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DEPRESSED SUPERCOOLING POINT AND INCREASED GLYCEROL CONCENTRATION IN OVERWINTERING ADULT TIGER BEETLES (*Cicindelida*)

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Abstract

BACKGROUND: Tiger beetles are a widely distributed group including species that may be exposed to sub-freezing temperature overwinter. Despite being well studied, little is known about tiger beetle cold tolerance. **OBJECTIVE:** We investigated seasonal changes in cold hardiness of two northerly distributed tiger beetle species (*Cicindela repanda* and *Cicindela limbalis*). **MATERIALS AND METHODS:** We monitored the supercooling point (SCP), glycerol concentration, and hemolymph osmolality of adult tiger beetles during a 3.5-month acclimation to winter. **RESULTS:** SCP decreased during winter acclimation for *C. repanda*, but not for *C. limbalis*. Both species modestly increased glycerol concentration, and *C. repanda* increased hemolymph osmolality by 38%. **CONCLUSION:** This initial investigation into the cold-hardiness of adult tiger beetles suggests that they are capable of lowering their SCP as winter approaches, which may help them survive sub-freezing winter temperatures. Further assessment of their chill and freeze tolerance and of their overwintering conditions in the field is needed to better understand their winter physiology.

Keywords: tiger beetle, overwintering, supercooling point, glycerol, chill-tolerance

INTRODUCTION

Tiger beetles are a group of predatory beetles within the family Carabidae that are distributed globally in a diverse range of ecosystems. They are known for their charismatic colouration, highly visual hunting behavior (23), and their use in studies of community ecology (2, 13, 21), insect predatory behavior (11, 20) and high-temperature physiology (7, 23, 25). Their inclination to be active during the hottest part of the day (22) has made tiger beetles particularly useful in examining how insects use a combination of behavior, physiology, and morphology to specialize in ecological niches driven by high temperature. In contrast, little attention has been paid to how tiger beetles tolerate low temperature. Several species of tiger beetles inhabit temperate climes (15, 30) and, depending on their microhabitats, may be exposed to sub-freezing temperature in the winter.

Although low temperature can be lethal to insects, many species, including other carabid beetles (9, 14, 24, 28), survive routine exposure to sub-freezing in the winter by either tolerating ice formation or by remaining supercooled, thereby avoiding freezing (19). Because the cold tolerance of freeze-avoiding insects is limited by their ability to remain supercooled, their cold hardiness can be assessed by measuring the temperature at which they freeze, termed the supercooling point – SCP (26). Many insects lower their SCPs by accumulating low-molecular weight compatible solutes, such as glycerol (1, 6, 26), thereby increasing their osmolality and depressing the freezing point of their body fluids.

Because so little is known about the overwintering biology of adult tiger beetles [but see Criddle (5) and Gwiazdowski et al. (12)], we investigated the cold hardiness of *Cicindela repanda* (bronzed tiger beetle) and *Cicindela limbalis* (common claybank tiger beetle). Both species exhibit the spring-fall life history in which larvae from eggs laid in spring overwinter in terrestrial burrows, pupate and eclose the following fall, and overwinter again as adults before mating the following spring. Thus, adults are active above ground in both the spring and fall (3, 23, 31). We measured both SCP and glycerol concentration of individual *C. repanda* and *C. limbalis*, and the hemolymph osmolality of *C. repanda*, during a 3.5-month acclimation to winter.

MATERIALS AND METHODS

Collection and care

Cicindela repanda were collected in August and September from a sandy beach along the Mississippi River, in Rock Island County, Illinois (41.6°N, 90.4°W), and *Cicindela limbalis* were collected in September from Knox County, Nebraska (42.8°N, 97.9°W). Beetles were then transported to Augustana College in Rock Island, IL, where they were separated by species, housed in small transparent polycarbonate vessels (Sigma, GA-7) in groups of three to four, and kept in an environmental cabinet (Tritech Research, DT2-MP-47L) that controlled temperature and light exposure. Each box contained ~5 cm of sand from the *C. repanda* collection site that had been autoclaved, dried, and rehydrated with deionized water to 2% (w:w). Beetles had continual access to a water source (Fluker's Cricket Quencher) and were fed commercially available Gryllidae *ad libitum* until they refused to eat in mid-autumn. To induce winter acclimation, beetles were exposed to daily temperature cycles approximating seasonal soil temperatures at 10-cm depth in the upper Midwest (4) and corresponding light cycles approximating those at the latitude of both collection sites (Fig. 1). Temperature ranged from 14-22°C to 2-5°C, and daily light exposure ranged from 12 h to 9.25 h.

