

# Cold tolerance strategy and cold hardiness of the invasive zigzag elm sawfly *Aproceros leucopoda* (Hymenoptera: Argidae)

Gábor Véték\*, Veronika Fekete\*, Márta Ladányi†, Elena Cargnus‡, Pietro Zandigiacoemo‡, Richárd Oláh\*, Martin Schebeck§<sup>1</sup> and Axel Schopf§<sup>1</sup>

\*Department of Entomology, Szent István University, Villányi út 29–43, H-1118 Budapest, Hungary, †Department of Biometrics and Agricultural Informatics, Szent István University, Villányi út 29–43, H-1118 Budapest, Hungary, ‡Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, via delle Scienze 206, I-33100 Udine, Italy and §Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna, BOKU, Peter-Jordan-Straße 82/I, A-1190 Vienna, Austria

- Abstract**
- 1 The invasive sawfly *Aproceros leucopoda* causes severe defoliation of various elm species and thus can be a major pest in forest stands and urban environments.
  - 2 The overwintering biology of *A. leucopoda* has not been investigated so far; therefore, the aim of this study was to determine the cold tolerance strategy and cold hardiness of hibernating *A. leucopoda* eonymphs.
  - 3 The supercooling points (SCPs) of overwintering individuals varied geographically, monthly and interannually and ranged between  $-12.14$  °C and  $-24.22$  °C.
  - 4 As none of the eonymphs survived once the SCP had been reached, *A. leucopoda* is classified as a freeze-avoidant species.
  - 5 Survival rates of overwintering eonymphs exposed to different sub-zero temperatures above the SCP ( $-1.6$  °C and  $-4.0$  °C for 10, 20 and 30 days and  $-10.5$  °C for 9 days) ranged between 89.2% and 100%, suggesting that *A. leucopoda* is not a chill-susceptible species.
  - 6 Our results suggest that low winter temperatures may not be expected to be an important limiting factor for the overwintering success of *A. leucopoda*.

**Keywords** Alien species, chill tolerance, freeze avoidance, overwintering, supercooling point, *Ulmus*.

## Introduction

The zigzag elm sawfly *Aproceros leucopoda* Takeuchi, 1939 (Hymenoptera: Argidae) is native to East Asia. First European records of this pest were reported from Poland and Hungary in 2003 (Blank *et al.*, 2010). Since then, *A. leucopoda* has spread to many countries of the continent and caused considerable damage to elm trees *Ulmus* spp., especially to Siberian elm *Ulmus pumila* L. in Hungary, Italy and Russia (Blank *et al.*, 2010; Véték *et al.*, 2010; Zandigiacoemo *et al.*, 2011; Artokhin *et al.*, 2012; Lengesova, 2012; Shchurov *et al.*, 2012; Blank *et al.*, 2014; Véték *et al.*, 2016; Martynov & Nikulina, 2017; Véték *et al.*, 2017). The pathways of its initial introduction into Europe, as well as the original sources of spread, remain unknown. For example, significant defoliations of elms by *A.*

*leucopoda* were observed in the European part of Russia as early as 2003–2004 (Shchurov *et al.*, 2012); at the same time, it was first recorded in Central Europe. In addition, first records of this pest in various Central European countries were reported at more or less the same time (Blank *et al.*, 2010). Therefore, its spread may not have involved a single origin, and a temporal pattern of its distribution might not be expected.

Elm trees repeatedly attacked by *A. leucopoda* may suffer severe or total defoliation that may cause the death of affected branches, as has been observed in many European countries (Blank *et al.*, 2010; Zandigiacoemo *et al.*, 2011; Lengesova, 2012; Lengesova & Mishchenko, 2013; Martynov & Nikulina, 2017). Due to the appearance and mass occurrence of this pest in the northern Cis-Azov region of Eastern Europe, even the potential complete loss of physiologically weakened *U. pumila* in some forest plantations has been predicted. These plantations usually suffer from drought and periodic mass attacks of the bark beetle *Scolytus kirschi* (Coleoptera: Curculionidae), thus being predisposed to additional stressors (Martynov & Nikulina, 2017).

