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RESEARCH ARTICLE

Chill coma temperatures appear similar along a latitudinal gradient, in contrast to divergent chill coma recovery times, in two widespread ant species

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ABSTRACT

Populations of widely distributed ectotherms demonstrate different cold resistance corresponding to the local climate. However, efficiently thermoregulating ectotherms could avoid divergence in cold resistance. Two species of ants, previously shown to even out latitudinal differences of mean summer temperatures in their nests, were used to test this hypothesis by comparing the temperature dependence of cold resistance in three distant populations (from 50°, 60° and 67°N). The species differ in habitat preferences, one (*Myrmica rubra*) being less stenotopic than the other (*M. ruginodis*). Therefore, three different predictions were made about their cold resistance: along the latitudinal gradient, it might be similar within the two species (because of thermoregulation within nests/habitats) or similar only in *M. rubra* (as a result of thermoregulation among habitats), or divergent at least in *M. rubra* (no effect of thermoregulation). Among populations of both species, neither differences nor latitudinal trends in chill coma temperature were statistically significant after 11 months of standard conditions, with or without cold hardening. In contrast, recovery time significantly differed among populations in both species, although its latitudinal trends were strongly curvilinear: in *M. rubra*, the intermediate population tended towards the slowest recovery, and in *M. ruginodis*, it tended towards the fastest. After 22 months, the patterns remained the same, except that *M. ruginodis* showed a significant linear latitudinal trend in chill coma temperature (with no significant populational differences). Hence, thermoregulation, both within and among habitats, apparently does keep chill coma temperatures similar. Recovery rate demonstrates divergence, but its curvilinear trends suggest a connection with climates experienced by ancestral populations.

KEY WORDS: Critical thermal minima, Acclimation, Hardening, Climatic adaptation, Thermoregulation

INTRODUCTION

Thermal niches of ectotherms inhabiting different latitudes evolve according to local climates (Angilletta, 2009; Sunday et al., 2011), as a part of a more general process of local adaptation (Kawecki and Ebert, 2004). Regarding the lower boundary of thermal niches in particular, multiple studies demonstrate that, within species, lower temperature limits for activity decrease with increasing latitude (Gibert et al., 2001; Hallas et al., 2002; Hoffmann et al., 2002), sometimes with minor departures from the general rule (Arthur et al., 2008; Collinge et al., 2006), indicating adaptation to lower temperatures. It remains to be shown, however, whether the same pattern is true for ectotherms with lifestyles substantially different from those of model organisms used in the studies cited above.

Avoidance of thermal variability lowers variation in traits of low-temperature physiology (Foray et al., 2013; Hawes et al., 2008), and it is possible that lower temperature limits will not diverge among populations of a hypothetical perfectly thermoregulating ectotherm (Huey and Pascual, 2009). Ants might be a particularly interesting group in this respect, because they usually dwell in thermally buffered microhabitats, which lower the temperature variation that their inhabitants face in nature, both within populations and among them. Colonies of *Myrmica* ants use diverse nesting substrates (Groden et al., 2005) and may, if necessary, demonstrate daily thermoregulatory relocations within nest layers. This type of thermoregulation has a seasonal character, which allows easy collection of virtually entire colonies in spring, but not in later seasons (Brian, 1972). Another type of thermoregulation in *Myrmica*, by choice of microhabitats within habitats, was shown to almost even out the latitudinal cline of average nest temperatures and to revert the cline of maximum temperatures in June and July along a gradient of 16° of northern latitude (Kipyatkov and Lopatina, 2010). Additionally, some widespread *Myrmica* demonstrate certain habitat versatility within their geographic areas, which also may prevent formation of latitudinal clines of cold resistance.

The preventive effects of thermoregulation on divergence in cold resistance among *Myrmica* populations first appeared likely after a study of critical thermal minima in two sister species, conducted in 2005 (Maysov and Kipyatkov, 2009). When distant populations (sampled from three localities along essentially the same latitudinal gradient) were compared pairwise (northern with southern and northern with intermediate within *Myrmica ruginodis* Nylander; northern with southern within *Myrmica rubra* Linnaeus), they showed only slightly lower chill coma temperatures in the north, and the differences were not statistically significant in *M. rubra*, the species whose northern population preferred open habitats instead of forested ones.

The statistically confirmed differences found in *M. ruginodis* also could not be readily attributed to divergence, but might be explained by reversible or irreversible phenotypic plasticity (Gibert and Huey, 2001; Hoffmann et al., 2005; Terblanche and Chown, 2006). Even clearly demonstrable thermoregulation may not result in a perfect compensation of body temperature across latitudes (Huey and Pascual, 2009), and, therefore, phenotypic plasticity may always give rise to latitudinal clines of cold resistance. Because of social interdependence among ant age cohorts within a colony, they cannot be easily separated, and, hence, ant samples in the pairwise

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Received 9 September 2013; Accepted 30 April 2014

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096958

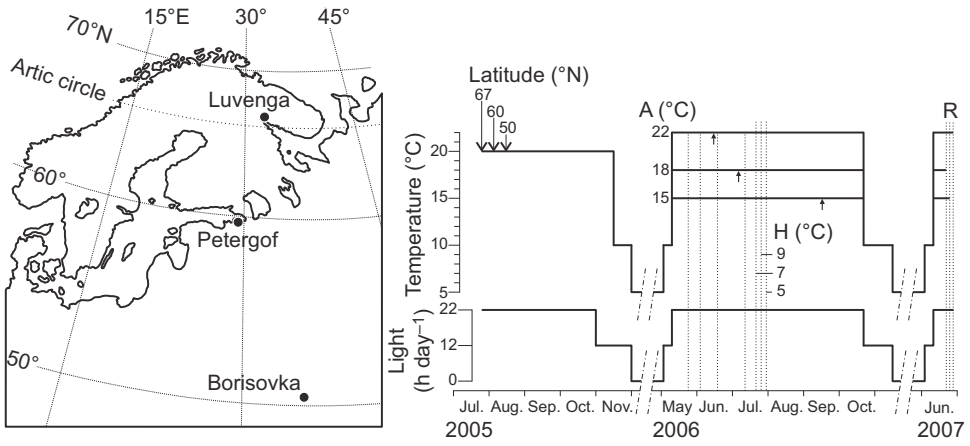

Fig. 1. Schematic diagram of the experiments. The map on the left shows the collection sites. The conditions (temperature and photoperiod) experienced by the ants are plotted against time (months and years) on the right. Downward arrows indicate times of collection (*N*=5 colonies per species per latitude). Acclimation regimes (A) were applied to a third of each colony; hardening treatments (H) were applied to ant samples from each such fraction. Upward arrows indicate the time when new adults started to eclose under each of the regimes. A full reciprocal transfer experiment is denoted by R (see Materials and methods for explanation). Dotted lines indicate median dates of experimental trials.

