Body Size, but Not Cooling Rate, Affects Supercooling Points in the Red Imported Fire Ant, *Solenopsis invicta*

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ABSTRACT The level of an animal's stress resistance is set by multiple intrinsic physiological and extrinsic environmental parameters. Body size is a critical intrinsic parameter that affects numerous fitness-related organismal traits including fecundity, survival, mating success, and stress resistance. The rate of cooling is a critical extrinsic environmental factor that can affect thermal stress resistance. Workers of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), display considerable variation in adult body size. Therefore, developing ecologically realistic models of thermotolerance in this species requires a consideration of body size. We tested the hypothesis that body size and cooling rate would interact to set the supercooling point in fire ant workers by exposing workers of a range of body sizes to three different cooling regimens: a very fast ramp of $-10°C/min$, an intermediate ramp of $-1°C/min$, and an ecologically relevant slow ramp of $-0.1°C/min$. Specifically, we asked whether large workers were more susceptible to differences in cooling rate than smaller workers. We found that body size had a considerable effect on supercooling point with the largest workers freezing at a temperature ~3°C higher than the smallest workers. Cooling rate had a very small effect on supercooling point, and there was no interaction between the two factors. Therefore, the allometry of supercooling points across the range of worker body sizes does not change with cooling rate.

KEY WORDS body size, cooling rate, supercooling point, body size $\times$ cooling rate interaction, fire ant

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Although many stress studies concentrate on a single environmental or physiological factor in isolation, stress resistance is the product of both extrinsic environmental factors and intrinsic physiological factors. The responses of stress resistance mechanisms are often correlated with each other and levels of environmental stressors (Chen and Denlinger 1992, Sømme 1995, Feder and Hofmann 1999, Hoffmann and Hercus 2000, Hoffmann et al. 2001, Bale 2002, Sinclair et al. 2003). Therefore, to understand an animal's stress resistance, it is important to consider both intrinsic physiological factors and extrinsic environmental factors, as well as interactions between intrinsic and extrinsic factors.

Mechanisms underlying the temperature at which ice forms in tissues, the supercooling point, have long been a subject of active inquiry in insect thermal stress biology. Many species of freeze-tolerant insects possess mechanisms that promote relatively high supercooling points, allowing them to decrease their probability of incurring damage caused by freezing at low temperatures and to conserve energy during the overwintering period (Zachariassen 1985, Irwin and Lee

2002, 2003). In contrast, for freeze-intolerant insects, the supercooling point represents a clear lower lethal limit for survival, and freeze-intolerant species typically depress supercooling point as part of their seasonal cold-hardening strategy (Lee 1991).

Body size is an important fitness correlate in most organisms, and in insects, larger individuals usually have greater survival, longevity, mating success, fecundity, and fertility (Roff 1992, Nylin and Gotthard 1998, Blanckenhorn 2000). Larger size is also typically associated with greater stress resistance. For example, large body size has been shown to confer desiccation resistance both within and between species of both ants and keritan beetles (Hood and Tschinkel 1990, Chown et al. 1999). Similarly, larger bodied individuals typically contain greater nutrient reserves and a lower mass-specific metabolic rate, conferring greater resistance to periodic starvation (Stockhoff 1991, Ohgushi 1996, Raubenheimer and Simpson 1997, Reim et al. 2006).

Large body size is also thought to confer added resistance to some types of thermal stress, such as above-freezing cold damage (Salt 1966a, Heinrich 1989, Sømme 1995, Chown and Nicolson 2004, Shepherd et al. 2008). However, previous studies of supercooling point have shown that larger individuals both within and among taxa typically have higher supercooling points than smaller individuals (Sømme 1982,

Pugh 1994, David 1996, Dautel and Knülle 1996, Sinclair and Chown 2005). For example, supercooling point changes with larval stadium in the mealworm *Tenebrio molitor*, where small early-instar larvae have lower supercooling points than larger later-instar larvae (Johnston and Lee 1990). This correlation is in agreement with physical studies of droplets of pure water wherein very small droplets can supercool to much lower temperatures than larger droplets, which have a greater probability of ice nucleation (Angell 1982). Therefore, body size is an important intrinsic factor for cold tolerance in insects, wherein larger individuals may be more resistant to declining performance and nonfreezing damage, but larger individuals are also more susceptible to freezing caused by their higher supercooling points.

