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# CRITICAL THERMAL MINIMA, THEIR SPATIAL AND TEMPORAL VARIATION AND RESPONSE TO HARDENING IN *Myrmica* ANTS

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## Abstract

In a changing environment the ability to adjust physiological functions to new conditions might be especially valuable for long-living ectothermic animals such as ants. With a simple method for estimating critical thermal minima of *Myrmica* ants we assessed variation of the minima and their response to lowered temperature. At a cooling rate of about 1°C per minute the ants first displayed knock-down (at temperatures 1.5 to −0.2°C) and only later immobilized completely (at −1.3 to −3.1°C). Pre-chilling of ants at 5°C for 1 h lowered the parameters significantly but not greatly (about 0.9°C). Constant laboratory conditions (20°C) raised the knock-down temperature and tended to lower the temperature of immobilization. In the same manner the two parameters changed in field conditions in June, but no significant change occurred through August and until the end of study season in mid-September. When populations from different geographic localities were compared, populations from the north showed lower knock-down temperatures. The magnitude of these apparently genetic differences among populations was comparable to the magnitude of plastic changes that occurred either naturally, or in experiments, or in laboratory culture. The little plasticity and low geographic variation of the critical thermal minima may indicate that the ability of the ants to withstand cold events and populate varying climates bases mainly on the protective properties of nesting substrate.

**Keywords:** chill coma, acclimation, hardening, plastic response, geographic variation, climate

## INTRODUCTION

Studies of physiological responses of ectotherms to changing temperature conditions are mostly conducted using model species (9, 24). Recently, researchers have studied the responses of non-model organisms (e.g. 42, 41) in order to test the universality of patterns found in organisms that are usually investigated. For this purpose, different ant taxa may be used as well, of which *Myrmica* ants are nearly the best choice, being very abundant and diverse in the Holarctic region (36) and easy to handle and maintain in laboratory. During their lifetime of up to two years (5, 37) the ants undergo multiple long- and short-term temperature fluctuations. Their colonies inhabit thermally buffered substrates (17) and may

avoid temperature extremes by within-nest relocations. However, the ants tend to aggregate right under heat-absorbing nest surface, especially in spring (6), where they inevitably experience occasional cold spells and repeated night-time chilling. Workers of *Myrmica* forage in both day and night (43), and plastic changes of their cold stress resistance might contribute to this ability. Finally, some species of *Myrmica* populate a wide range of climatic zones (36, 17). Populations of widely distributed solitary insects demonstrate adaptive clines of ability to resist stressful temperatures (19, 22) and may differ by capacity of plastic changes of the resistance (25, 8). Widespread *Myrmica* species can provide a test for a generalization following from such studies that populations of colder climates should express higher cold resistance.

As a starting point for studies of responses to cold in ants, activity-based metrics of cold resistance, such as critical thermal minimum, have several advantages. Measured at higher temperatures than survival-based metrics of cold tolerance, they are often more ecologically meaningful (1, 38) and easier to estimate; in addition, cold resistance correlates with cold tolerance (e.g. 15, 22). We devised a simple method for cooling *Myrmica* workers gradually and estimating their critical thermal minimum (CTmin). Here we report the results obtained in 2005, the first year of our measurements. Using the method, we tackled the following questions: (a) What is cold hardening capacity of *Myrmica*? (b) Does their CTmin demonstrate acclimational changes in natural conditions? (c) Is it affected by climatically differing habitats? (d) Do populations of the ants from distant localities differ by CTmin in a manner predicted by climate?

## MATERIALS AND METHODS

*Species, populations and habitats*

Main study objects were two Eurasian *Myrmica* species, the most common *M. rubra* (Linnaeus 1758) and *M. ruginodis* (Nylander 1846), both collected in 2005 near three localities: Borisovka (50° 36' N, 36° 01' E), Petergof (59° 53' N, 29° 54' E) and Luvenga (67° 06' N, 32° 42' E), Russia (Fig. 1). Within this range of latitudes the species differ in habitat preferences. Colonies of *M. ruginodis* always prefer shadowed habitats (various forests). In contrast, *M. rubra* gradually shifts its preference from exclusively shadowed habitats near Borisovka (broad-leaved forests) to exclusively open ones near Luvenga (sphagnum-covered stony areas, particularly those that border the tidal zone of the White Sea's Kandalaksha Gulf and are partly flooded in high tides). In the vicinity of Petergof *M. rubra* dwells in both shadowed habitats (forests) and open ones (meadows, human-made revegetating clearings, etc.). In the latter it coexists with *M. rugulosa* (Nylander 1849), the third species studied near Petergof (Table 1).