Correspondence: Gábor Véték. Tel.: +3613057518; e-mail: vetek.gabor@kertk.szie.hu

<sup>1</sup>These authors are equally contributing senior authors.

*Aproceros leucopoda* seems to be feeding and developing exclusively on elms *Ulmus* spp. (Blank *et al.*, 2010; Yu *et al.*, 2011; Martynov & Nikulina, 2017). Females are thelytokous. They insert their eggs into the leaf edge, and the hatching larvae start feeding from the leaf margin in a characteristic zigzag pattern (Wu, 2006; Blank *et al.*, 2010; Lengesova & Mishchenko, 2013; Martynov & Nikulina, 2017). Larvae develop through six instars, typically consuming the entirety of leaves except for thick midribs. Non-overwintering individuals pupate in loosely spun, light-coloured cocoons attached to abaxial surfaces of leaves or woody parts of host plants or on the ground (Blank *et al.*, 2010; Papp *et al.*, 2018). This sawfly can produce four (Wu, 2006; Blank *et al.*, 2010) or even more generations per year (Mol & Vonk, 2015; Mol, 2017; Papp *et al.*, 2018).

*Aproceros leucopoda* overwinters as an eonymph in a brown, double-walled cocoon in the litter or upper soil layer (Wu, 2006; Blank *et al.*, 2010; Lengesova, 2012; Martynov & Nikulina, 2017). This cocoon is structurally different from that produced by the non-overwintering generations. While the latter one has a loose, net-like structure, the overwintering cocoon has a parchment-like inner wall and is covered with an outer wall composed of a strong grid of silk strands, additionally containing soil and organic particles. The winter cocoon has a barrel-like shape and is about 7–8 mm in length and 4 mm in width (Blank *et al.*, 2010; Martynov & Nikulina, 2017; Véték, personal observation). Cold winters with little snow cover have been considered to significantly reduce *A. leucopoda* populations, for example, in the Middle Volga region (Lengesova, 2012; Lengesova & Mishchenko, 2013); however, detailed studies on the cold tolerance strategy and cold hardiness of this species are lacking. Such data would add basic knowledge to the biology of *A. leucopoda* and would be important to understand winter survival of this invasive sawfly.

Insect cold tolerance encompasses a range of adaptations to cope with low temperatures, for example, dealing with freezing of body fluids, also including cryoprotective dehydration, avoidance of chill injuries, entering chill coma or performance of rapid cold hardening (Sinclair *et al.*, 2015). It is generally agreed that there are three basic types of responses to cold temperatures, corresponding to the primary types of insect cold tolerance: (i) chill-susceptible (or chill-intolerant) species die due to direct effects of low temperatures without internal ice formation; (ii) freeze-avoidant (or freeze-intolerant) insects can survive cold as long as ice does not form within their body; and (iii) freeze-tolerant insects tolerate extracellular ice formation in their haemolymph (Bale, 1993, 1996; Lee Jr, 2010; Sinclair *et al.*, 2015). Many insects evolved adaptations to cope with low sub-zero temperatures. For example, they can lower the freezing point of body fluids by producing antifreeze agents and stay in a supercooled state. The temperature when spontaneous ice formation finally occurs is called the supercooling point (SCP) and corresponds to the lower lethal temperature in freeze-avoidant species as ice formation is intracellular (Lee Jr, 2010; Sinclair *et al.*, 2015). In insects for which knowledge on cold tolerance strategies is scarce, measuring the SCP is an initial step to obtain insights into the overwintering biology (Sinclair *et al.*, 2015). As these basic data have not yet been determined for *A. leucopoda*, our main objectives were to (i) estimate SCPs of the overwintering eonymphs of the non-native zigzag elm sawfly

in different European countries and years; (ii) identify the cold tolerance strategy of the species; and (iii) determine whether the overwintering eonymphs are chill-susceptible. Our results add fundamental knowledge to the life history of the species and are important for both basic and applied fields.

