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Cold hardiness of horseradish flea beetle (*Phyllotreta armoraciae* (Koch))

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Abstract

Supercooling ability and cold hardiness in the horseradish flea beetle *Phyllotreta armoracia* (Koch) (*Coleoptera*: *Chrysomelidae*: *Alticinae*) was investigated. Mean supercooling points (SCP) varied from −10.1 to −23.9°C depending on the season: SCP started to decrease in August, achieved their lowest value in mid-January after four months at 5°C, increased drastically in February and stayed at this high level until May. In contrast, some beetles had a distinctly high SCP during overwintering and survived freezing at their SCP. The beetles acquired sufficient cold hardiness already in September–October with lethal time for 50% mortality of 448 h; the temperature necessary to kill 50% of beetles after 24 h exposure was −12.2°C. The beetles were able to survive very low temperatures only during diapause period. Their cold tolerance decreased on the fifth month of hibernation with Ltemp$_{50}$ above −8°C. At the same time the beetles retained a relatively good ability to withstand the moderately low temperature of −6°C for longer periods after termination of diapause as over 60% of them survived 312 h exposure in February; however, all the feeding beetles in May were dead already after 96 h exposure.

Key words: *Phyllotreta armoraciae*, cold hardiness, supercooling, mortality, seasonality.

Introduction

The horseradish flea beetle *Phyllotreta armoracia* (Koch) (*Coleoptera*: *Chrysomelidae*: *Alticinae*) is a univoltine monophagous species feeding in nature only on horseradish, *Armoracia rusticana* G.M. and Sch. (*Brassicaceae*) (Nielsen et al., 1979). In the laboratory, the beetles can be reared on several glucosinolate-containing cruciferous plants, such as *Brassica napus* L., *Sinapis alba* L., *Brasssica nigra* Koch, and they show no preference between *B. nigra* and *A. rusticana* (Vig, Verdyck, 2001). However, in choice tests, beetles have preferred feeding on their host plant, horseradish (Hagerup et al., 1990). Horseradish is widely cultivated in Europe as a spice for pickles or as a side dish. It also tends to naturalize outside the cultivated areas (on abandoned fields). The horseradish flea beetle is the most prevalent and most damaging insect pest of the horseradish plant. The beetles are irregularly distributed: in some habitats plants are densely colonized by them and the leaves entirely perforated, whereas the leaves remain intact throughout the growing season in other locations. According to our long-term observations, the first beetles emerge from their overwintering sites on a sunny, warm day at the end of April and start eating immediately when the horseradish has barely sprouted. The major damage is caused by adults in springtime. Females lay their eggs on the petioles of young leaves in May, and the larvae burrow into the petioles (Nielsen et al., 1979). The new generation beetles emerge at the end of July and in the beginning of August and feed on the same plant for about one month. In mid-September, they start leaving the plants for their overwintering sites. The adults overwinter in debris and soil crevices not far from the host plants (Vig, 2002). In one of our observation stations, in herb collection garden where horseradish has been grown for many years, the plants were continuously heavily damaged, irrespective of the severity of winter. As a dispersed weed in open fields, plants were much less damaged.

So far, there are no reports about hibernation and cold hardiness in the horseradish flea beetle. Generally, insects survive low temperatures by using freeze tolerance or freeze avoidance strategies (Zachariassen, 1985), but some species are known to employ both (Duman, 1984; Gehrken et al., 1991). It is not known which strategy enables *P. armoraciae* to survive long and unstable winters; therefore this study focused on factors influencing supercooling ability and cold hardiness in this species.

Our study aimed to assess: 1) seasonal changes in supercooling points (SCP) of adults, 2) seasonal changes in cold tolerance of beetles after exposure to constant subzero temperature for different periods of time, 3) seasonal changes in cold tolerance of beetles after exposure of beetles to different subzero temperatures for a constant period of time.

Material and methods

Beetles. The experiments were performed from 2008 to 2009. The horseradish flea beetles were collected by a manual aspirator from horseradish in the medical herb garden of Tartu University (Estonia) (58°18′ N, 26°41′ E). For supercooling points measurements, the beetles were picked from the host plants in August and from soil crevices 1–3 m from the beds or from different weeds on which they were resting in September, when most had already left the food plant. Part of the beetles collected in September (a pre-winter group) were kept in a freezer (RF) with no access to food, at 5 ± 1ºC and absolute darkness in 1-l glass jars which were half-filled with lightly moistened peat until the experiments. Only a few beetles dug into the soil whereas most of them stayed on the walls and covers of the jars.