Figure 1. Collection sites for the ant colonies.

*Collection and maintenance*

Within a habitat we sampled the species along more or less straight random paths. Conspecific colonies that we selected on a path were considerably remote from each other (the shortest distance between two nests was 150 m). A fraction taken from each nest was large enough to simulate a mature colony: 1-2 queens, 250-300 workers and a comparable quantity of brood. We collected them by exhauster into capped 50-ml plastic vials with a moistened sponge lining. Upon transporting the vials to laboratory, we settled the fractions in closed plexiglas formicaries (29) consisting of three sections, of which the largest one provided darkness and high relative humidity, imitating ant nest interior. The ants were then kept at 20°C, under 22 L : 2 D daily photoperiod and fed on pieces of moistened raisins and laboratory-cultivated insects: cockroaches (*Nauphoeta cinerea*) and flies (*Calliphora vicina*). Food was renewed every third day.

*Equipment*

We chilled ant samples in duralumin cylindrical containers on a thermogradient device. Temperature of the containers was measured once per second by digital thermometers with external thermosensor (0.1°C, "Digi-thermo", Brannan, UK). The cylinders (height: 12 mm, diameter: 39 mm) had a blind horizontal channel drilled in lateral surface (5 mm from upper surface), where a thermoprobe was inserted. Six small chambers for ants (7 mm diameter and depth) were drilled in the upper surface of the cylinders. The chambers were arranged in two rows along the inserted probe, as close to its sensor as possible. Lids of 40-mm Petri dishes prevented ants from escaping. The thermogradient was maintained on a horizontally fixed

duralumin plate (1500×305×15 mm) with a 50 mm-thick styrofoam insulation and a bare measurement area (1000×120 mm) on the upper surface. The area, with a gradient of temperatures 5 to −5°C, was isolated from the air in the room by a glass cover.

*Measurement procedure*

To sample from a colony, we placed formicaries in small basins with fluon-covered walls and opened the inhabited section. In such a case workers roughly segregated into older individuals (who left the formicary) and younger ones (who stayed and cared for the brood). Then we selected ants haphazardly from both age cohorts to include in the sample the maximum possible variation of CTmin present in a colony.

In each chamber we placed a worker ant by an artist's brush, haphazardly distributing workers of the different age cohorts among the chambers. Each colony was measured twice: a control sample and a pre-chilled one. Random sequences of colony numbers and treatments were used to define the order of measurements. In the control treatment samples were placed on the measurement area, cooled to 5°C and then moved along the gradient stepwise, with a cooling rate about 0.8-1.0°C/min. The same was done in the pre-chilling treatment, except that we kept samples at 5°C (±0.3°C) for 1 h before further cooling. The long plastic arm of the tightly inserted thermoprobe served as a handle to move the containers. Whilst moving them we slightly lifted the glass cover for a short while, so that the cooling process remained unaffected. If the cooling rate increased (in very few cases), we always could return it to the required level (~1°C/min) the next minute.

When chill coma approached, ants would knock down (or just have an unsteady posture that indicated inability to walk) and later totally immobilize. Thus, at the cooling rate of ~1°C/min, the CTmin of *Myrmica* naturally divided in two parameters: the knock-down temperature (abbreviated $T_{kd}$) and the immobilization temperature ($T_{im}$).

We monitored the ants continuously and voice-recorded observed events, such as knocking-down, immobilization, resuming movement or upright posture. The labels of respective chambers (A-F) and container temperature were also recorded. A measurement stopped in 1.5 min after the last individual immobilized. After this we returned the ants to their formicary, to an isolated section with a moistened sponge, checked them in 1 h for possible cold shock mortality and verified mortality data after 24 h. Later we replayed the recordings and converted the temperature data to numerical form.