The rate of temperature change is a critical extrinsic factor affecting cold tolerance in insects (Worland and Convey 2001, Bale 2002, Sinclair et al. 2003, Shreve et al. 2004). Typically, faster rates of cooling lead to decreased resistance to above-freezing damage, such as LT_{50} values for a 1-h exposure, and decreases in performance parameters, such as knock-down temperature or CT_{min} (Bale et al. 1989, Kelty and Lee 1999, 2001, Powell and Bale 2006). Temperatures in most habitats decrease relatively slowly, and it is thought that improved survival and performance at slower, ecologically relevant, cooling rates is reflective of the induction of rapid cold hardening mechanisms that insects may use during natural thermoperiodic cycles (Kelty and Lee 2001, Sinclair et al. 2003). In contrast to above-freezing damage, slower rates of cooling generally have a negative impact on supercooling ability wherein ice nucleation occurs at higher temperatures when organisms are cooled more slowly. For example, Salt (1966b) showed that the supercooling points of diapausing larvae of the wheat stem sawfly, *Cephus cinctus*, decreased linearly with increasing cooling rate from 0.008 to 8°C/min. Slow cooling rates increase the time of exposure to temperatures below the melting point, which leads to an increased probability of ice nucleation occurring and consequently higher supercooling points at slower cooling rates (Salt 1966c, Miller 1978, Shimada and Riihimaa 1988, Lee et al. 1996).

Both body size and cooling rate can affect an insect's supercooling point through different yet complementary mechanisms influencing the probability of ice nucleation. Therefore, understanding supercooling in species with a wide range of body sizes requires understanding the relative roles of body size and cooling rate on supercooling point and whether any interaction occurs between these key intrinsic and extrinsic factors. We expect that larger individuals will have a proportionally greater increase in supercooling point with slow cooling rates than small individuals. To test the hypothesis that body size and cooling rate interact to set supercooling points, we determined the relationships between body size, cooling rate, and supercooling point in workers of the red imported fire ant, *Solenopsis invicta* Buren, an invasive pest species with substantial continuous body size variation among workers within a colony (Porter and Tschinkel 1985).

Supercooling points of *S. invicta* workers have been examined by several authors in a number of contexts including comparisons across fire ant species (Francke et al. 1986, Diffie and Sheppard 1989, James et al. 2002), seasonal acclimation (Taber et al. 1987), geographic variation (Quarles et al. 2005), and infection with microorganisms (Landry and Phillips 1996, James et al. 2002). Both Francke et al. (1986) and James et al. (2002) studied the role of worker size on supercooling point, each finding that small workers had lower supercooling points than large workers, as expected. However, in all of these previous studies, workers have been cooled at rates in excess of 5°C/min, which is much more rapid than the temperature fluctuations that these ants would experience in either their natural or introduced ranges. Supercooling points in the above studies have all been between −6 and −12°C, and it has been observed that these are temperatures much lower than one would expect ants to experience in their mounds in the soil. Our current work extends the previous research on supercooling capacity of *S. invicta* workers by studying the relationship between worker body size and supercooling points at several cooling rates, including a very fast rate of 10°C/min, an intermediate rate of 1°C/min that is the standard for many cold-tolerance studies, and a slower, more ecologically relevant cooling rate of 0.1°C/min. We asked the following specific questions. First, does cooling rate affect the supercooling points of workers in this species? Specifically, does cooling workers at a slow rate increase worker supercooling points to temperatures that might be experienced in the soil in nature? Second, does cooling rate affect the allometry of supercooling points across the body size range in *S. invicta*? Specifically, do different cooling rates affect large workers more than small workers? Third, is there any significant interaction between cooling rate as an extrinsic environmental factor and body size as an intrinsic physiological factor?

Materials and Methods

Five monogyne colonies of *S. invicta* were maintained under a 16 L:8 D photoperiod at 30 ± 2.0°C and 60% humidity. Each colony was reared from a single queen in a similar laboratory environment. All colonies were kept in open-top trays (52 by 40 by 13 cm) lined with Fluon to prevent escape. To provide sugar to the colonies, KimWipes were soaked in 2 M sucrose solution and dried. Water was added to the dried KimWipes daily to permit sugar consumption, and the ants were fed frozen crickets Monday through Friday. All the colonies had continuous ad libitum access to both sugar and water and ad libitum access to crickets 5 d/wk.