Supercooling points (SCP). The SCP of the beetles were measured by a chromel-alumel thermocouple-thermometer "RS-232 Data Logger Thermometer" ("TES Electrical Electronic", Taiwan). The low temperatures were attained by deep-freeze HF-103 (−30ºC). Prior to supercooling, the beetles were slightly anaesthetized with ether to render them immobile and then fixed individually by a thin Vaseline layer to the top of the thermocouple and sealed in a plastic tube. Tubes were placed in a cotton-lined container and transferred into the freezing chamber. A cooling rate of 1°C min^{-1} was used. The temperature at which freezing produced a release of latent heat was taken as the SCP of the individual. After a sudden spike in the measured temperature the beetles were removed from the freezer and left at room temperature to recover for 24 h before the assessment of their condition. The beetles were considered fit if they were active and able to move in a normal manner. Beetles not responding to tactile stimulation were considered dead.

Supercooling measurements included the beetles of the same generation. We started with summer beetles in August 2008 and finished with the overwintered beetles in May 2009. SCP were measured once a month, except in November. In August and September only, field-fresh beetles were used and before starting the experiment they were held for 24 h at room temperature (23 ± 1°C), with no access to food to allow them to empty their guts. In all other measurements beetles stored at 5 ± 1°C were used. The number of beetles (N) in each assessment varied from 16 to 20. After SCP measurements the sex of beetles was determined; 40% were male and 60% female. As sex did not affect their SCP, mixed sex beetles were used in all experiments.

Cold tolerance. The cold tolerance of beetles from various seasonal groups was assessed. In the first experiment, the effect of long-term exposure to the mortality at constant temperature of −6ºC was determined. Batches of beetles were wrapped into glass vials lined with filter-paper and transferred into the thermostat for 24, 48, 72, 96, 120, 144, or 312 h. In the second experiment, the beetles were exposed to various sub-zero temperatures: −6, −8, −10, −12, −14, −16, −18 or −20ºC for a constant period of time, 24 h. Steady cooling and warming rates were achieved using a liquid thermostat "Ministat 230 w-2" ("Huber", Germany). Each vial was supplied with thermocouple to register the temperature. The temperature fluctuation inside the vials did not exceed 0.5ºC. The beetles were cooled at a rate of 1ºC min^{-1} down to the required temperature and warmed up to 20ºC at the same speed after the termination of the exposure. After transferring from thermostat the beetles were placed in Petri dishes lined with lightly moistened filter-paper and left at room temperature to recover for 24–48 h; after which survival was recorded.

The cold tolerance was estimated at different dates: 1) in September when the beetles were ending their pre-winter feeding (field-fresh beetles), 2) at the end of October, 3) in December and 4) at the end of February (non-feeding beetles stored in RF at 5 ± 1°C and absolute darkness), 5) in May after emergence from overwintering sites when the food was already available (field-fresh beetles). Each group of beetles was replicated three times. The total number of beetles is represented in Tables 1 and 2.

Statistical analyses. Analyses of variance (one-way *ANOVA*) were used to assess the effect of seasonality on SCP of beetles. Differences in SCP values between measurement dates were analysed with Fisher LSD test. Differences in mortality of beetles between seasonal groups depending on exposure time or temperature were compared by two-way *ANOVA*. Differences in mortality depending on exposure time in February were compared by one-way *ANOVA*. Lethal time to reach 50% mortality of beetles (Ltime$_{50}$) after exposure to constant −6°C, and lower lethal temperature at which 50% of beetles died (Ltemp$_{50}$) when exposed for a constant 24 h period were estimated by *Probit Analyses* (*SAS/STAT version 9.1*, SAS Institute Inc., USA).