All experimental dates shared a common measurement scheme: on every such date we compared either two species from a locality or two populations of a species (Table 1).

*Statistical analysis*

To address the questions set out in the introduction, we subjected the data to the analysis of variance (ANOVA). The method requires analysed observations to be independent, normally distributed and homoscedastic (39). For this reason it could be applied only within a species, as multiple-species data do not satisfy the assumption of independence (14). Initially, we tested the significance of factors such as population, treatment and date by analysing entire sets of conspecific observations. However, because on most experimental dates only one population was tested, combinations of this factor with others (interaction terms) could not be constructed in these general ANOVAs. Combinations involving treatment were indispensable for our study, because they tested differences in the hardening capacity. Hence we tested the significance of all the factors by analysing smaller subsets within the conspecific data.

Firstly, measurements taken soon after collection and those from the same colonies after a period of maintenance allowed to test the effect of laboratory acclimation on CTmin. We treated such data separately for the early study season (June) and the late study season (from 28 July on), because *Myrmica* colonies are endogenous-heterodynamic and tend to enter

diapause in August-September even under laboratory simulated mid-summer conditions (28, 27). Diapause state and increased cold tolerance may be linked (10), and therefore lowering of CTmin was also likely for late-summer colonies, in contrast to early-summer ones, for which an increase in CTmin was expected. Secondly, using the early-season data on *M. rubra* we also compared measurements from a forest to those from a neighbouring revegetating clearing. Thirdly, to follow temporal dynamics of $T_{kd}$ and $T_{im}$ in field conditions we used data on Petergof colonies measured soon after collection. Finally, we performed intraspecific comparisons for each of the last three experimental dates (Table 1).

Table 1. Dates of measurements. For all species/populations n = 5 colonies, except for *M. rubra* on 16 and 30 June: n = 4 colonies.

| Date | Species | Collected | Population | Habitat |
|---|---|---|---|---|
| 6 Jun | *M. rubra*, *M. ruginodis* | 2 Jun | Petergof | mixed forest |
| 22 Jun | as above | -"- | -"- | -"- |
| 16 Jun | *M. rubra*, *M. rugulosa* | 8 Jun | Petergof | grassy clearing near a motorway |
| 30 Jun | as above | -"- | -"- | -"- |
| 6 Jul | *M. rubra*, *M. ruginodis* | 4 Jul | Petergof | mixed forest |
| 28 Jul | *M. rubra* | 21 Jul | Luvenga | tidal borderline |
| | *M. ruginodis* | | | coniferous forest |
| 8 Aug | *M. rubra*, *M. ruginodis* | 3 Aug | Petergof | mixed forest |
| 14 Sep | *M. rubra*, *M. ruginodis* | 12 Sep | Petergof | mixed forest |
| 21 Sep | *M. ruginodis* | 21 Jul | Luvenga | coniferous forest |
| | | 3 Aug | Petergof | mixed forest |
| 5 Oct | *M. rubra* | 21 Jul | Luvenga | tidal borderline |
| | | 13 Aug | Borisovka | broad-leaved forest |
| 12 Oct | *M. ruginodis* | 21 Jul | Luvenga | coniferous forest |
| | | 13 Aug | Borisovka | broad-leaved forest |

To satisfy the assumptions of independence and normality, for the ANOVAs we used colony means of $T_{kd}$ and $T_{im}$ obtained by averaging individual data from each sample of ants. Sample size per species per population thus equalled the number of colonies used (Table 1). Homogeneity of variances in the colony means was confirmed by Levene's test. Where it was not confirmed, we applied rank ANOVA (Scheirer-Ray-Hare extension of Kruskal-Wallis test), otherwise we applied parametric ANOVA with fixed effects (39). We performed the analysis using the GLM command in the SPSS Syntax Editor (40). In the ANOVAs, treatment was always the second main factor, so that interaction terms tested the difference in the hardening capacities either among populations, or subpopulations (in the case of two habitats), or times of measurement. Where necessary, we employed Tukey's HSD test for multiple comparisons.