## Materials and methods

### *Sampling locations and time of SCP measurements*

In autumn/winter (October, November, January and February) of 2013/2014, double-walled cocoons of *A. leucopoda* were collected once per month from soil samples of a mixed forest plantation, comprising black locust *Robinia pseudoacacia* L. and Siberian elm *U. pumila*, located near Kecskemét (46° 59' 23"N; 19° 40' 15"E), Hungary. Moreover, in March 2015, cocoons from the same Hungarian location were sampled. Furthermore, two additional soil samples were taken to obtain cocoons from a Siberian elm forest plantation located near Udine (46° 01' 15"N; 13° 13' 35"E), Italy, and from a forest belt of mixed vegetation dominated by Siberian elm located close to Gonars (45° 54' 47"N; 13° 13' 39"E), Italy, also in March 2015. Finally, further collections were conducted in March and April of 2017, again at the Kecskemét site (for details see Table 1). Prior to sample collection, severe defoliation of elm trees by *A. leucopoda* had been observed at each location for several years, indicating a relatively large number of cocoons for subsequent experiments. Collection and storage of sawfly cocoons lasted for a few hours or days in the field and in the laboratories of Szent István University (SZIU) and University of Udine. Afterwards, the cocoons were transferred to the laboratory of BOKU Vienna to measure SCPs of *A. leucopoda* eonymphs. The sawfly cocoons that were collected in Hungary and Italy in March 2015 were not personally carried but express-mailed to BOKU in cooling packages in which the temperatures did not decrease below 2 °C before the measurements. In the case of Hungarian samples, cooling packs ensured that the temperature did not exceed 13 °C until 17 March (arrival at BOKU). Subsequently, these cocoons were stored in a climate chamber (Sanyo MIR-553) set at 3 °C until the next day (18 March) when SCPs were measured. The samples from Udine and Gonars were sent in one cooled package on 16 March. Inside the polystyrene box with ice, the temperature was recorded using an iButton data logger (DS1922L). In this case, the temperature range in the box was 2.1–5.6 °C between 16 and 17 March (i.e. the date of arrival and SCPs measurements at BOKU).

### *Determination of SCPs and cold tolerance strategy*

Details on specimens used for cold tolerance experiments are provided in Table 1. Prior to SCP measurements, each cocoon was cautiously dissected, and the eonymph was carefully removed from it under a stereo-microscope (Wild M3). Living and healthy eonymphs were selected based on their vigorous abdominal movements. Pronymphs, when found, were distinguished from eonymphs by the presence of the pupal compound eyes shining through the more or less transparent head capsule (compound eyes are not visible in eonymphs) (Pschorn-Walcher, 1982). Here, only eonymphs were used for our experiments as it is the overwintering stage.

**Table 1** Sites and dates of collections of *Aproceros leucopoda* cocoons (HU – Hungary; IT – Italy), dates of supercooling point (SCP) measurements and numbers of overwintering eonymphs examined

Location	Date of collection	Date of SCP measurement	Number of eonymphs
Kecskemét (HU)	14.10.2013	17.10.2013	36
	19.11.2013	27.11.2013	35
	15.01.2014	20.01.2014	43
	26.02.2014	05.03.2014	48
	16.03.2015	18.03.2015	39
	23.03.2017	24.03.2017	19
	04.04.2017	07.04.2017	38
Gonars (IT)	15.03.2015	17.03.2015	4
Udine (IT)	15.03.2015	17.03.2015	32

SCP measurements were performed with a type-T (copper-constantan) thermocouple attached to a recording potentiometer, and temperature data were recorded using the programme 'Personal DaqView' (version 2.0.4, Measurement Computing GmbH). The tip of the thermocouple wire was adhered to the surface of the specimen with a small amount of petroleum jelly ('Vaseline') and subsequently placed in a 1.6-mL microcentrifuge ('Eppendorf') tube. This was inserted into a larger plastic tube ('Falcon') to provide better insulation and to ensure a constant cooling rate of  $2.5 \pm 0.4$  °C/min (mean  $\pm$  SD; below 3 °C). Ice formation in the insect body is indicated by the release of latent heat of crystallization, and the lowest temperature that precedes this exotherm is the SCP (Sinclair *et al.*, 2015).

To determine the cold tolerance strategy of *A. leucopoda* (i.e. freeze-avoidant or freeze-tolerant), eonymph survival was assessed after their individual SCPs. Eonymphs were either immediately removed from the freezer when the SCP had been reached or until a temperature of about  $-30$  °C to record a potential second exotherm. Afterwards, samples were stored at room temperature for a minimum of 12 h and examined for survival, that is, abdominal movement when touched.