Chill tolerance and SCP

We assessed chill tolerance, the ability to survive low-temperature exposure in the absence of internal freezing, of *C. repanda* in late August and early September by exposing beetles to -3°C (n=10), -6°C (n=10), or -8°C (n=10), noting whether they froze, and monitoring their recovery. Each beetle was weighed to the nearest mg and

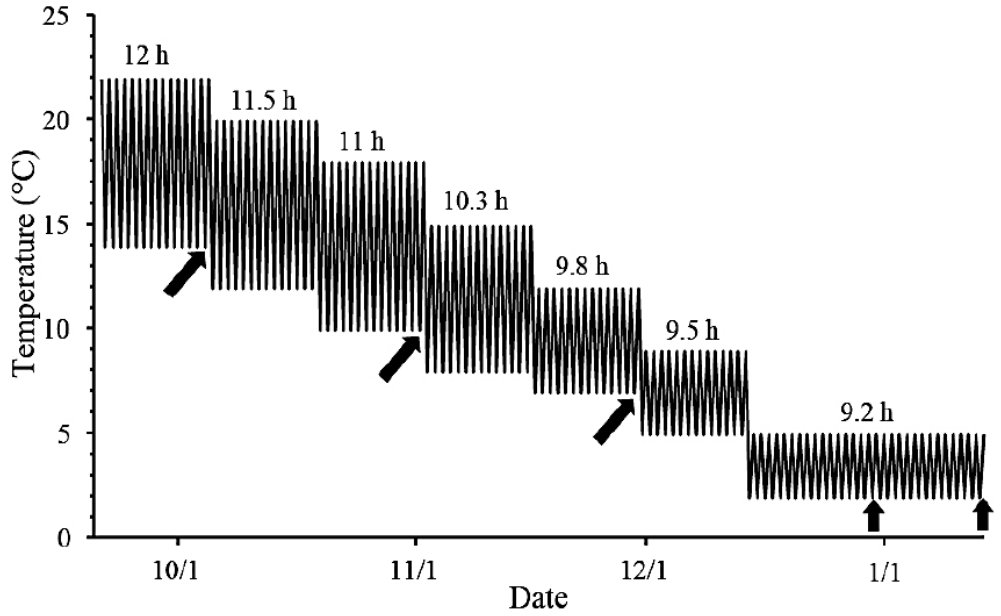

Figure 1. Thermal and photic regimen to which *Cicindela repanda* and *C. limbalis* were exposed during acclimation. Temperature was cycled daily and decreased every two weeks until reaching the final range of 2-5°C in mid-December. Daily light-dark cycles were synchronized with temperature cycles, and day length ranged from 12 h in early fall to 9.2 h in winter. Daily light durations are listed above the trace. Arrows denote sampling dates.

placed individually inside clean, dry, 0.6-mL microcentrifuge tubes. A 26-gauge type-T thermocouple was inserted into each tube and the tube's opening was then plugged with plastic foam. During cooling, temperature was recorded at 5-s intervals using a data logger (Omega, TC-08). Each tube was then placed individually inside a 50-mL centrifuge tube that was immersed in a programmable ethanol bath (Neslab, RTE 10) and cooled at 1°C h^{-1} from 10°C to its target temperature, held at the target temperature for 24 h, and rewarmed to 10°C at 1 °C h^{-1}, after which, the beetles were allowed to recover at ~20°C. During the ensuing 48-h recovery period, each beetle had access to water and was assessed for voluntary movement and response to tactile stimuli.

SCP of *C. repanda* and *C. limbalis* were measured periodically during a 3.5-month winter acclimation (Fig 1). For each sample date, beetles (n=10, except on 5 October where n=9 for *C. repanda*, and on 14 January where n=11 for *C. repanda* and n=5 for *C. limbalis*) were treated as above and cooled at 0.5°C/min from 15°C (5 October and 2 November), 10°C (30 November), or 5°C (30 December and 14 January) until spontaneous freezing occurred. The SCP, defined as the recorded temperature immediately preceding the freezing exotherm, was determined for each beetle. Each beetle was then stored at -80°C until it was used in a glycerol assay (see below).