We tested interspecific differences by the meta-analytic procedure (18), which is a valid alternative in the case of two- and multiple-species data (14). We included in the meta-analysis those dates on which two species were measured, considering each date a replicate. Our data provided enough such replicates only for comparison of *M. rubra* and *M. ruginodis*. Data from 5-12 October were treated as a single observation and also included in the list of replicates. The resultant eight suitable observations (n = 8 dates) represented different localities, so we performed a mixed-model meta-analysis (18), which was more realistic in this case. The analysis tested whether the difference ($d_+$*, or combined effect size) between the mean $T_{kd}$ or $T_{im}$ of the two species is significantly different from zero.

## RESULTS

The number of insects that died in the experiments was negligibly small: one on 16 June, two on 22 June, and three on 28 July and 5 October. Among these were ants of all the three species, but always from pre-chilled samples, indicating that longer chilling elevates chances for cold shock, at least for some colony members (by their appearance, the most aged ones). Data from such ants were included in the calculations of colony means.

The general analyses demonstrated significant effects of treatment (all species), population (*M. ruginodis*) and date (*M. rubra* and *M. ruginodis*). The subsequent analyses of smaller datasets (detailed below) confirmed these results.

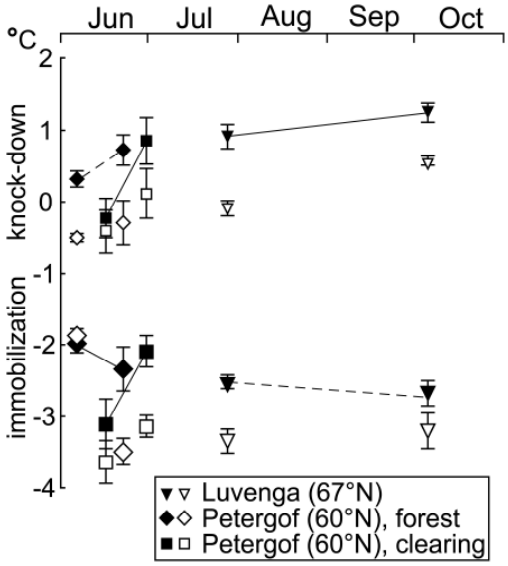


Figure 2. CTmin in fresh and laboratory-acclimated *M. rubra*. Mean temperatures of knock-down and immobilization (± SEM; squares: n=4, otherwise n=5 colonies). Solid markers - conrol, open markers - pre-chilling. Lines connect the control means before and after laboratory acclimation, solid lines denote significant differences ($p<0.05$, main factor "date" in a two-way ANOVA).

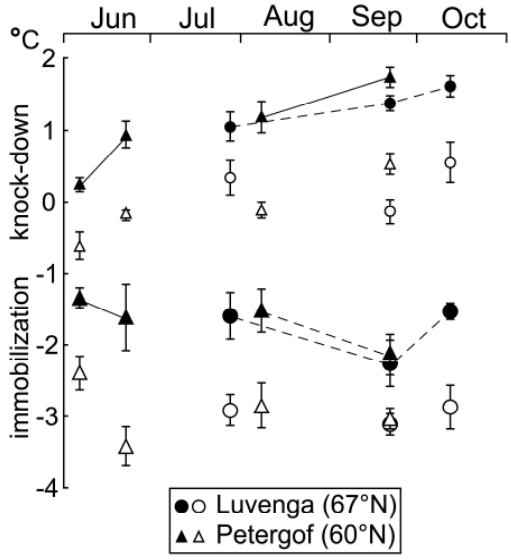


Figure 3. CTmin in fresh and laboratory-acclimated *M. ruginodis*. Mean temperatures of knock-down and immobilization (± SEM; n=5 colonies). Notation as in figure 1.

*Habitat differences, laboratory acclimation and pre-chilling effect*

In fresh *M. rubra*, ants from the grassy clearing were more resistant to immobilization than those from the forest. After 20-22 days in laboratory conditions the resistances of the subpopulations approached each other and became almost equal (Fig. 2). Laboratory-acclimated *M. rubra* from the clearing both knocked down and immobilized at temperatures that were higher than those soon after collection. Otherwise laboratory acclimation tended to increase knock-down temperatures, but decrease those of immobilization in all species (Fig. 2 and 3), although in *M. rugulosa* the changes lacked statistical significance (data not shown).