#### Studies on chill tolerance

Soil samples from the mixed forest plantation at Kecskemét were collected on 26 October 2018 and transferred to SZIU. Double-walled cocoons, each containing a living eonymph of *A. leucopoda*, were obtained from these samples in the laboratory between 26 and 30 October. To preserve the original protective structure of the cocoons and avoid any damage during manipulation, each cocoon was carefully examined under a stereo-microscope (Alpha STO-4LED) without dissecting it. The eonymph inside was considered living, based on our previous experiences, if it had a green, not-shrunk body, occupying the whole cocoon inside its inner wall. Pre-freeze mortality caused by chilling was tested in a total of 219 specimens at six different experimental conditions, using 35–37 eonymphs per trial (for details, see Table 2). Cocoons were placed in 5.5-L plastic boxes in climate chambers (Sanyo MIR-553) at BOKU Vienna. Cocoons in three separate boxes were exposed to  $-1.6 \pm 0.1$  °C (mean  $\pm$  SD) for 10, 20 and 30 days, whereas

the other three groups were treated at  $-4.0 \pm 0.1$  °C for the same periods. All treatments were performed in constant darkness. At the end of the cold treatments, boxes were transferred to a climate chamber (Memmert IPP260plus) at 24 °C (and 16L:8D) and kept overnight. The temperatures in the boxes were recorded with data loggers (LOG32 T). To assess survival rates of eonymphs, cocoons were carefully dissected, and specimens were checked for signs of movement. Eonymphs were classified as 'alive' (evident body movement) or 'dead' (no movement). Alive eonymphs were tested again 2 days after the first examination to confirm survival.

To assess the effects of shorter periods with even colder temperatures on the survival of overwintering eonymphs, 49 cocoons were collected from soil samples taken at Kecskemét on 5 February 2019. They were placed in a 5.5-L plastic box in a climate chamber (RUMED thermostat 1001) at  $-10.5 \pm 0.1$  °C (mean  $\pm$  SD; constant darkness) for 9 days. The temperature in the box was recorded with a data logger (Greisinger T-Logg 160). Thereafter, the insects were rewarmed in a climate chamber (Memmert IPP260plus) at 22 °C (and 16L:8D) and examined for survival during the following days, as described above.

#### Statistical analyses

As a step of data cleaning, outliers were detected by standardization. According to the 1sigma-2sigma-3sigma rule, standardized values of normally distributed variables higher than 1.96 (or lower than  $-1.96$ ) can be considered outliers. We set the cut-off at 2.2. Assuming that SCP values have normal distribution, the probability of an SCP value with a standardized value above this threshold is 0.014. In this way, we excluded 18 extreme high SCP values (out of 312) – probably caused by unintentional lesions of the nymphal cuticle during preparation of samples. Mean SCP values were compared among collection dates by one-way ANOVA. Normality of the residuals was proven by their skewness (skew) and kurtosis (kurt) as their absolute values abs(skew) and abs(kurt) were both below 1. As the homogeneity of variances was violated (Levene's test;  $P < 0.05$ ), robust ANOVA tests (Brown-Forsythe and Welch) were also performed, and a post-hoc test was finally run using Games-Howell's method.

The survival rates of *A. leucopoda* eonymphs cold-treated in seven batches (i.e. chilling experiments) were compared by Marascuilo's procedure (Marascuilo, 1966). Statistical evaluations were performed with IBM SPSS (v25) and MS Excel 2016.