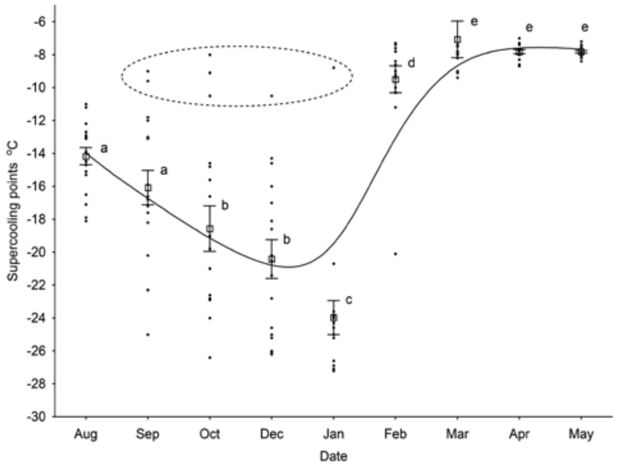
Results

Seasonal changes in supercooling ability of horseradish flea beetles. Figure 1 illustrates the seasonal changes in SCP of horseradish flea beetles. The mean SCP varied significantly over the period August to May ($F_{8;144} = 38.16$, $p < 0.001$); it decreased gradually from August to January, increased drastically in February and then remained at a high level until May.

In mid-August, during the pre-winter feeding, the mean SCP of the beetles was −14.2ºC. In September, when feeding had finished, the mean SCP decreased, although there were no statistically significant differences compared with the previous period. The SCP values tended to decrease further during October–December, when the beetles exhibited significantly lower SCP than in September. The lowest mean SCP, −23.9ºC as well as the lowest individual SCP, −27.2ºC was attained in mid-January.

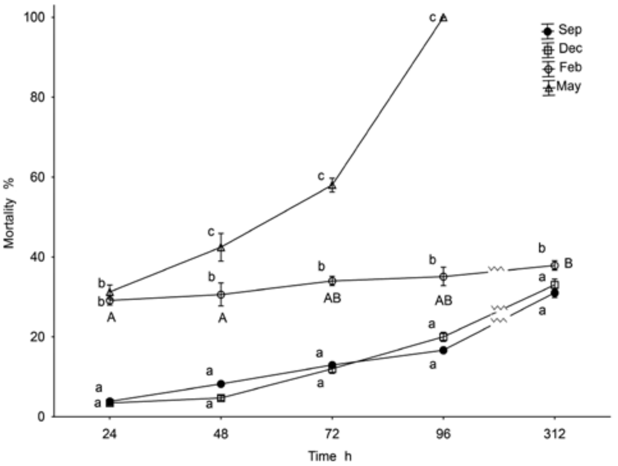
From August to February, a broad range of SCP values was observed. Individuals with SCP values of over −10°C survived their SCP (marked with a dotted circle on Fig. 1); after re-warming, these beetles were able to move in a normal way and gathered on the white mustard leaves we offered them. The beetles with SCP below −10°C did not survive the SCP measurement. In February, the SCP of most beetles had increased and achieved a mean value of −10.1ºC; by this time, only one beetle maintained a very low SCP (−20.1ºC). From March to May, the mean SCP did not change, remaining constant at about −8ºC with very small individual variability. All these beetles survived their SCP (Fig. 1).

Effect of duration of exposure on mortality of horseradish beetles from various seasonal groups at constant −6ºC. The mean mortality of beetles from different seasonal groups after exposure to −6ºC for different periods of time is shown in Figure 2; it changed significantly over time. The probit analysis results, the time required to cause 50% mortality of beetles (Ltime$_{50}$) are presented in Table 1. The overlapping of their 95% fiducial limits indicated no significant differences in mortality between estimating dates.



Note. Different letters above columns indicate significant differences between the means (Turkey's test, $P < 0.001$); N = 20 for both groups in May and August, N = 17 for each group from September to January, N = 16 for each group from February to April.

Figure 1. Seasonal changes of supercooling points (°C, mean ± SEM) in adult *Phyllotreta armoraciae*



Notes. Bars (mean ± SEM) with different lowercase letters within the same exposure duration are significantly different (Fisher LSD test, $P < 0.05$). Different upper case letters below the bars indicate the significant difference at various exposure durations within the same evaluation date.