Pre-chilling increased resistance to cold stress of all species, except that fresh forest cultures of *M. rubra* did not become more resistant to immobilization (Fig. 2, see also Fig. 4). This response became pronounced only after some period in laboratory.

*Natural dynamics in forest species*

In the early summer ants displayed the most distinct level of both parameters, lowest for $T_{kd}$ and highest for $T_{im}$ (Fig. 4). Later $T_{kd}$ increased and $T_{im}$ decreased in both species, and then in *M. ruginodis* again returned closer to the early-summer level. Again, lowering of $T_{im}$ after pre-chilling was significant only in *M. ruginodis*.

*Intraspecific comparisons*

Ants from populations of more southerly localities knocked down at higher temperatures, although the difference was significant only in *M. ruginodis* (Fig. 5). Differences by resistance to immobilization were all insignificant.

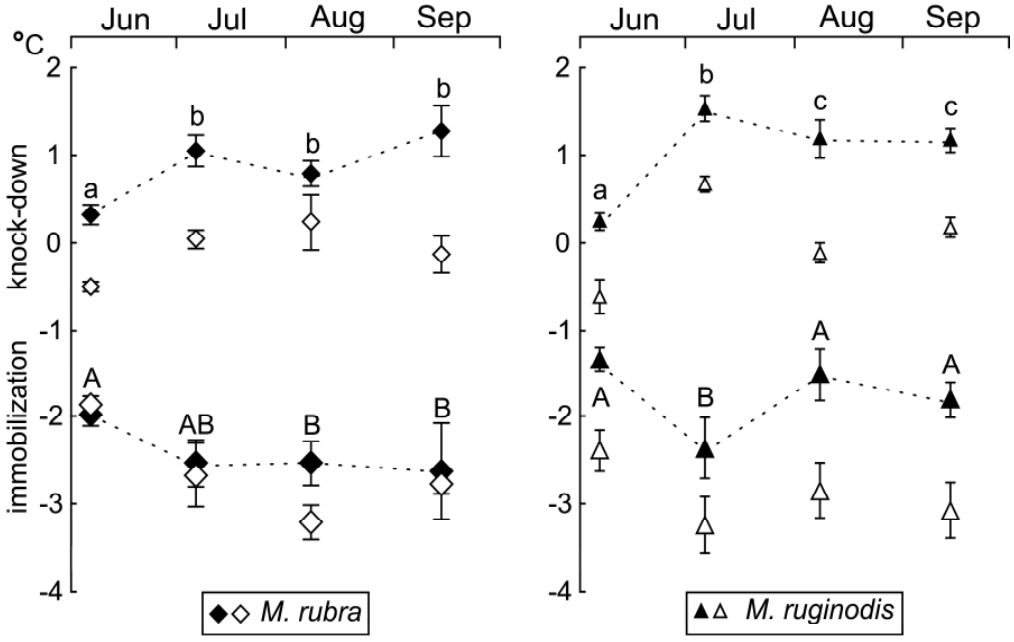


Figure 4. Natural dynamics of CTmin in *Myrmica* (Petergof, forest). Mean temperatures of knock-down and immobilization (±SEM, n=5 colonies). Solid markers - control, open markers - pre-chilling. Within a species, different letters denote dates that differ by mean $T_{kd}$ (lowercase) and/or $T_{im}$ (uppercase) significantly ($p<0.05$, main factor "date" in a two-way ANOVA followed by Tukey's HSD test).

*Interspecific comparisons*

On average, *M. rubra* knocked down and immobilized only at slightly lower temperatures than *M. ruginodis* (Fig. 6). The difference by $T_{kd}$ in control was moderate and insignificant, and even more so it became after pre-chilling. The species significantly differed by $T_{im}$ in control, but after pre-chilling the difference virtually disappeared.

Although for species of the grassy clearing there were not enough data to allow meta-analytical testing, it must be noted that *M. rugulosa* was always less resistant to immobilization by cold than *M. rubra*. In all cases, the mean $T_{im}$ of the former species was higher by 0.4-1.8°C.