## Results

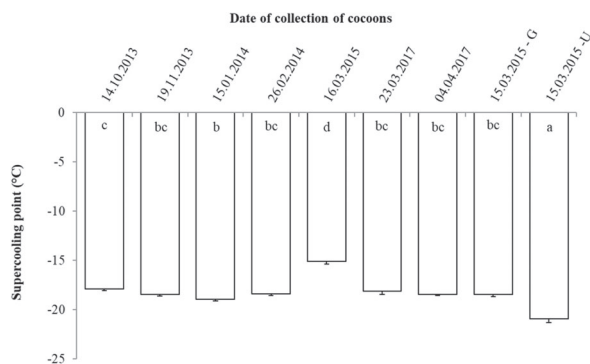
#### Supercooling points of *A. leucopoda* eonymphs

Overwintering eonymphs of *A. leucopoda* responded to cold conditions by extensive supercooling. There were significant differences between SCP values among different observations (ANOVA:  $F(8;285) = 49.34$ ;  $P < 0.001$ ; Brown-Forsythe:  $F(8;54.33) = 25.51$ ;  $P < 0.001$ ; Welch:  $F(8;163.31) = 51.38$ ;  $P < 0.001$ ). Games-Howell's post-hoc test results are shown in Fig. 1.

For the autumn/winter period of 2013/2014, the cold hardness of eonymphs from Kecskemét increased significantly from October ( $-17.89 \pm 0.98$  °C; mean  $\pm$  SD) to January

**Table 2** Survival rates of overwintering *Aproceros leucopoda* eonymphs in cocoons collected at Kecskemét, Hungary, in October 2018 (treatments at  $-1.6\text{ }^{\circ}\text{C}$  and  $-4.0\text{ }^{\circ}\text{C}$ ) and February 2019 (treatment at  $-10.5\text{ }^{\circ}\text{C}$ ) after various periods of exposure to sub-zero temperatures

Temperature ( $^{\circ}\text{C}$ )	Period of cold treatment (days)	Total number of cold-treated eonymphs	Number of alive eonymphs		
			1 day after the end of cold treatment	3 days after the end of cold treatment	Survival (%)
$-1.6$	10	37	33	33	89.2
	20	37	35	35	94.6
	30	35	35	33	94.3
$-4.0$	10	36	36	36	100
	20	37	36	36	97.3
	30	37	34	34	91.9
$-10.5$	9	49	49	49	100

**Figure 1** Mean supercooling points with standard errors (indicated by error bars) of overwintering eonymphs of *Aproceros leucopoda* collected between 2013 and 2017 and at different locations (G – Gonars; U – Udine, Italy; all the other samplings took place at Kecskemét, Hungary). Bars with different letters are significantly different from each other (Games-Howell's,  $P < 0.05$ ).

$(-18.95 \pm 1.04\text{ }^{\circ}\text{C})$ , when the lowest mean SCP was measured. SCPs in November 2013 ( $-18.45 \pm 0.90\text{ }^{\circ}\text{C}$ ) and February 2014 ( $-18.42 \pm 1.09\text{ }^{\circ}\text{C}$ ) showed intermediate values and were not significantly different from each other, nor from those measured in October and January.

Mean SCPs of eonymphs collected at Gonars in March 2015 and at Kecskemét in March and April 2017 ( $-18.44 \pm 0.50\text{ }^{\circ}\text{C}$ ;  $-18.12 \pm 1.45\text{ }^{\circ}\text{C}$ ;  $-18.43 \pm 0.82\text{ }^{\circ}\text{C}$ ) were not significantly different, neither from each other nor from any SCP values measured in 2013/2014. Interestingly, the samples from Udine, March 2015, had the lowest mean SCP value ( $-20.93 \pm 2.11\text{ }^{\circ}\text{C}$ ), and those from Kecskemét, March 2015, had the highest ( $-15.09 \pm 1.66\text{ }^{\circ}\text{C}$ ). These latter two differed significantly from all the other SCPs. The range of SCPs of all the measured eonymphs ( $n = 294$ ) of *A. leucopoda* was  $-12.14\text{ }^{\circ}\text{C}$  to  $-24.22\text{ }^{\circ}\text{C}$  (Fig. 1).

Survival tests after SCP measurements revealed that none of the specimens was alive 1 day after experimental trials. Thus, overwintering *A. leucopoda* eonymphs are freeze-avoidant.