comparisons necessarily consisted mainly of individuals developed to adults in the climate of their original localities. The above-mentioned effects of microhabitat choice on average and maximal nest temperatures in June–July are not observed in other months (Kipyatkov and Lopatina, 2010), and, in fact, minimal temperatures might have a stronger influence on cold resistance variation. At the same time, even those adults that eclosed in common garden conditions during the nearly 2 months before the comparisons were carried out experienced the conditions only for a short part of their juvenile stage. Therefore, population differences had to be re-evaluated after a longer standardization period that would allow colonies to grow new adults, preferably so that these become the majority in samples. This would minimize the effects of phenotypic plasticity and might standardize cross-generational effects, another factor capable of obscuring genetic differences (Kawecki and Ebert, 2004).

Re-evaluation of cold resistance in these species after their substantially longer maintenance in common garden conditions could indicate different variants of interplay between thermoregulation and natural selection resulting in local adaptation. If both species showed similarity of cold resistance along the latitudinal gradient, it would indicate that thermoregulation within nests and habitats allowed the ants to avoid the selection and prevent divergence in cold resistance. If only *M. rubra* demonstrated similarity of cold resistance among its populations, it would suggest that only by additionally changing their habitat preferences were the ants able to negate the selection and prevent formation of a genetically based cold resistance cline. Finally, if both species (or only *M. rubra*) displayed such a cline, that would conflict with the thermoregulation hypothesis and question the results of the previous study (Maysov and Kipyatkov, 2009).

Here, I describe outcomes of experiments in which two cold resistance traits, the temperature of chill coma onset (measured as the temperature of knock-down under gradual chilling) and the time of subsequent recovery of ants, were scored on the above-mentioned population samples after 11 and 22 months of common conditions (Fig. 1), and then analysed for latitudinal trends. If trends existed, they were more likely to be clines, because of the mostly linear differences in temperature minima observed among the localities during the warm season (Fig. 2). The temperature dependence of the two traits was also examined, because it gives an opportunity to compare populations not only by their cold resistance but also by their physiological plasticity. The working hypothesis for the temperature dependence of cold resistance was that at ecologically relevant temperatures it would also be linear. I provide evidence for almost complete equality among the populations in the chill coma temperature and somewhat unexpected differences in the recovery time, which nevertheless might be explained by recently established details of the species phylogeography.

RESULTS

Populations

After being held under common garden conditions for 11 months, *M. rubra* and *M. ruginodis* demonstrated neither significant population differences nor significant latitudinal trends in their chill coma temperatures (Fig. 3; supplementary material Tables S1, S3, S4).

In contrast, chill coma recovery times significantly differed among populations within each species (supplementary material Tables S5, S6), with both linear and curvilinear trends significant in *M. rubra* and only the curvilinear one significant in *M. ruginodis* (supplementary material Table S1). The results for *M. rubra* were explained by faster recovery in its northern population than in the others (with a tendency of the intermediate population towards the slowest recovery at the end of the acclimation period). In *M. ruginodis*, the northern population was the slowest to recover, while the intermediate one was the fastest. Unlike *M. rubra*, which after hardening only partly changed the order of recovery (the intermediate population became the slowest), hardened *M. ruginodis* displayed no population differences or trends in recovery times (Fig. 4; see also Fig. 5 for the effects of hardening temperatures).

After 22 months, the above findings were generally confirmed, with a single difference: although knock-down temperatures in *M. ruginodis* still did not differ among populations (full-factorial unreduced general linear model on log-transformed individual data: $F_{2,12.683}$=1.144, P=0.349), the linear latitudinal trend for them was highly significant (linear contrast L=−0.028±0.010, P=0.006; quadratic, Q=−0.002±0.009, P=0.855; Fig. 6).

Wintering population samples differed neither in their knock-down temperature nor in their recovery time (supplementary material Figs S1, S2), although, in the case of recovery, necessary methodological differences (supplementary material Fig. S3) prohibited comparisons of the results with those for active ants.

Temperature dependence

Acclimation temperatures always caused a very highly significant linear trend in both traits (Figs 3, 4). In addition, quite often curvilinearity was also very highly significant (supplementary material Table S2). Hardening temperatures produced only curvilinear trends in the traits (Fig. 5).

Analysis of the recovery data revealed a significant interaction between the temperature factors (acclimation and hardening). In *M.*