## DISCUSSION

*Methodology*

The use of thermogradient adds to the numerous earlier-described methods for measuring critical thermal minimum (24), and the repeatability of results indicates that parameter estimation was reliable. However, the weak point of the method was its labour-intensity, resulting in both small number of experimental trials and small samples. One measurement session (including handling the insects, cleaning the thermogradient of moist and hoarfrost, etc.) took 15-20 min, and thus more than 20 measurements (effectively, replicates) in a day were infeasible. Small number of replicates lowers the power of statistical tests, most of all for interaction terms. Therefore, differences of two groups of insects by hardening capacity become difficult to detect. The present results strongly indicate such differences within a species only for immobilization in *M. rubra*, between fresh and laboratory cultures.

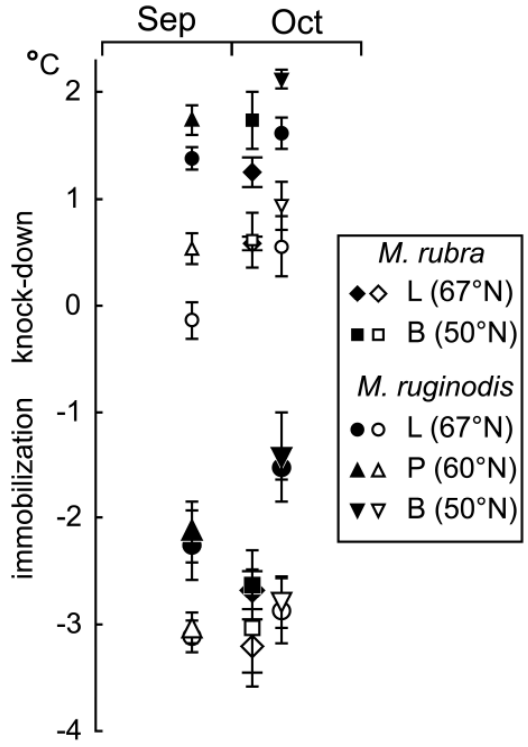


Figure 5. Temperatures of knock-down and immobilization in *Myrmica* populations (L - Luvenga, P - Petergof, B - Borisovka) after at least 1.5 months in laboratory. Means±SEM, n=5 colonies; solid markers - control, open markers - pre-chilling.

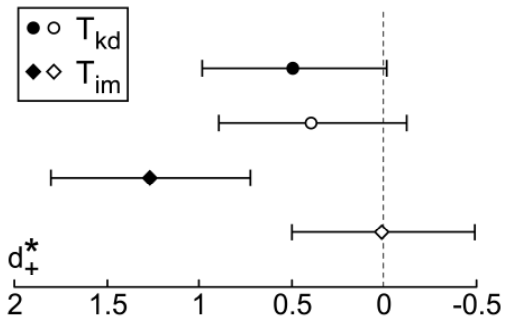


Figure 6. Difference (±95-% confidence limits, n=8 dates) between CTmin of *M. rubra* and *M. ruginodis* (a meta-analysis). Calculated for mean temperatures of knock-down or immobilization ($T_{kd}$ or $T_{im}$). Solid markers - control, open markers - pre-chilling. Differences whose confidence limits do not cross zero are significant.

*The effect of habitat*

Our results confirm that different habitats modify cold resistance of the same population of ants (see Petergof on Fig. 1). After a period of standard laboratory conditions the ants eventually achieve similarity in resistance level, and, more importantly, in plastic responses to cold. The finding once again proves the consistency of the method, and suggests that a more stressful environment lowers $T_{im}$ of *M. rubra*. Apparently, to a large degree the stress was from harsher thermal conditions (i.e. sharper daily temperature fluctuations) of open areas. Some additional stress from road pollutants was likely, because the grassy clearing

neighboured a road with a light suburban traffic. To clarify this uncertainty, we need measurements in a more natural open habitat.