#### Chill tolerance of *A. leucopoda* eonymphs

The rates of survival of overwintering *A. leucopoda* eonymphs in cocoons exposed to different sub-zero temperatures above the

SCP for a set of different periods are shown in Table 2. Across all experimental set-ups, the survival rates of *A. leucopoda* eonymphs ranged between 89.2% and 100% in the batches collected in October 2018. This rate reached 100% in the case of cocoons collected in February 2019 and treated at the lowest sub-zero temperature ( $-10.5\text{ }^{\circ}\text{C}$ ). Except for a single case ( $-1.6\text{ }^{\circ}\text{C}$ , 30 days), the number of alive individuals did not change between the first and the third day as they were being rewarmed at  $22/24\text{ }^{\circ}\text{C}$  after the end of the cold exposure (Table 2).

The survival rates of *A. leucopoda* eonymphs cold-treated in these batches were not significantly different among the seven experimental set-ups, either 1 or 3 days later (Marascuilo's  $\chi^2(6) < 4.48$ ;  $P > 0.61$ ).

## Discussion

Here, we performed the first formal investigation on the cold tolerance of the invasive zigzag elm sawfly *A. leucopoda*. To the best of our knowledge, cold tolerance strategies of sawflies belonging to Argidae have not been investigated so far. Thus, the present study is the first investigation on cold tolerance for this hymenopteran family.

Supercooling points of overwintering *A. leucopoda* eonymphs were determined in samples from three different locations in two European countries (Hungary and Italy), collected between 2013 and 2017. The presence of extensive supercooling ability, accompanied with the inability of eonymphs to survive once the SCP was reached, strongly indicates that *A. leucopoda* is a freeze-avoidant (i.e. freeze-intolerant) species. Supercooling is an important component of overwintering for many temperate insects (Bale, 2002). Moreover, freeze avoidance is the major cold tolerance strategy used by insect species in temperate regions of the northern hemisphere (Sinclair & Chown, 2005), where *A. leucopoda* is native to and occurs as an invasive alien species (Takeuchi, 1939; Véték *et al.*, 2016). Interestingly, some other sawfly species, such as the small spruce sawfly *Pristiphora abietina* (Hymenoptera: Tenthredinidae) and the mountain spruce sawfly *Pachynematus montanus* (Hymenoptera: Tenthredinidae), which occur in European spruce forests and also overwinter in the forest litter as a nymph in a cocoon (Pschorn-Walcher, 1982), are freeze-tolerant (Rosner & Führer, 1996; Schebeck *et al.*, 2015). As these two species belong to

another sawfly family, differences in cold tolerance strategies within Symphyta might be explained by different evolutionary histories.

Variations of SCPs among various months might reflect environmental conditions at the different sampling locations and in different years. Our results of the autumn/winter period of 2013/2014 in Kecskemét seem to indicate some dynamics of SCP variation during an overwintering season. The highest mean SCP value in October and the lowest in January, with two intermediate values in November and February, reflect an increase from autumn to winter followed by a decrease in cold hardiness after January. However, a change of only about 1 °C in the mean SCPs through the entire season was observed. The general pattern of SCPs – first a decrease from autumn to winter, followed by an increase with the onset of spring – has been reported from various insect species, such as the bark beetles *Ips typographus* and *Pityogenes chalcographus* (Coleoptera: Curculionidae) (Košťál *et al.*, 2011, 2014). Fluctuations in SCPs of *A. leucopoda* as a plastic response to varying environmental conditions might have been affected by ambient temperatures in the field. Sobek-Swant *et al.* (2012) found that the SCPs of winter-acclimatized freeze-avoidant emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae) prepupae increased after being exposed to mid-winter warm spells. Similar phenotypic plasticity may also apply to *A. leucopoda* under variable seasonal temperature events. This might also have resulted in the statistically significant differences of mean SCPs of the eonymphs collected at the same time but from three different locations in 2015.

According to Sømme (1982), geographical variations in supercooling ability within species should be expected, particularly in species exposed to fluctuating air temperatures. Ditrich *et al.* (2018) reported a strong association between winter climatic conditions and SCPs in geographically different populations of the linden bug *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae). Rochefort *et al.* (2011) observed considerable changes in the SCPs of hemlock looper *Lambdina fiscellaria* (Lepidoptera: Geometridae) eggs during the autumn/spring period but no significant effect of populations of different geographical origin. Similarly, our results show only minor differences in *A. leucopoda* cold hardiness among various geographic locations. Furthermore, Rochefort *et al.* (2011) found that, even in October and April, similar to our findings, individuals of *L. fiscellaria* could show a relatively strong supercooling capacity. Crosthwaite *et al.* (2011) also observed this latter phenomenon in *A. planipennis*. Further investigations are needed to reveal how different thermoperiods – typically experienced in the overwintering microhabitat – affect changes in cold tolerance, as well as the underlying physiological mechanisms in hibernating *A. leucopoda*.