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096958

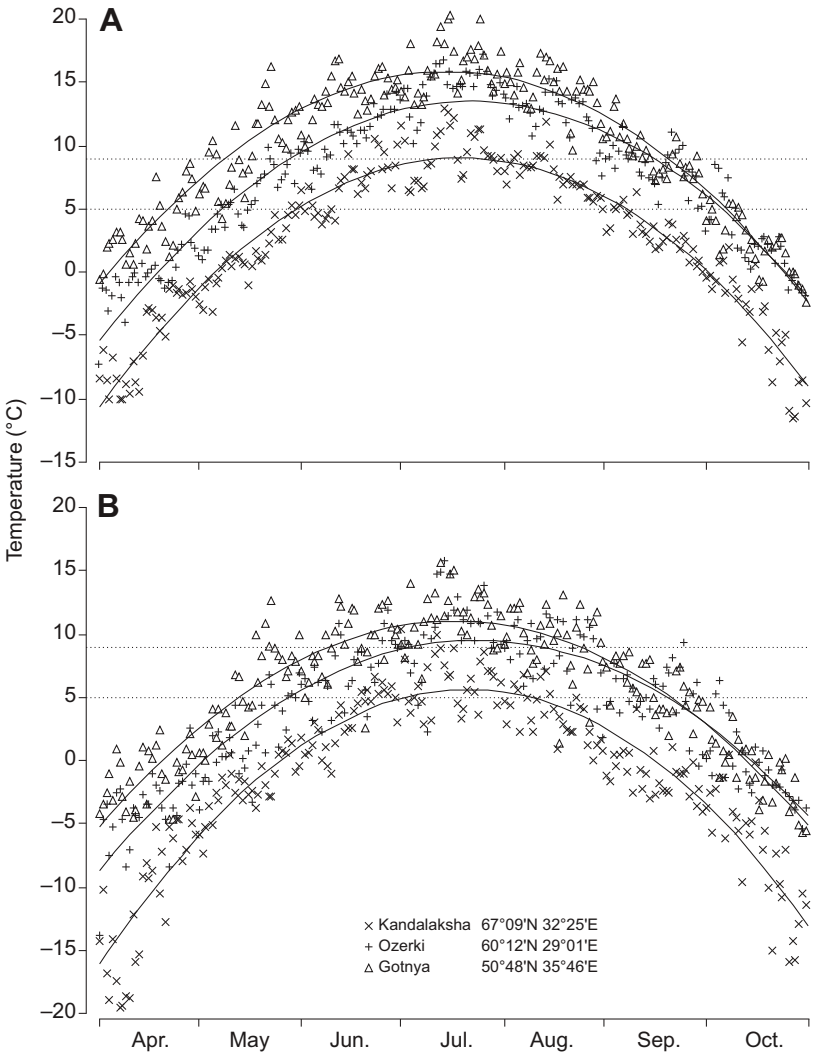

Fig. 2. Daily minima of air temperatures observed over 2005–2012 along the latitudinal gradient. (A) Average values; (B) lowest values. Data are from weather stations located closest to the collection sites (source: www.rp5.ru). Solid lines are second-order polynomial regressions added to facilitate visual comparison of the data. Dotted lines bracket the interval of hardening temperatures used in the present study.

rubra, it was significant only when the analysis was performed on individual observations ($F_{4,381}$=4.227, P=0.002), and not when averages obtained from colony fractions (fraction means) were analysed (supplementary material Table S6; see 'Statistical analysis' for details). Nevertheless, acclimation at 15°C enabled ants to recover faster after pre-chilling, whereas individuals from higher temperatures demonstrated a weaker response, no response or an inverse response (Fig. 5). Additionally, in *M. rubra* there was a significant interaction of population and acclimation temperature during the acclimation period (supplementary material Table S5). Apparently, in the southern population of this species, temperature dependence of recovery time was lower, as it became less pronounced over time (Fig. 4, last acclimation trial).

Size and colony factors

Ant size rarely had an effect on the studied parameters (supplementary material Table S6), unlike the factor 'colony', which was always significant for knock-down (supplementary material Tables S3) or close to significant for recovery (supplementary material Tables S5), indicating a hereditary component of variation, stronger for knock-down than for recovery. There was no significant effect of ant size after 22 months (either trait), while colony effect again was very highly significant (knock-down only, as fraction means were used for analysis of recovery).

DISCUSSION

Populations

This study compared cold resistance in ant populations along a latitudinal gradient, to test for possible adaptive differences. Higher cold resistances in colder climates would support the notion of local adaptation of populations. Equal cold resistances might support one of the variations on the thermoregulation hypothesis outlined in the Introduction. As a result, after 11 months of common conditions, the populations showed no difference in chill coma temperature, including populations of *M. ruginodis* that in 2005 differed significantly in this trait. This indicates a reversible, environmentally induced character of those differences, which were previously thought to be determined only genetically (Maysov and Kipyatkov, 2009). Therefore, the results might be viewed as supporting the hypothesis of within-habitat thermoregulation preventing divergence in chill coma temperature of the ant populations. Importantly, however, a significant linear trend appeared in knock-down temperatures of *M. ruginodis* after 22 months of common conditions, although this species showed no significant population differences in knock-down temperatures. One must conclude therefore, that some selection for stronger resistance to chill coma does take place in colder climates. The importance of this particular conclusion becomes clear if we take into account that plasticity in chill coma temperature of adult ants in a colony is beneficial for

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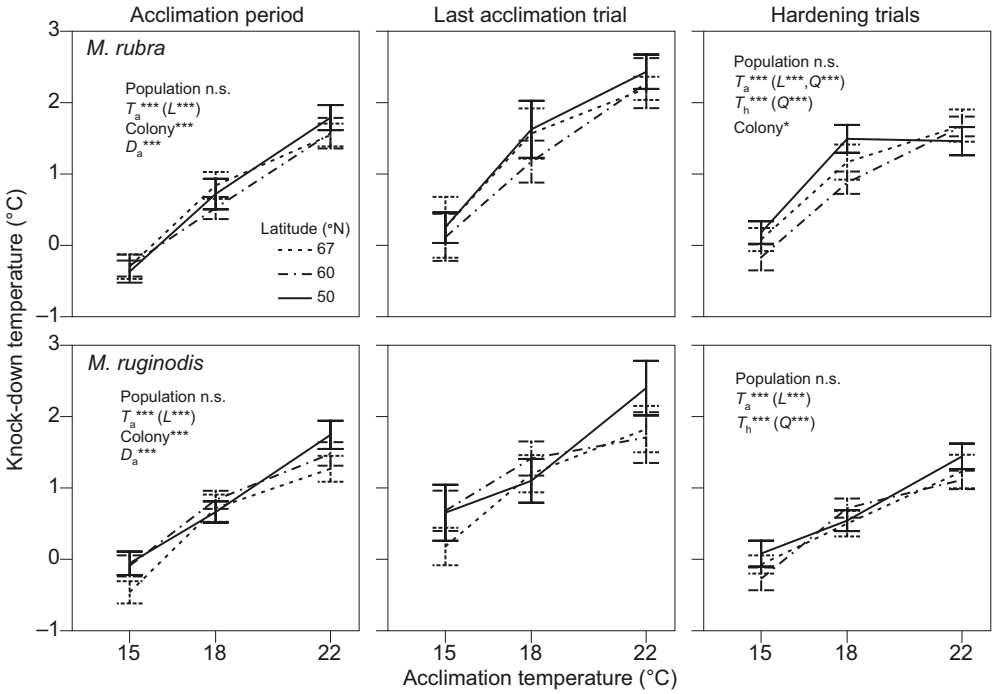