Figure 2. Mean mortality (%) of *Phyllotreta armoraciae* beetles from different seasonal groups after exposure to −6.0°C for different periods of time

Table 1. Time required to cause 50% (Ltime_{50}) of *Phyllotreta armoraciae* beetles at a constant temperature −6°C

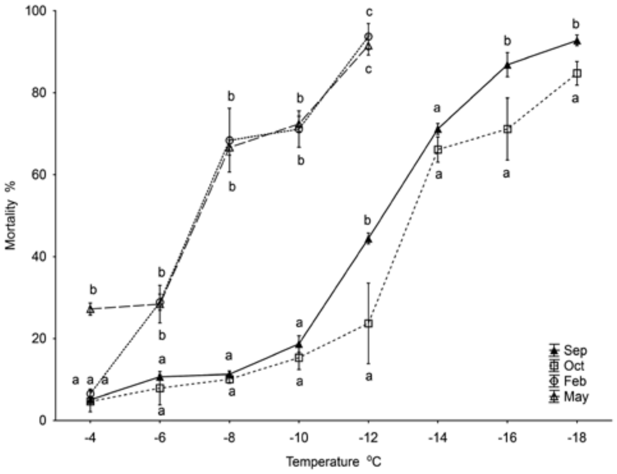
Estimation time	N	Ltime_{50} h	95% fiducial limits h
September	285	448 a	337.5 to 748.2
December	282	408 a	321.7 to 599.3
May	236	50 b	42.8 to 56.0

Note. Ltime_{50} of different seasonal groups followed by same letters are not significantly different based on the overlap of their 95% fiducial limits.

The season ($F_{3;49} = 40.36$, $p < 0.001$) as well as the duration of exposure ($F_{1;49} = 341.27$, $p < 0.001$) had a significant effect on the mortality of beetles: the longer the exposure, the higher the mortality in all groups. There was also a significant interaction between season and duration of exposure ($F_{3;49} = 79.03$, $p < 0.001$).

The beetles acquired high cold tolerance already in autumn. The highest cold tolerance with lowest mortality was observed in October; when more than 60% of beetles survived 312 h exposure at −6°C and calculated Ltime_{50} was 448 h. In December cold tolerance of beetles did not change substantially; there was no statistically significant difference from the previous period ($p > 0.05$), their 95% fiducial limits of calculated Ltime_{50} values overlapped. In February, the beetles started losing their cold tolerance and the mortality of beetles was significantly higher than in October or December ($p < 0.001$), still significantly lower than in May ($p < 0.001$). LTime_{50} value in February is not shown; the data did not enable calculation of their fiducial limits, because mortality rate remained almost on a plateau at different exposure durations, although the percentage of mortality increased slowly and significantly with prolonged exposure ($F_{4;10} = 3.50$, $p = 0.049$). Most sensitive to low temperatures were the overwintered feeding beetles in May with no surviving 96 h exposure at −6ºC, Ltime_{50} equalled to 50 h.

Effect of low temperatures on mortality of horseradish beetles from various seasonal groups after constant 24 h exposure. Mortality of beetles from various seasonal groups after 24 h exposure to different low temperatures is presented in Figure 3 and Table 2. Mortality increased with the decrease of temperatures in all groups irrespective of the season ($F_{1;30} = 426.85$, $p < 0.001$). The effect of measuring date was not significant ($F_{1;30} = 1.98$, $p = 0.12$), however the interaction between date and temperature was significant ($F_{3;30} = 6.13$, $p < 0.001$).



Note. Different letters above the data points indicate statistically significant difference between the different seasonal groups at the same temperature (Fisher LSD test, $P < 0.05$).

Figure 3. Mortality of *Phyllotreta armoraciae* beetles from different seasonal groups after 24 h exposure to various subzero temperatures

The beetles acquired a high capacity to survive very low temperatures for a short period already in September when they had stopped feeding. In October, the cold tolerance of beetles increased slightly; nevertheless there was no significant difference in mortality at most temperatures in comparison with the previous period. In the frame of this experiment, the most tolerant to low temperatures were the beetles in October with Ltemp$_{50}$ = −13.8 h with over 10% of beetles surviving −18°C. In February, their cold hardiness decreased notably, as significantly fewer beetles survived at all temperatures; the temperature necessary to kill 50% of beetles was −7.7°C. The tolerance of beetles remained low until the spring. In May the Ltemp$_{50}$ was −7.1°C. The lowest temperature the beetles could survive as well in February as in May was −12.0°C.

Table 2. Temperature required to cause 50% mortality *Phyllotreta armoraciae* beetles (Ltemp$_{50}$) after 24 h exposure

Estimation time	N	Ltemp$_{50}$ °C	95% fiducial limits °C
September	546	−12.2 a	−12.7 to −11.7
October	570	−13.8 a	−14.7 to −12.9
February	282	−7.7 b	−8.3 to −7.2
May	307	−7.1 b	−7.7 to −6.4

Note. Ltemp$_{50}$ of different seasonal groups followed by different letters are significantly different based on the lack of overlap of their 95% fiducial limits.

