

daily minimum of  $-11.7\text{ }^{\circ}\text{C}$  was measured on 13 February 2021, and the highest daily maximum was measured 2 weeks later on 26 February 2021 with  $20\text{ }^{\circ}\text{C}$ . In the central location Szolnok, the lowest daily average was measured on 13 February 2021 with  $-6.09\text{ }^{\circ}\text{C}$  ( $\pm 4.13\text{ }^{\circ}\text{C}$ ). The lowest daily minimum was  $-12.4\text{ }^{\circ}\text{C}$  measured on the same day. The highest daily maximum was recorded on 31 March with  $21.1\text{ }^{\circ}\text{C}$ . In Mátrafüred, which was the northernmost location at the highest elevation, the lowest daily average was on 12 February 2021 with  $-9.08\text{ }^{\circ}\text{C}$  ( $\pm 2.08\text{ }^{\circ}\text{C}$ ), the lowest daily minimum was 1 day later with  $-14.1\text{ }^{\circ}\text{C}$ . The highest daily maximum with  $20\text{ }^{\circ}\text{C}$  was recorded at the end of March. Taken together, all three locations experienced similar temperature patterns with coldest conditions in mid-February and temperatures started to increase in spring by March (Figure 3).

## DISCUSSION

Overwintering adults of OLB have a high tolerance to withstand cold temperatures and the supercooling capacity remained high over the entire winter season from November 2020 to March 2021, which is similar to another invasive species, the elm sawfly *Aproceros leucopoda* (Vétek et al., 2020). Although no significant differences among mean SCPs over time were found here (in none of the three locations), we observed a seasonal pattern in the highest and lowest SCP values. For example, in Gyula and Szolnok the lowest individual SCPs were measured in mid-winter, that is, December 2020. The highest SCPs in these two locations, however, were found by the end of winter, that is, February 2021. This is in contrast to the northernmost site Mátrafüred where the lowest SCP was recorded in February 2021 and the highest value in March 2021. This finding might be explained by the coldest environmental temperature in this location in February 2021, as insect cold tolerance can be a plastic response and individuals can respond

quickly to ambient conditions (Sobek-Swant et al., 2012). Moreover, our findings show that cold hardiness remains high also in spring, an adaptation to respond to late cold spells at the end of winter, as it was also shown in other species (Rocheftort et al., 2011; Vétek et al., 2020).

Our findings are similar to those of a closely related species, the sycamore lace bug *Corythucha ciliata* (Ju et al., 2010). For example, this species employs the same cold-tolerance strategy as OLB, that is, freeze avoidance, which might be explained by a shared evolutionary trajectory. Moreover, this species also shows a high tolerance to withstand cold conditions in its natural environment (Ju et al., 2010).

Although no strong variation in mean SCPs during the overwintering period was found here, we observed a significant difference in these values among sample locations. This might be explained by local habitat conditions, as lowest mean temperature values were found in the same location, that is, Mátrafüred, as the lowest SCPs. Mátrafüred is the northernmost site and is located at the highest elevation. Furthermore, there might be another effect of environmental parameters on SCPs. The tree species composition in the three locations is quite different, consisting of various oak species. As it was reported in other species (Liu et al., 2007; Liu et al., 2009) there might be an influence of different host plants, that is, *Quercus* spp., on the nutritional status of overwintering OLB, resulting in a variation to withstand cold temperatures.

It is important to keep in mind that SCP values are obtained from short-time laboratory studies and provide information on the physiological responses to low temperatures (i.e., ice formation of body fluids) and on the limits for survival under experimental conditions (Bale, 1993; Lee, 2010; Sinclair, 1999). Thus, these data might not necessarily reflect the performance of an overwintering insect in its natural habitat, however, they are the basis for understanding responses to cold or to evaluate seasonal variations and differences among various environments (Sinclair et al., 2015). Including different experimental approaches (e.g., SCP measurements and chilling injuries, as performed here) and field observations (Paulin et al., 2021) can help to draw ecologically meaningful conclusions on insect cold hardiness.

Overwintering *C. arcuata* probably have the potential to survive longer periods at higher sub-zero temperatures. Survival at both  $-3$  and  $-5\text{ }^{\circ}\text{C}$  was quite high at the beginning of winter as well as in mid-winter, even after 3 weeks, with mortality rates below 10%. Interestingly, mortality rates increased by the beginning of spring (March 2021), suggesting some temporal variation to cope with cold conditions. These data show that OLB is very likely to be well adapted to survive Central European winter conditions, even for longer time (especially, as continuous sub-zero laboratory conditions tested here, for up to 3 weeks, are not observed in nature, see Figure 3).

Taken together, our results suggest that cold winter temperatures might not be a major limiting factor for the establishment and spread of OLB in wide parts of its invaded European range, supporting the data of previous field observations in Hungary (Csepelényi et al., 2017; Paulin et al., 2021). As the native range of *C. arcuata* spans wide parts of North America (Drake & Ruhoff, 1965), it can be assumed that it is well adapted to harsh winter conditions.

Future studies should focus on additional abiotic and biotic factors that might affect the establishment and spread of *C. arcuata* in Europe. For example, the presence/lack of natural enemies, upper lethal temperatures or dry summer conditions, precipitation in the growing season, or overexploitation of food sources might have adverse effects on the performance of this bug. Moreover, additional studies on the overwintering biology will add important knowledge on the life history of this insect. For instance, intraspecific variation in the ability to withstand cold across a huge geographic range, additional experimental conditions to assess chilling-related mortality (even colder temperatures), behavioural strategies to seek favourable microhabitats, the physiological basis of hibernation or other environmental conditions affecting survival would provide valuable information for both cold tolerance research as well as applied forest management.

#### AUTHOR CONTRIBUTIONS

**Márton J. Paulin:** Conceptualization; data curation; formal analysis; investigation; resources; writing – original draft; writing – review and editing. **Csaba B. Eötvös:** Data curation; formal analysis; investigation; validation; visualization; writing – review and editing. **Petr Zabransky:** Formal analysis; investigation; writing – review and editing. **György Csóka:** Conceptualization; funding acquisition; project administration; supervision; validation; writing – review and editing. **Martin Schebeck:** Conceptualization; data curation; formal analysis; investigation; supervision; validation; writing – original draft; writing – review and editing.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

All raw data of this study are provided in the supplementary material.

#### ORCID

Martin Schebeck  <https://orcid.org/0000-0001-7376-4878>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1.** Dates of sample collection and sample sizes for supercooling point measurements of *Corythucha arcuata*.

**Table S2.** Dates of sample collection, as well as duration, conditions and sample sizes of chilling experiments with *Corythucha arcuata*.

**Table S3.** Results of Tukey's post-hoc tests comparing overall mean chilling mortality rates of adult *Corythucha arcuata* from three study sites (Mátrafüred, Szolnok, Gyula).

**Data S1.** Supporting information.

**Data S2.** Supporting information.

**Data S3.** Supporting information.

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