Fig. 3. Chill coma temperature of *Myrmica* under different acclimation regimes. Averages of fraction means ± s.e.m. (N=5 colony fractions for the acclimation assays and 15 colony fractions for the hardening trials). Populations are coded by latitude. The last acclimation assay of the acclimation period is singled out as a control for the hardening trials (here, data are shown regardless of hardening temperatures; for their effects, see Fig. 5). Statistical significance of the factor 'population' is indicated for the acclimation period and for the hardening trials (*P<0.025, **P<0.005, ***P<0.0005; n.s., not significant); other factors, contrasts and covariates are listed only if significant. T_a, acclimation temperature; D_a, duration of acclimation; T_h, temperature of hardening; L, linear contrast; Q, quadratic contrast. Full GLM results can be found in supplementary material Table S3 (acclimation) and Table S5 (hardening).

juvenile ants of that colony. As ants at juvenile stages depend completely on the care provided by adults, it means that the latitudinal similarity of chill coma resistance might theoretically be explained not by thermoregulation but by the trait's high plasticity, which could be initially (i.e. before distribution to the north) selected for naturally because of its possible benefit resulting from the eusociality of the studied insects. But the fact that temperatures of chill coma onset, while remaining similar along the latitudinal cline, show signs of climatic adaptation only in the more stenotopic of the two species speaks in favour of thermoregulation as an explanation for the observed similarity. It is thus likely that both within-habitat (including within-nest) and among-habitat mechanisms of thermoregulation are operating, but only in combination do they prevent any divergence in chill coma resistance.

In contrast to chill coma temperature results, populations of both ant species demonstrated a strong divergence in the time of recovery after chill coma (not studied in 2005). However, only in *M. rubra* did the population differences loosely correspond to geographic situation after 11 months of common conditions, indicating a stronger selection for the trait in the north. Thus, the generalization that ectotherms at higher latitudes evolve higher cold resistance, as measured by chill coma recovery time (Arthur et al., 2008; Collinge

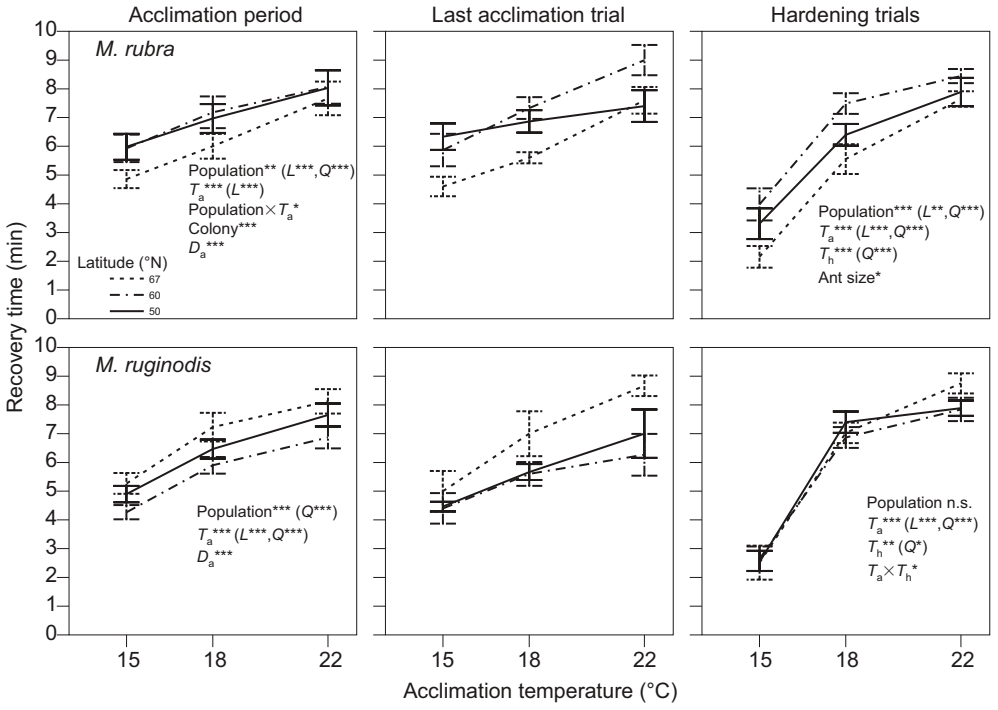

Fig. 4. Recovery time of *Myrmica* under different acclimation regimes. Averages of fraction means ± s.e.m. (N=5 colony fractions for the acclimation assays and 15 colony fractions for the hardening trials). Populations are coded by latitude. The last acclimation assay of the acclimation period is singled out as a control for the hardening trials (here, data are shown regardless of hardening temperatures; for their effects, see Fig. 5). Statistical significance of the factor 'population' is indicated for the acclimation period and for the hardening trials (*P<0.025, **P<0.005, ***P<0.0005; n.s., not significant); other factors, contrasts and covariates are listed only if significant. T_a, acclimation temperature; D_a, duration of acclimation; T_h, temperature of hardening; L, linear contrast; Q, quadratic contrast. Full GLM results can be found in supplementary material Table S4 (acclimation) and Table S6 (hardening).