*Plasticity of CTmin*

After a period in the laboratory *Myrmica* became less resistant to knock-down, but were able to stir at lower temperatures after knocking down (except *M. rubra* from the grassy clearing). As the pattern was the same both in the early and late summer, it follows that transfer to laboratory conditions caused the alterations. These might be brought about by changes in diet and temperature regimes, both known as modifiers of cold resistance for laboratory insects (16, 42). Nutritional status of laboratory ant cultures was improved, as they never utilized their food completely, in contrast to newly-collected ones. Temperature regime changed to constant, which was reported to lower cold resistance, when compared with simulated daily thermoperiods (23). Our hypothesis is that knock-down in the ants reflected primarily the changes in temperature regime, while immobilization depended more on nutrition. At any rate, the ecological meaning of $T_{im}$ is limited, because *Myrmica* hardly face such temperatures naturally even in winter. Temperature loggers, placed in 1996-1997 within three typical nests in an area close to Petergof, recorded no sub-zero temperatures in nest chambers as deep as 15 cm (a usual depth for the ants to hibernate) (Fig. 7). Lastly, it is unclear if the ants might immobilize after knock-down even without further cooling, but available publications report on a slow recovery of insects in such a case (33).

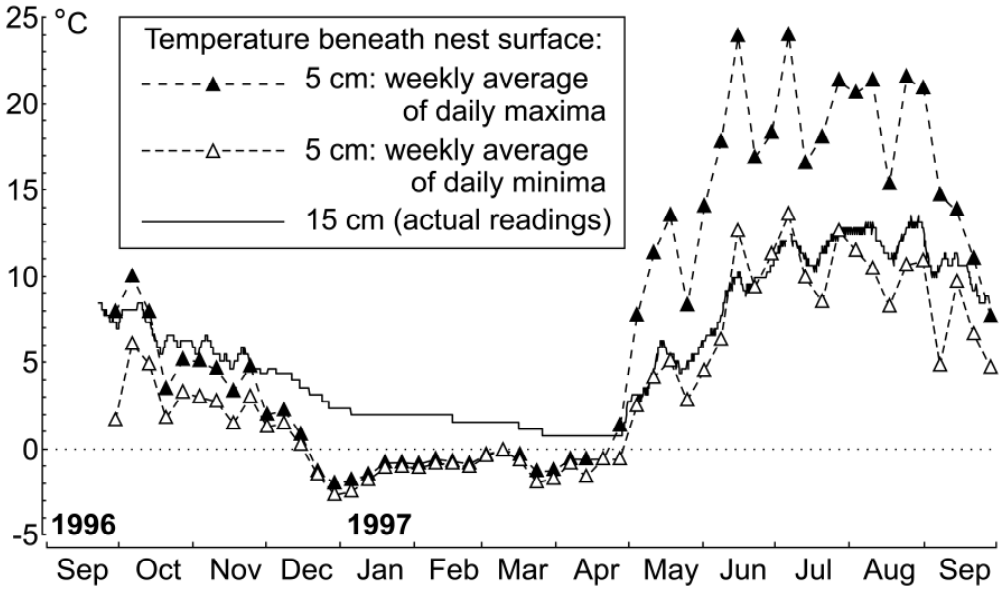


Figure 7. Thermal environment of a *Myrmica* colony inhabiting a moss tussock in a pine forest (55 km SSE from Petergof). In winter months the nest faced the strongest chilling of total three studied nests (V. E. Kipyatkov, unpublished data).

As in laboratory conditions, in the field the slow early-summer changes of the two measures of CTmin were opposite: increase of $T_{kd}$ and decrease of $T_{im}$. Lower mean daily temperatures and depleted nutritional reserves in early-summer ants might account for the distinct 'starting' levels of the parameters. Contrary to expectations, in the end of study season ants showed no clear increase of cold resistance either with the onset of diapause or in response to September temperatures (acclimation). Possibly, for *Myrmica*, like for other ectotherms living in thermally buffered microhabitats, the gradual decrease of environmental temperature becomes relevant only later, with the start of cold season (2).

Rapid decrease of the stress temperatures (hardening) in pre-chilled ants was the most repeated and obvious result. Some constraint on the hardening for immobilization was evident in freshly-collected *M. rubra*, but it was lifted in laboratory culture (again due to improved nutrition?).