Glycerol concentration & hemolymph osmolality

Whole-body glycerol concentration was measured for each beetle used in the supercooling point trials. An additional subset of *C. repanda* (n=8) was taken directly from the holding boxes at each sample date, except on 14 January, for glycerol measurement. Each of those beetles was weighed to the nearest mg, frozen in liquid N_2, and stored at -80°C. Whole-body glycerol concentration was measured by homogenizing each beetle at 4°C in a Bullet Blender tissue homogenizer (NextAdvance, BBY24M); first in the absence of a homogenization solution to pulverize the exoskeleton, and then in the presence of ice-cold 1-N $HClO_4$ to denature metabolic enzymes. The homogenate was then centrifuged at 2000*g*, the supernatant neutralized with ice-cold 1-N KOH, and a colourimetric assay (Sigma, MAK117) was performed to measure glycerol concentration. Hemolymph was collected from *C. repanda* taken directly from the holding boxes on 2 November (10 beetles), 30 November (8 beetles), and 30 December (9 beetles). Due to a limited number of beetles, hemolymph was not collected on 5 October or 14 January. Hemolymph osmolality was measured using 5-μL samples of hemolymph on a vapour-pressure osmometer (Wescor, model 5500) calibrated with NaCl standards. In cases where less than 5 μL of hemolymph was collected from an individual beetle, samples from two beetles were combined such that there were six independent osmolality values for each sample date. No individual beetle was represented more than once in the osmolality measurements.

Statistical analysis

Statistical analyses were performed using Sigma Plot 11.1; significance was accepted at $P \leq 0.05$. In cases where necessary assumptions were met, parametric tests were used, whereas non-parametric tests were used otherwise. Body mass was compared among sample dates within a species using one-way ANOVAs. SCPs were compared among sample dates within a species using one-way ANOVAs on ranks followed by Dunn's post hoc tests. A two-way ANOVA revealed no significant difference in glycerol concentration between *C. repanda* that had been used in the SCP trials and those taken directly from their holding boxes, so their values were pooled within each sample date. Glycerol concentrations were compared among sample dates within a species using one-way ANOVAs followed by Student-Newman-Keuls post hoc tests. Spearman's rank-order correlation was used to investigate the relationship between glycerol concentration and supercooling point for beetles used in both assays. Hemolymph osmolality of *C. repanda* was compared among sample dates using a one-way ANOVA followed by Student-Newman-Keuls post hoc tests.

RESULTS

Beetles of both species readily constructed burrows at the beginning of the study and shuttled between them and the substrate surface. By mid-December, beetles were found exclusively within burrows. Because of this behavior, care was taken during the first three sampling dates to include beetles found on the surface and those found within burrows. Both species routinely fed on the available crickets until late November when the beetles began ignoring them and became aphagic. Mean body mass of *C. repanda* used in the study

Table 1. Mean (± SEM) body mass, whole-body glycerol concentration, and hemolymph osmolality of tiger beetles during winter acclimation.

Date (M/D)	10/5	11/2	11/30	12/30	1/14
			Body mass (mg)		
C. repanda	66 ± 3 (17)	68 ± 2 (28)	70 ± 2 (26)	67 ± 2 (27)	70 ± 3 (11)
C. limbalis		109 ± 6 (10)	122 ± 6 (10)	114 ± 5 (10)	104 ± 5 (5)
			Glycerol (μmol g^{-1})		
C. repanda	0.9 ± 0.1a (17)	1.2 ± 0.1a,b (18)	1.2 ± 0.1a,b (18)	1.5 ± 0.1b (18)	2.1 ± 0.2c (11)
C. limbalis		1.0 ± 0.1 (10)	1.4 ± 0.1 (10)	1.4 ± 0.1 (10)	1.5 ± 0.04 (5)
			Hemolymph osmolarity (mosmol kg^{-1})		
C. repanda		436 ± 20a (6)	540 ± 33b (6)	601 ± 24b (6)	

Values not sharing a letter within a row are significantly different. Sample size is reported parenthetically.

was 68 mg (range 43-90 mg; n=109), and that of *C. limbalis* was 113 mg (range 71-146 mg; n=35), and it did not vary significantly among sample dates in either species (*C. repanda*, P=0.666; *C. limbalis*, P=0.194; Table 1).