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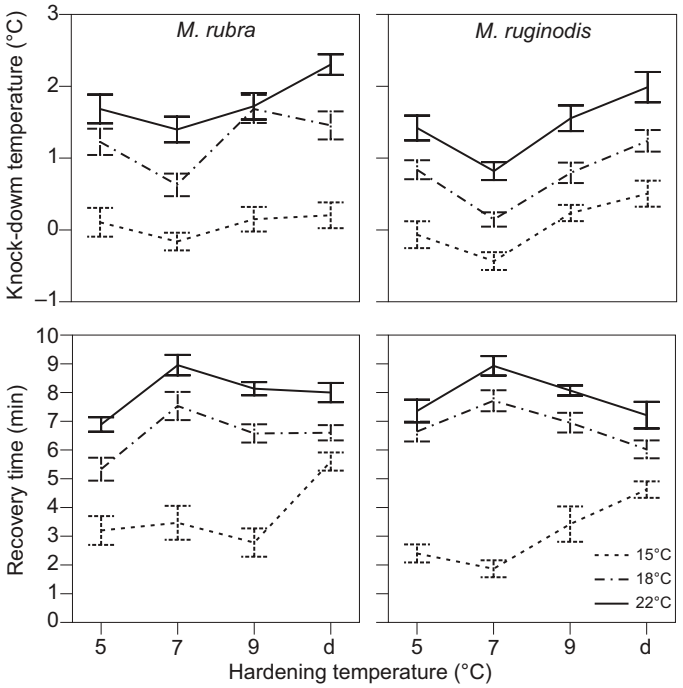

Fig. 5. Chill coma temperature and recovery time of *Myrmica* after hardening (regardless of populations). Averages of fraction means ± s.e.m. (*N*=15 colony fractions). Line styles denote acclimation temperatures. The data are the same as for the hardening trials in Figs 3 and 4. Direct transfer (d, for which *N*=5 colony fractions) shows data from the last trial of the acclimation period, for comparison.

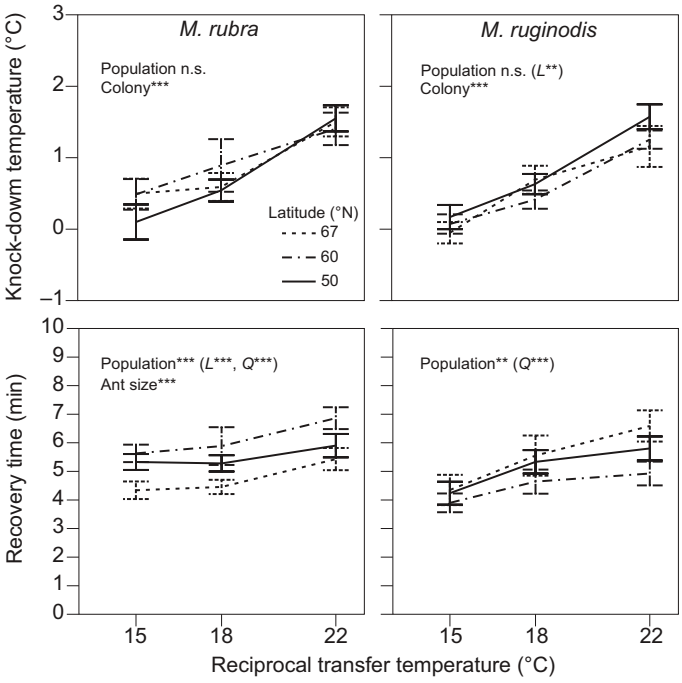

Fig. 6. Chill coma temperature and recovery time of *Myrmica* after reciprocal transfer (regardless of acclimation temperatures). Averages of subfraction means ± s.e.m. (*N*=15 colony subfractions). Populations are coded by latitude. Statistical significance of the factor 'population' is indicated (*P<0.05, **P<0.01, ***P<0.001; n.s., not significant); other factors, contrasts and covariates are listed only if significant (except for reciprocal transfer temperature, which was always very highly significant). *L*, linear contrast; *Q*, quadratic contrast.

et al., 2006; Hallas et al., 2002; Hoffmann et al., 2002; Huey and Pascual, 2009), appears to be applicable only to *M. rubra*. Even in this species, however, climatic differences cannot explain the latitudinal pattern that the populations demonstrated after 22 months of common conditions (Fig. 6). Although the initial similarity between the southern and intermediate populations of *M. rubra* (Fig. 4, acclimation period) might be explained by the similarity of temperature minima at their corresponding localities in September–October (Fig. 2), such an explanation would imply an inordinate importance of the autumnal period for activity in these populations, whereas in nature they form a diapause by this time (Kipyatkov and Lopatina, 1997). Perhaps, one has to consider the recovery results obtained in 2007 to be more conclusive and take into account that they resemble those obtained in 2006 by the end of the acclimation period (Fig. 4, last acclimation trial).

An explanation for the patterns of variation in chill coma recovery time along the transect might partly lie in the history of distribution of the species over their ranges. From recent studies of the *Myrmica* phylogeography, partly based on samples from the same three localities (Leppänen et al., 2013), it follows that the southern population of *M. rubra* formed through almost a completely separate route of colonization, because it is more closely related to populations in areas southwest of it (the region of the Balkans and southern Carpathians). Therefore, its faster recovery after chill coma might reflect a higher selection for the trait in those montane areas. At the same time, in *M. ruginodis*, the path of formation for the northern population was also largely separate, more western (connecting Central Europe and Finland). The slowest recovery in this population might therefore reflect a weaker selection for the trait in less continental climates.

The disparate patterns of latitudinal variation in the cold knock-down and recovery emphasize their independence from each other. This is in line with conclusions from studies on fruit flies, where the traits showed a positive correlation that was weaker at the extremes of the relationship (Ransberry et al., 2011). Investigation of the mechanisms behind the traits (MacMillan and Sinclair, 2011; MacMillan et al., 2012; Sinclair et al., 2013) might clarify the causes of their dissimilarities. Interestingly, in *M. ruginodis* the pattern of recovery along the latitudinal gradient inversely corresponded to the pattern found in the dynamics of expression of a heat-shock protein (Maisov et al., 2007). Namely, populations that recovered faster after cold treatment also responded to the treatment by slower expression and lesser amounts of the protein HSP70, thus confirming the expression to be an inverse indicator of adaptation to a stress factor (Sørensen et al., 2003) and disproving an earlier suggestion of a positive role of HSP70 for climatic adaptation of *M. ruginodis* in northern areas (Maisov et al., 2007). Nevertheless, as data on *M. rubra* show no similar inverse relationship, overall there is no trade-off between the expression of HSP70 and the mechanism of chill coma recovery.