It is also known from studies on the cotton bollworm *Helioverpa armigera* (Lepidoptera: Noctuidae) that different nutrient levels provided by different host plant species may affect overwinter survival (Liu *et al.*, 2007, 2009). Verdú *et al.* (2010) also found that food quality influenced the SCPs of the dung beetle *Thorectes lusitanicus* (Coleoptera: Geotrupidae). On the contrary, Rochefort *et al.* (2011) revealed no major effect of various hosts on the SCP of the geometrid *L. fiscellaria*. In our study, the host plant of all *A. leucopoda* specimens was the same (*U. pumila*) in all locations. Therefore, the plant taxon itself (but

not potential differences in the nutrient levels, which were not examined here) is not considered to have influenced differences in SCPs.

Soil moisture, as well as other soil parameters, may also affect the cold hardiness and overwinter survival of insects as it has been shown in adults of the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), which overwinters in burrows in the ground (Costanzo *et al.*, 1997). As the overwintering eonymph of *A. leucopoda* is covered with a double-walled cocoon in the soil, a relatively moderate influence of these factors on its supercooling capacity might be assumed. The cocoon may act as a barrier to inoculative freezing (Sømme, 1982; Sakagami *et al.*, 1985; Danks, 2000). This phenomenon occurs when an insect is in contact with environmental ice, which seeds the freezing of body water through natural body orifices or directly through the cuticle (Lee Jr, 2010). According to Rosner and Führer (1996), the cocoon of the sawfly *P. abietina*, although built up of three layers with the innermost being a dense one (Rosner, 1994), is permeable to water, and hence, contact of nymphs with external ice cannot be excluded. However, experiments showed that the freeze-tolerant *P. abietina* could withstand inoculative freezing (Rosner & Führer, 1996). Therefore, additional investigations on the water permeability of the double-walled cocoons of *A. leucopoda* are needed to assess if inoculative freezing is avoided in this species.

Here, survival of *A. leucopoda* eonymphs in cocoons collected in mid-autumn and late winter and exposed to prolonged periods at sub-zero temperatures above the SCP was tested to assess the level of potential pre-freeze mortality. Our results suggest that constant low temperatures may not be expected to cause substantial mortality, and overwintering eonymphs can tolerate several weeks of cold above the SCPs determined. Therefore, *A. leucopoda* is not considered chill-susceptible based on the classification by Bale (1993, 1996). However, further investigations on the survival of eonymphs collected at different times between late autumn and early spring (i.e. during overwintering) and exposed to various sub-zero temperatures above the SCP, even for longer periods than we tested here, are needed. In addition, and more importantly, the sub-lethal effects of chilling that may result in later death or in negative effects on development and performance of the affected individuals should be further studied to characterize the chill tolerance of *A. leucopoda* in detail.

Eonymphs of *A. leucopoda* overwinter in a cocoon in the forest litter or in the upper soil layers. Therefore, the overwintering site, together with the insulating properties of the soil, litter and also potential snow (MacKinney, 1929) – but less likely of the insect cocoon (Sakagami *et al.*, 1985) – are important considerations to assess survival of cold in this specific microhabitat. Temperatures above and below ground level may differ largely (Bale, 1987). Hence, *A. leucopoda* might experience buffered temperature conditions in nature that remain above the SCP data reported here. These factors, as well as effects of periods of prolonged cold, likely determine the overwintering success of this species. Additional studies on survival at low temperatures in the laboratory and in the field, combined with air and soil temperature records, along a wide range of habitats will help to understand the multifaceted overwintering biology of this invasive sawfly.

Basic data on the overwintering biology of *A. leucopoda*, such as its cold tolerance strategy and cold hardiness, improves our understanding of the performance of this sawfly during cold. Moreover, our results add another important piece to the knowledge on the life history of a rapidly spreading invasive insect.

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