Discussion

Supercooling points (SCP) measurements in combination with survival data indicated that the cold hardiness and supercooling ability of horseradish flea beetles changed seasonally. This phenomenon has been reported by many authors for different insect species (Sømme, 1982; Koštal, Šimek, 1995; Vernon, Vannier, 2002; Koch et al., 2004, Worland, 2010). SCP as well as cold tolerance are primarily influenced by two processes: cold acclimation and development of diapause and with the physiological changes accompanying these processes like the accumulation of cryoprotectants and antifreezes, dehydration of organism, inactivation of ice-nucleating agents etc. (Zachariassen, 1985; Denlinger, 1991; Block, 2003; Hodkova, Hodek, 2004; Koštal, 2006). The gradual lowering in SCP and increase in cold tolerance in horseradish flea beetles started with the onset of the diapause in August when the temperature decreased and days shortened, deepened as the autumn progressed and the beetles stopped feeding (acclimation and diapause development) and achieved the maximum in mid-January (deep diapause). In February SCP of beetles increased drastically and simultaneously, whereas cold tolerance decreased.

Horse radish flea beetles had high supercooling ability and were able to survive a short (24 h) exposure to relatively low temperatures or longer exposures to a moderately low constant temperature (−6°C) from September to January during pre-diapausing and diapausing periods. Relatively low mortality of beetles was observed in this period. Generally in most of the beetles the diapause is terminated before the end of January (Koštal, Šimek, 1996). Based on specification of insect diapause (Koštal, 2006) horseradish flea beetles belong to the species whose diapause reached its end spontaneously despite keeping them at constant above freezing temperature and resumption of their development followed immediately. In February, after fifth months at 5°C when some of the overwintering beetles were transferred to room temperature and provided with white mustard leaves they started eating and, in a little while mating. By this time SCP of beetles increased drastically and remained unchanged until May; at the same time temperature at which 50% of beetles would die (Ltemp$_{50}$) increased. Still, probit analyses data did not enable estimation of the fiducial limits for lethal time (Ltime$_{50}$), because mortality did not change much with extension of exposure duration at moderately low temperature in February when the development was prevented only by low temperature. Evidently, the cohort of beetles tested at different dates consisted of individuals with different viability. On inspection of the beetles overwintering at 5°C we found that the less viable beetles (nearly 25%) had been dead by February because of undetermined reasons, definitely not because of the low temperature and only the stronger individuals with a chance of survival until spring were included in the trial. Active feeding beetles collected in May prove to be the most cold sensitive, none of which survived 96 h exposure to −6°C.

The overwintering insects would die not only because of the freezing. Several physiological, biochemical and metabolic dysfunctions induced by cold are described by Renault (2011). According to Danks (2005), mortality in many insects results from the effect of cold rather than freezing. Long-term exposure takes weeks or months and leads to significant mortality in the absence of freezing (Rojas, Leopold, 1996). The temperature of −6ºC used for prolonged exposure was higher than the mean SCP of horse radish beetles in all seasonal groups. That is why we suppose that mortality in beetles could result from the cumulative effect of chill injuries during exposure times not from the freezing. Despite the increased mortality after termination of diapauses, a relatively large proportion of the beetles maintained their ability to withstand moderately low temperature for a longer period of exposure. This reflects the adaptation of beetles to the long period they must spend in unfavourable cold conditions before the onset of warm weather.