Regardless of physiological mechanisms of the two traits, their relationship with climate is clearly different. Although both of them demonstrated considerable plasticity in response to constant temperature (this study), it was shown in a parallel investigation conducted on field-fresh ants (Maysov and Kipyatkov, 2011) that their recovery time does not closely correlate with seasonal temperature changes, although it is capable of quick alterations. In other words, recovery time is more dependent on some intrinsic factors. Possibly this is one of the reasons why, while distributing over their ranges, these species of ants eventually undergo a

selection for recovery time (whose smaller intercolonial variation may be interpreted as supporting this idea). Even this selection, though, is not immediate, as the climates presently experienced by the ant populations only partly explain the latitudinal variation found in recovery times, and the phylogeographic explanation implies that in some areas populations still preserve genotypes selected by climates in which ancestral populations lived (another consequence of thermoregulation?).

Currently, the available literature provides only one example of a latitudinal cline for the temperature of chill coma onset in an arthropod (Castañeda et al., 2004). Given the thoroughness of other available publications on latitudinal variation of cold resistance in ectotherms, it may be surprising that these articles report data only on chill coma recovery. If the preference is solely because of measurement feasibility, the present work provides reassurance that, of the two traits, chill coma recovery probably is more useful as an indicator of adaptation, because it is more responsive to selection by climate even in rather effectively thermoregulating insects. Data on chill coma temperature in other ectotherms may be valuable here, because they may either support the thermoregulation hypothesis (if more latitudinal clines are described in other species) or contradict it (if evidence is revealed for no such clines).

Pre-chilling the ants modified latitudinal trends for recovery time, indicating that the populations also differ in their hardening capacities. For instance, the northern population in *M. ruginodis* apparently demonstrates the strongest hardening response. In contrast, in *M. rubra*, the strength of the response is obviously lowest in the intermediate population (Fig. 3). Therefore, this type of plasticity is not traded off for cold resistance in these insects, unlike, for instance, in the case of heat resistance in some other arthropods (Stillman, 2003; Stillman and Somero, 2000), but might be determined by different selection pressures in their current environments and perhaps also complicated by selection pressures faced by their ancestral populations. These findings may be rather important, taking into account that a comprehensive study of *Drosophila* did not support the concept of plasticity increasing with temperature variability (Overgaard et al., 2011).

Temperature dependence

Widening the scope of test temperatures in the present study provided insight into the temperature dependence of the studied aspects of cold resistance. The dependence on acclimation temperature in both ant species was remarkably uniform: always strongly linear (as expected), but often disproportionally. Generally, the ants confirm the fact that thermal limits in ectotherms shift according to changes in viable temperatures (Angilletta, 2009).

There was a significant interaction between population and acclimation temperature in the recovery data in *M. rubra*, suggesting varying temperature dependence of recovery among its populations; specifically, a lower dependence in the southern population. This may be interpreted as an additional argument for a stronger past selection for recovery rate in this population, whose representatives are still faster to recover even when acclimated to higher temperatures.

The temperatures of hardening produced strictly curvilinear trends in the traits. This was especially obvious for the onset of chill coma, one possible explanation being that in constant laboratory conditions the ants became physiologically less prepared to withstand chilling injuries at temperatures closer to zero (at 5°C). But the lack of randomization of hardening treatments (see Materials and methods) makes it possible that the curvilinear trend for chill coma temperatures reflects changes in some uncontrolled factor resulting in the highest cold resistance of the insects in the beginning of the hardening trials (the treatment of 7°C). This seems unlikely, because of the close timing of the trials (each treatment took 2–3 days to complete, with 1 day in between), and because recovery times suggest, on the contrary, the lowest cold resistance for that treatment (at least for ants from 18 and 22°C). However, it is more important that this drawback does not undermine the conclusions about trends among populations and acclimation temperatures, or about general effects of hardening on either trait.

The effect of pre-chilling on recovery in ants from 15°C differed qualitatively from that in ants from the other regimes. This raises the concern that fresh adults in samples from 18 and 22°C (see Materials and methods) could be responsible for the difference. This is unlikely, because random sampling could not yield ant groups consisting only of such adults. Interestingly, a situation in which insects grown at temperatures closer to optimum were less able to withstand cold treatments has previously been described in *Drosophila* flies, which are free from the problem of overlapping generations or age cohorts, presented by the ants (Rako and Hoffmann, 2006). However, in the flies the described effect was found in their survival after a cold treatment, and not in their recovery time.

Finally, the results clearly indicate that constant laboratory temperatures may be poor models of climate. Judging by brood development, the regimes of 22 and 18°C resulted in first adult eclosion timings (Fig. 1) closely approximating those in natural populations at 50°N and 60°N, respectively (Kipyatkov and Lopatina, 1997). Nevertheless, judging by chill coma recovery, the regimes are not equivalents of the corresponding climates. In a simultaneously studied field population at 60°N, recovery in *M. ruginodis* was clearly faster than in *M. rubra* (Maysov and Kipyatkov, 2011). The absence of such interspecific differences in the present study (Fig. 4) suggests that in the field they might be primarily caused by differing responses of these species to daily temperature variation. Apparently, not only repeated exposure to stressful temperatures (Marshall and Sinclair, 2012) but also daily cycles of temperature within viable limits may be able to modify chill coma recovery time in ectotherms and reveal subtle interspecific differences in their climatic adaptation. Therefore, some laboratory emulation of daily thermoperiods would be necessary to make inferences about the effects of climate change on chill coma recovery or its plasticity in the ants. However, both studies show that temperatures of chill coma onset in these species are highly plastic and rather similar, under either constant or field conditions. Thus, it may be safe to assume that the laboratory results adequately reflect possible responses of chill coma temperature in the natural ant populations to climatic changes in their environment.

Conclusions

Chill coma recovery times were clearly divergent among ant populations, giving no support for the hypothesis of thermoregulation negating divergence in cold resistance. Support for the hypothesis (specifically, for the variant emphasizing among-habitat thermoregulation) was found in the similarity of chill coma onset temperatures, with a significant linear contrast in *M. ruginodis*. The disparity of latitudinal patterns of variation between the traits suggests that their usefulness as indicators of climatic adaptation is not equal. This suggestion may be either confirmed or disproved by data on latitudinal variation of chill coma temperature in other ectotherms.