Acclimation and hardening, the plastic changes of temperature tolerance and resistance, are widely recognized as adaptive responses of insects to varying environmental temperature (20, 3). Acclimation slowly increases cold survival of insects kept at the lower boundary of their normal viable temperature limits. It is viewed as a model of seasonal increase of cold tolerance occurring in autumn (30, 11). Hardening rapidly lowers cold shock mortality (34, 31, 7, 32, 45, 42) and is able to lower CTmin as well, thus being a model of physiological adjustment to daily temperature fluctuations and unpredictable cold snaps (26). Our study demonstrates that at least hardening can also be observed in *Myrmica* and used for comparison of species and populations. In the active season this response may allow *Myrmica* to switch their foraging activity to colder periods of day, especially when dominant ant species are present (43), while during hibernation it apparently diminishes the probability of cold shock in wintering colonies. As we used a relatively high cooling rate compared to those that can be naturally observed (26), we should expect some overestimation of the stress temperatures and underestimation of hardening capacity.

*Intraspecific differences*

Both *M. rubra* and *M. ruginodis* displayed expected geographic pattern of cold stress resistance, i.e. its lower level in populations from sites of lower latitudes. In *M. ruginodis* significant differences by resistance to knock-down persisted after a long maintenance in standard conditions, suggesting a genetic source of the geographic variation. Another plausible source is irreversible plasticity of the trait merely because of different developmental temperatures (15, 23) that the populations faced on different latitudes. The latter possibility is ruled out to some extent, taking into account the initially significant but reversible among-habitat differences in the other species (see above). Versatility in the choice of habitat in *M. rubra* could be responsible for the lower divergence and therefore insignificant differences of its distant populations. However, one should note that comparing such populations demands non-traditional statistical approaches (14), like the one we used for interspecific differences. The data available from our study were too scarce and allowed to apply only conventional analytic methods.

*Interspecific differences*

Of the two forest species *M. rubra* tended to be slightly more resistant to knock-down, and we believe that the difference escaped statistical confirmation due to the small number of suitable observations. At the same time, *M. rubra* was definitely more resistant to immobilization by cold, while *M. ruginodis* achieved the same resistance only after 1-h chilling. This finding is surprising, because it contradicts the notion of *M. rubra* as a more thermophilic species. The latter view is supported by the preference of this species for better insolated patches of colony settlement (4, 13, see also 12 for taxonomic correction). In our sampling sites colonies of *M. rubra* were also usually found on patches under openings in forest canopy. In addition, *M. ruginodis* distributes much further to the north than *M. rubra* does (44, 17). We can only try to explain the contradiction by suggesting differences in larval and adult physiologies of *M. rubra*: better insolated patches may be necessary for raising brood, whereas higher cold stress resistance of adult ants may fit sharper temperature fluctuations (and greater impact of cold spells) in such patches.

However, it cannot explain why *M. rubra* was still more resistant to immobilization than *M. rugulosa*, an exclusively open-habitat species. The reason might be merely the different

size classes of worker ants: body length of 5-6 mm in *M. rubra* (and *M. ruginodis*) versus 4-5 mm in *M. rugulosa*. It has long been known that in the smaller animals heat loss rate is faster due to the lower body volume/surface ratio (46). Nonetheless, the relationship of size and a specific temperature-related physiological trait is not always so straightforward. Evidence exists that in ants the supercooling point, another measure of cold tolerance, may vary considerably with little variation in body size (35). From a more general viewpoint, congeners are always likely to have physiological differences resulting from genetic processes that accompany speciation (14). This view is especially relevant for our study, because it generally helps explain consistent differences of closely related species sharing the same type of habitat, where they face fairly similar selection pressures.

*Concluding remarks*

Overall, the results illustrate the necessity to assess temporal and local spatial variability of a physiological trait when studying its wide-range geographic patterns, as all the potential modifiers (season, habitat, laboratory) directionally affected cold resistance in the ants. We favour the use of knock-down temperature as a more ecologically relevant measure of CTmin in the ant populations, but note that immobilization by cold can reveal physiological differences of sister species. On the other hand, regardless of the differences found, variation in both parameters falls within a narrow range only about 2°C in magnitude. This leads to a suggestion that nesting substrates and soil, actively used by the ants as thermal buffers, eliminate the need for greater plasticity or geographic variation of cold resistance. Recent evidence for a trade-off between plasticity of cold tolerance and the avoidance of thermal variability in some soil ectotherms (21) reinforces the idea that geographic variation may be affected by the avoidance as well. The critical test for the suggestion would be a study of plasticity and variation of cold tolerance in the ants.

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