Generally, insects survive low temperatures by the use of freeze tolerance or freeze avoidance strategies (Zachariassen, 1985; Lee, 1991). Freeze avoidance usually involves a relatively low SCP, whereas freeze tolerant insects usually have a high SCP (Turnock, Fields, 2005). High SCP of feeding insects is explained by food particles in their alimentary tract which induce ice formation at relatively high temperatures (Zachariassen, 1985) and with high water content in its organism (Block, 2003). Overwintering horseradish flea beetles are mostly freeze avoiding with low SCP in the diapausing period, however, at the same time, a few specimens had a relatively high SCP irrespective of the season or feeding rate (Fig. 1). Individuals with SCP up to −10°C survived their SCP even though their gut contents were not causing freezing since they were in non-feeding state. Soon after re-warming they were able to move in a normal way; individuals with a SCP substantially lower got killed. This suggests that this species may simultaneously possess two different strategies. In the literature, some species employing both strategies are described (Gehrken et al., 1991; Koštal, Havelka, 2000; Sinclaire et al., 2003). For example, freeze-tolerant sugar-beet maggot would become freeze avoidant as a result of microhabitat selection (Rinehart et al., 2009). According to Bale et al. (2001), the response to multiple freeze-thaw cycles involves bet-hedging strategy with some individuals retaining their initial freeze tolerance, whereas others become freeze avoiding. The horseradish flea beetle overwinters in debris and soil crevices a few centimetres below the soil surface not burrowing deeply in the ground (Vig, 2002). In snowless, unstable winters they are highly exposed to repeatedly fluctuating temperatures and freeze-thaw cycles, so two different strategies would enable them to survive unpredictable winters.

Winter survival of insects is greatly related to the choice of overwintering sites and on the severity of a given winter. One reason for an occasionally high population density in Nordic temperate areas could probably be the presence of suitable overwintering sites nearby; for instance in our experiment, horseradish bed had a hedgerow located within 10 m of it and a trench filled with debris closely surrounding it. In large open fields with less suitable microhabitats the plants were almost undamaged.

Conclusions

1. Supercooling ability as well as cold hardiness of horseradish flea beetles changed seasonally. The lowest mean supercooling points (SCP) (−27ºC) were measured in January; the rapid increase of SCP were observed in February, when the mean SCP rose to −11ºC. The highest cold tolerance with lowest mortality was observed in October when the time required to cause 50% mortality (Ltime$_{50}$) at a constant temperature −6°C equalled 448 h; temperature required to cause 50% mortality (Ltemp$_{50}$) after 24 h exposure equalled −13.8°C. The beetles are most susceptible to low temperatures in springtime after termination of the diapause.

2. Horseradish flea beetles have two different overwintering strategies: beetles with low supercooling ability (SCP −7…−12ºC) during diapause period belong to the freeze tolerant group and beetles with high supercooling ability (−18…−24ºC) belong to the freeze avoiding group.

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Krienų spragės (*Phyllotreta armoracia* (Koch)) atsparumas šalčiui

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Santrauka

Tirta krienų spragės *Phyllotreta armoracia* (Koch) (*Coleoptera*: *Chrysomelidae*: *Alticinae*) geba ištverti žemą temperatūrą ir atsparumas šalčiui. Priklausomai nuo sezono, vidutinė žemiausia temperatūra, kurią toleravo spragės, buvo nuo −10,1 iki −23,9° C. Žemiausia toleruotina temperatūra ėmė mažėti rugpjūčio mėnesį, žemiausias reikšmes pasiekė sausio mėn. viduryje, po keturių mėnesių ekspozicijos esant +5° C temperatūrai, staigiai padidėjo vasario mėnesį ir tame pačiame lygmenyje išliko iki gegužės mėnesio. Atvirkščiai, kai kurie vabalų individai žiemojimo metu turėjo aukštą toleruotiną žemiausią temperatūrą ir išgyveno juos šaldant jų toleruotinoje žemoje temperatūroje. Krienų spragės pakankamą atsparumą šalčiui įgijo jau rugsėjo–spalio mėnesiais: 448 valandų ekspozicija buvo letalinis laikas, lėmęs 50 % spragių mirtingumą; temperatūra, būtina žūti 50 % spragių po 24 valandų ekspozicijos, buvo −12,2° C. Spragės labai žemoje temperatūroje sugebėjo išgyventi tik diapauzės metu. Jų atsparumas šalčiui sumažėjo penktą žiemojimo mėnesį, Ltemp_{50} esant daugiau nei −8° C šalčio. Kartu spragės išlaikė gana gerą gebėjimą ištverti vidutiniškai žemą −6° C temperatūrą ilgesnį laiką pasibaigus diapauzei, nes daugiau nei 60 % spragių išgyveno 312 valandų ekspoziciją vasario mėnesį. Tačiau gegužės mėnesį visos besimaitinančios spragės žuvo jau po 96 valandų ekspozicijos.

Reikšminiai žodžiai: *Phyllotreta armoraciae*, atsparumas šalčiui, didelis atšaldymas, mirtingumas, sezoniškumas.