Chill tolerance and SCP

All *C. repanda* exposed to -3°C or -6°C for 24 h in late summer remained unfrozen and recovered immediately, exhibiting voluntary locomotion and responding to tactile stimuli, upon return to ~20°C. Conversely, all of those exposed to -8°C froze, with SCP ranging from -6.7°C to -7.2°C, and died from that exposure, exhibiting no voluntary or responsive movement after 24 h. During winter acclimation *C. repanda* exhibited a significant (P<0.001) decrease of 3.6°C in median SCP, whereas no significant change was found for *C. limbalis* (Fig. 2).

Glycerol concentration and hemolymph osmolality

A modest, but significant, increase of 0.5-1.2 μmol g^{-1} in whole-body glycerol concentration during winter acclimation was found for both *C. repanda* (P < 0.001) and *C. limbalis* (P = 0.037; Table 1). Despite significant variation in *C. limbalis* glycerol concentration during acclimation, post hoc tests did not reveal any significant differences (P>0.05) between any two sample dates. Glycerol concentration was negatively correlated (rs = -0.30, P = 0.034, n = 50) with SCP in *C. repanda*, but was not correlated (rs = -0.17, P = 0.333, n = 35) with SCP in *C. limbalis*. Hemolymph osmolality of *C. repanda* significantly increased (P = 0.002) during acclimation, rising by 38% from early November to late December (Table 1).

DISCUSSION

Our findings provide insight into the cold-hardiness mechanisms employed by two species of the spring-fall clade of North American tiger beetles during adult overwintering. Both species stopped feeding in mid-November when daytime temperature was still relatively high (12°C), but apparently remained active for another month until daytime temperature dropped to 5°C. This difference may indicate that the thermal threshold for predatory behaviour is above that for general activity. In the field, maintaining activity at relatively low temperatures might allow beetles to seek warmer microhabitats or basking spots in which they could warm above the predatory thermal threshold. Consistent with that observation, Dreisig found that adult *Cicindela hybrida* in May emerged from burrows only at ambient temperatures above ~19°C (7) and in a