MATERIALS AND METHODS

Species and populations

Myrmica rubra and *M. ruginodis* are common Palaearctic ant species that diverged about 9 million years ago, without further speciation (Jansen et al., 2010); they are relatively abundant over a wide range of latitudes and very often share the same habitats (Leppänen et al., 2013). *Myrmica* colonies are non-territorial (Savolainen et al., 1989), polygynous, and display a prolonged type of annual cycle (Kipyatkov, 2001), whereby worker ants either grow from eggs to adults within one warm season or delay their development until the next year by entering a diapause at the last (third) larval instar. The phenological phases of the cycle, such as the presence of eggs or eclosing adults in a colony, become shorter in colder areas (Kipyatkov and Lopatina, 1997) and the northernmost populations demonstrate only the delayed brood development (Kipyatkov, 2006). For this study, I used the same colonies that were first investigated in 2005; methods of their maintenance were detailed in the corresponding paper (Maysov and Kipyatkov, 2009). Places of collection were located along a latitudinal transect (Fig. 1). Because of the gradual habitat switch that *M. rubra* demonstrates along the transect, in the north, I collected this species in tidal shore areas with peat bog-like vegetation; otherwise, I collected all colonies in forests.

Temperature dependence of cold resistance

Measurements of temperature dependence of cold resistance followed in 2006: first, on colony fractions acclimating for 2 months at three different viable temperatures, and second, on ants subjected after the acclimation period to three different stressful temperatures for 1 h. Like the number of original localities, the number of levels in both temperature factors was chosen to make the investigation feasible for one experimenter. The maintenance regimes and experimental treatments are summarized in Fig. 1; their details are provided below.

The initial colonies experienced similar periods of laboratory-simulated summer (until 1 November 2005), during which a part of their brood, collected with them at the respective sites, developed into adults. Artificial autumn, winter and spring were identical for all populations, roughly imitating thermal conditions that colonies face in nature in North-West Russia (Maysov and Kipyatkov, 2009). The ants overwinter as adults and third instar larvae in a diapausing state (Kipyatkov, 2006); therefore, no food was provided during the winter maintenance.

After the artificial spring (on 11 May 2006), I divided each colony in thirds, randomly distributing the juveniles and adults among three separate formicaries. For a few colonies with fewer than three queens, some of the fractions remained without a queen, a situation regularly observed in natural colonies. Then, I distributed fractions from each colony among three acclimation regimes, 15, 18 or 22 (±0.5)°C, and maintained them at these regimes, measuring ant cold resistance, in total four times during 2 months, at approximately similar intervals in both species. The acclimation temperatures spanned a viable gradient from those nearly optimal for the development of brood (Elmes and Wardlaw, 1983) to those greatly slowing it: at 15°C, the pupal period alone was expected to be over 40 days (Lopatina et al., 2002).

To choose hardening treatments, I first tested several above-zero temperatures on a natural mid-summer population of Petergof (previous studies of hardening in *Myrmica* employed only 5°C). In the pilot hardening test (conducted on 13–14 July), a number of field-fresh ants showed knock-down immediately on transfer to 3°C and did not recover during 3 h at this temperature, indicating it was too stressful. Therefore, 1 h treatments of 5, 7 and 9 (±0.1)°C were applied to laboratory ants in actual hardening trials (conducted on 21–31 July). Available climatological data suggest that these temperatures undoubtedly may occur in the described period at the original localities (Fig. 2), and are ecologically relevant at least for forager ants.

In 2007, I evaluated cold resistance of *Myrmica* in a reciprocal transfer experiment; that is, after a redistribution of material from each acclimation regime among the same regimes. Namely, after 22 months of common conditions (with a second artificial wintering), all colony fractions were acclimated (for 6 days) at their initial regimes, after which they underwent another division into three subfractions, one of which remained at the initial temperature, while the others were acclimated (for 2 days) at one of the other regimes. The studied traits were measured once for each subfraction, in the same way as described below for the acclimation period (see 'Measurements'). This experiment, whose results will be fully described elsewhere, again produced comparative data on the populations, provided here only with information about the final regimes.

Additionally, I made some population comparisons using wintering and vernal ants, immediately before (in 2006–2007) and during an artificial spring (in 2006).

Despite the common garden conditions, irreversible phenotypic plasticity might still affect among-population variation of the cold resistance traits in 2006, but could not be present in the samples measured in 2007 (see 'Measurements', below). Some possibility of maternal effects, due to differences in climates experienced by queens, still remained in 2007. Given the lifespan of these ants, maternal effects on any physiological parameter would be especially difficult to remove: indirect estimates (by measuring the coefficient of genetic relatedness within colonies) indicate that, on average, queen longevity constitutes about 1.5 years (Seppä, 1994), which is in agreement with earlier direct measurements of about 2 years maximum for worker females (Brian, 1972). In such animals, maternal effects (if any) might persist for at least 3, possibly 4 years. Nevertheless, the following considerations suggest that maternal effects are not to be expected here. Maternal effects provide a benefit only if the environment of the maternal generation correlates with that of the offspring (Whitman and Ananthakrishnan, 2009). Because of the long lifespan, worker individuals have to experience certain (probably full annual) variation of nest temperatures, plus, when they start foraging, some additional variation of outside temperatures during the active season(s). In contrast, gynes, who either found their own nest or return to the maternal one upon mating (Seppä, 1994), spend their lives almost entirely within nesting substrates, unless their colonies are forced to move by territorial ant species (Savolainen et al., 1989).

Measurements

For measuring the traits of cold resistance, I followed a procedure developed in 2005–2006 and described in detail subsequently (Maysov and Kipyatkov, 2011). Briefly, worker individuals, in triplicate per fraction, were photographed once a minute while being cooled at a rate of 0.17°C min^{-1} (from 5 to −3°C) in metallic well containers on a thermogradient device (supplementary material Fig. S4); workers were maintained at −3°C for 10 min and then photographed again once a minute while recovering upon immediate transfer to 20°C. Timings of container temperatures were recorded during the cooling phase over 0.5°C intervals. Later, I used the time stamps of digital photographs, first, to find the temperature at which photographed knock-downs occurred (by comparison with the temperature timings) and, second, to calculate the time of photographed recoveries. For hardening tests, ants were sampled in triplicate per fraction for each temperature of pre-chilling; after 1 h at that temperature, measurements were taken as described above. The hardening took place on the thermogradient, whose short working surface and high thermal inertia did not allow formation of the entire range of temperatures from 9 to −3°C or easy switching from a colder to a warmer mode. Therefore, hardening treatments were not randomized but applied separately in this order: 7, 9, 5°C. For the treatments at 7 and 9°C, after 1 h in those modes, I had to switch the device to a more intensive cooling to achieve −3°C on its colder end.