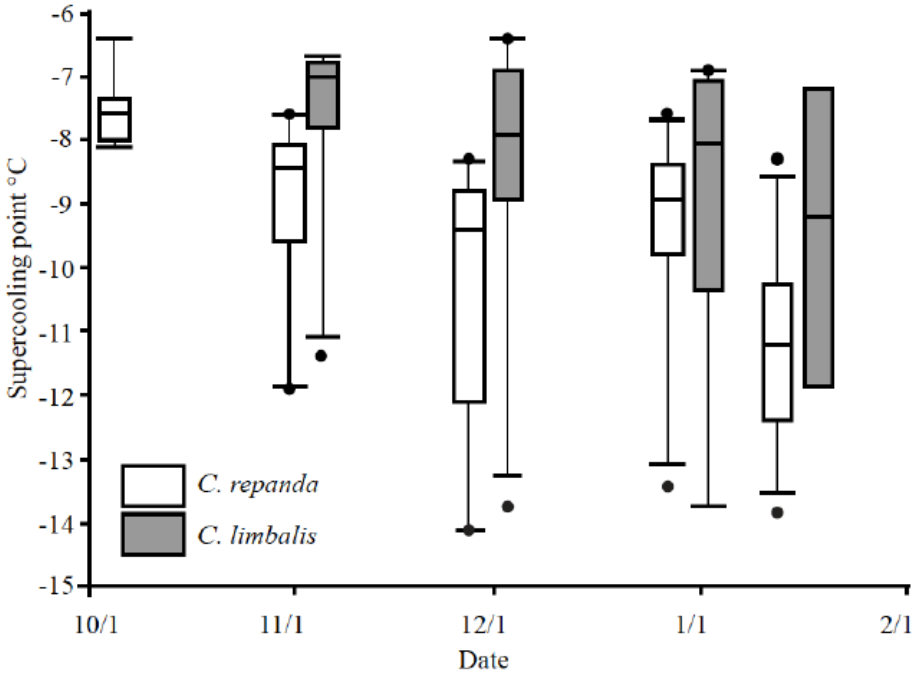

Figure 2. Box plots of supercooling points of individual *C. repanda* (n=10 at each sampling date, except on 5 October where n=9 and on 14 January where n=11) and *C. limbalis* (n=10 at each sampling date except on 14 January where n=5) throughout winter acclimation. The horizontal line in each box represents the median value. *Cicindela repanda* showed a significant (H4=26.3, P<0.001) decrease in supercooling point as winter acclimation progressed, whereas *C. limbalis* did not (H3=5.3, P=0.148). For *C. repanda*, sampling dates not sharing a letter are significantly different (P<0.05). Boxes are offset for clarity.

different study (8) found that up to 80% of their time at 20°C was spent sun-basking, presumably to increase body temperature prior to hunting.

In this study we assessed freeze tolerance and chill tolerance in late summer for *C. repanda*. This investigation suggest that prior to winter the beetles are tolerant of sub-freezing temperature, but not of internal ice formation. Many insects develop freeze-tolerance in preparation for winter (19, 26), and it is possible that tiger beetles do the same. A systematic study of freeze tolerance and chill tolerance in winter-acclimated tiger beetles is needed. However, if the chill tolerance and freeze intolerance found in late summer persists through winter acclimation, then lowering the SCP would be an effective strategy for tiger beetles to survive sub-freezing winter temperatures. For example, we found that *C. repanda* lowered its median SCP from -7 to -11°C during winter acclimation which, though modest compared to that of some other adult beetles (16), may reduce overwinter mortality. *Cincindela limbalis* had generally higher SCPs than did *C. repanda*, perhaps owing to its larger body size, and did not significantly lower its SCP during winter acclimation. Because little is known about the location and depth of the adult burrows in winter, the ecological significance of the SCPs reported here is unknown. However, both of these species inhabit regions where the frost line may extend 0.5-1.0 m into the ground (27) suggesting that unless adults burrow below that depth, they are likely exposed to sub-freezing temperature. Field and laboratory investigations into both the winter burrowing behavior and microhabitat conditions of adult tiger beetles are needed to better understand their overwintering biology.

It is unclear what physical and/or physiological changes precipitated the decrease in SCP, but cessation of feeding likely played a role by removing potential ice nucleating agents from the gut (32). Additionally, as SCP decreased during winter acclimation in *C. repanda*, whole-body glycerol increased. This ~1 μmol g^{-1} increase is on par with that found in some insects, e.g., *Alphitobius diaperinus* (18), but up to 100-fold lower than that found in others, e.g., *Eurosta solidaginis* (29). Although the modest increase is too small for a meaningful colligative effect,

glycerol may confer non-colligative protective effects at low temperature, such as membrane and protein stabilization (26).

In contrast to the modest increase in glycerol, hemolymph osmolality of *C. repanda* increased substantially. That increase may be due to the accumulation of some other unmeasured osmolyte, possibly one with cryoprotective properties. Multi-cryoprotectant accumulation strategies have been well documented (26), and several metabolites have been shown to aid in reducing cryoinjury through a variety of means, such as membrane and protein stabilization (17). Alternatively, the increase in hemolymph osmolality could be due to the concentrating effect of whole-body water loss, which we did not measure. We think that unlikely, however, because the beetles had continual access to water, often occupied burrows that would retard evaporative water loss, and did not vary in body mass over the course of winter acclimation. Gwiazdowski et al (12) also found little change in body mass (~7%) in *C. repanda* overwintered in the laboratory, suggesting that the beetles are not particularly susceptible to dehydration.

Although this initial investigation into the seasonal development of cold hardiness by adult tiger beetles is insightful, much remains unknown. Subsequent study should focus on determining the microhabitat conditions of overwintering tiger beetles and further assessing their chill and freeze tolerance (10). Vogler and Goldstein (30) hypothesized that successful adult overwintering by spring-fall *Cicindela* species partially explains the bias in range distributions of tiger beetles across North America where more spring-fall species occur in high latitudes and alpine regions than do the summer species whose adults do not overwinter. Developing a more complete understanding of the spring-fall overwintering strategy may better help us predict and model the response of spring-fall active tiger beetles at high latitudes and in the alpine distributions in North America to environmental changes

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