Distribution of the populations along a container was always uniform and alternating. Within a population, pre-established randomized sequences of colony numbers and acclimation temperatures defined the position of ant triplicates in a container.

I sampled ant individuals from the fractions randomly, avoiding only freshly eclosed calf ants (distinguished by very light coloration), known from the pilot test to be knocked down by temperatures sometimes even as high as 9°C. By the time of the hardening trials in 2006, many of the fresh adults (first to eclose around mid-June at 22°C and during the first 10 days of July at 18°C, in all populations) had already passed the calf stage and darkened enough to be within the normal range of variation in adult coloration; therefore, samples from these regimes were expected to have more laboratory-reared adults than those at 15°C (at which temperature fresh adults started to emerge only in mid-September, in all populations).

Comparison of ant sizes (widths of head measured under a binocular microscope, magnification ×2 for the objective, ×8 for the ocular) before and during the hardening trials confirmed this (in the trials, ants from the higher temperature regimes tended to be smaller), making the data from 18 and 22°C more comparable, and suggesting a need for separate analysis.

A 5 day outage of the conditioning system in the experimental room, starting 17 June, resulted in an increase of the room temperature from 20 to 23 (±0.5)°C and hence exceptionally fast recoveries of the insects in the acclimation assays on 17 and 19 June. These recovery data caused statistical problems (see below). Knock-down data remained unaffected, because the thermogradient device was well isolated.

In 2007, all acclimation regimes could provide ants grown completely in the laboratory conditions; to make sure that triplicates consisted only of such ants, I intentionally sampled lighter-coloured individuals (as the periods of acclimation here were short, no new ants eclosed).

Cold resistance of active ants was measured in the same way as in 2006. However, to measure recovery time in wintering ants (supplementary material Fig. S2), I made a single methodological adjustment to be able to get more variation in the data: instead of immediate transfer of ants from −3 to 20°C (which resulted in nearly simultaneous recovery within the second minute), natural warming of containers, returned from −3 to 20°C, was used (supplementary material Fig. S3). The slightly different resultant parameter could not be compared with recovery in active ants, but allowed comparison of wintering populations.

Statistical analysis

As each experimental factor (latitude and temperatures of acclimation and hardening) had three levels, which may be viewed as insufficient for regression analysis, I employed general linear models (GLMs) with *a priori* polynomial contrasts to investigate the trends that the factors induced in the cold resistance of the ants. Such one-degree-of-freedom contrasts are able to detect significance of linear and curvilinear trends even for a main factor with no significant differences among its levels (Sokal and Rohlf, 1995).

The GLMs always included the relevant fixed-effect factors and initially also a random-effect factor of colony, nested within latitude. Two major sets of data from 2006 were analysed separately: the acclimation period and the hardening trials. GLMs were applied to the sets intraspecifically, using the GLM command in SPSS 12.0RU (SPSS Inc., 2003). Models for the hardening trials included one covariate, head width, while those for the acclimation period additionally had the duration of acclimation (in days) as a second covariate. Unequally spaced levels of two factors (latitude and acclimation temperature) were specified in the SPSS syntax for the software to make corrections. As the SPSS output does not provide mean squares of the contrasts, which were also impossible to work out manually because of the unequal level spacing and often non-orthogonal design, the contrasts are not shown in resultant GLM tables, but summarized separately (supplementary material Tables S1, S2). After analysing full datasets, I also repeated the tests without data from 15°C. Standard significance levels were adjusted according to the Bonferroni method (divided by two, the number of repetitions for each dataset).

The assumption of homoscedasticity, which was tested by Levene's test simultaneously with executing the models, was often violated, especially with data on recovery. Therefore, the main task was to find a model that satisfied this assumption, which is of a greater importance than that of normality: simulation studies have shown that even severe violations of the latter do not affect the performance of this type of tests (Quinn and Keough, 2002). In cases of significant heteroscedasticity, I attempted to use data transformations, model reductions (removals of insignificant interaction terms) or analyses of fraction means, in that order, until a model showed acceptable homogeneity of variance. Sometimes, variation in the models became homogenic only if the fraction means were transformed and all interaction terms removed (supplementary material Table S6). Moreover, no homogeneity could be achieved with recovery data for the acclimation period unless the exceptionally fast recoveries caused by the outage of the room conditioning system were removed from the datasets.

Type IV sums of squares were employed for *M. ruginodis* whenever individual data (i.e. not fraction means) were analysed, because in those cases there was a missing cell in model layouts due to escape of an entire fraction from a cracked formicary before the first measurement.

Analyses of data from 2007 were analogous to the above, with minor differences (main factors were latitude, temperature of acclimation and reciprocal transfer temperature).

Acknowledgements

The staff of two nature reserves, Kandalakshskiy in Luvenga and Belogorye in Borisovka, kindly provided access to their territories. The staff of the Laboratory of Entomology (St Petersburg State University) in Petergof provided various help, including maintenance of the photothermostats in working condition, diversification of ant diet and creation of customized equipment. Two anonymous referees gave valuable criticisms, comments and suggestions on earlier versions of the text, greatly improving the final result. Julia Koricheva checked the final manuscript for readability. This paper is dedicated to the memory of Professor V. E. Kipyatkov, who kindly allowed me to substantially elaborate and extend the design of the study, which he initially suggested to be conducted as a 1 year, one-temperature experiment.

Competing interests

The author declares no competing financial interests.

Funding

The study was partly financed by the Russian Foundation for Basic Research (RFBR) [grants 03-04-48854 and 06-04-49383] and the Federal Program 'Universities of Russia' [grant 07.01.327].

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.096958/-/DC1

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