Individual ants occurring outside the nest enclosures were haphazardly selected within each colony for chilling. Individuals were chilled using a computer-controlled Thermo Neslab RTE-740 water bath containing a 1:1 solution of water and ethylene glycol.

Each individual was subjected to chilling at one of three discrete rates: a direct plunge into a bath equilibrated at −35°C, which yielded an approximately linear cooling rate of −10°C/min from 28 to −25°C, tapering off in rate from −25 to −35°C, a controlled ramp of −1°C/min from 28 to −35°C, and a controlled ramp of −0.1°C/min from 28 to −35°C. None of the supercooling points measured in the direct plunge treatment were below −25°C, so this treatment effectively ramped linearly at −10°C/min.

The supercooling point of each ant was measured using a 36-gauge copper/constantan thermocouple (Omega Engineering) linked to a data logging thermometer (Extech EA15) recording at 1-s intervals. The tip of each thermocouple extended through the cap of a 2-ml plastic screw-cap vial that was used to house each ant during chilling. A wooden dowel was attached to the inside of the vial cap with hot-melt glue and served as an anchor point. Each ant selected for chilling was temporarily anesthetized with N_2 and attached at the abdomen to the tip of the thermocouple using a very thin layer of petroleum jelly. Ants were fastened to the dowel with a very thin strip of modeling clay. The supercooling point for each ant was defined as the lowest temperature before a substantial (>1.0°C) exotherm was observed. After chilling, specimens were dried in a 50°C oven for at least 24 h, and their head widths, a typical measure of body size in ants, were quantified (in mm) using a wedge micrometer (Porter 1983).

Data were analyzed using analysis of covariance (ANCOVA), with size (head width) and cooling rate as explanatory variables and colony of origin as a random variable blocked across treatments. We specifically tested for allometric effects of body size on the response of supercooling point to cooling rate (i.e., are larger workers more susceptible to changes in cooling rate than smaller workers) by studying the interaction term between body size and cooling rate in the full-model ANCOVA. Because we were particularly interested in this interaction, both models with and without this interaction are shown. A stepwise procedure was used to eliminate all nonsignificant terms from the reduced model (Zar 1989). Figures show the adjusted means for each parameter of interest holding all other parameters in the model constant (AKA least squares means). All statistical analyses were performed using the JMP software package (SAS Institute 2004).

Results

Both ANCOVA models were highly significant. There was no significant interaction between body size and cooling rate in the model containing this term (Table 1, model a), so the interaction was dropped from the reduced model (Table 1, model b). Therefore, there was no allometric affect of body size on an individual's supercooling response to cooling rate (i.e., larger workers were not more or less affected by cooling rate than small workers). There was also no significant two-way interaction between colony and

Table 1. Two reduced ANCOVA models for the effects of body size, cooling rate, colony, and interactions on supercooling point

Source	df	F	P
Whole model a	17	4.62	<0.001
Body size (head width)	1	3.75	0.053
Cooling rate	2	2.78	0.063
Colony	4	3.20	0.013
Body size × cooling rate	2	1.21	0.298
Cooling rate × colony	8	2.69	0.007
Error	410		
Total	427		
Whole model b	15	5.07	<0.001
Body size (head width)	1	40.96	<0.001
Cooling rate	2	2.85	0.059
Colony	4	3.19	0.014
Cooling rate × colony	8	2.63	0.008
Error	412		
Total	427		

The first model contains the interaction term between body size and cooling rate, which was not significant and was eliminated from the second model.

body size or three-way interaction in the full model, so these terms were dropped from both reduced models and are not reported. Head width was the greatest contributor to the reduced model ($F = 40.96$, $P < 0.0001$). As expected, body size had a significant effect on supercooling point, with smaller individuals having lower supercooling points than larger individuals across all cooling treatments (Fig. 1). There was a difference of ≈3°C between the supercooling points of the smallest and largest workers. The effect of cooling rate on the reduced model was only moderately significant and constituted a minor part of the whole model ($F = 2.854$, $P = 0.059$). Surprisingly, individuals cooled at the intermediate rate of 1°C/min had the highest supercooling points, although the difference between the body size and colony-adjusted means of all three cooling rate treatments was <1°C (Fig. 2). Colony of origin also had a significant effect on supercooling point, and there was a considerable interaction between cooling rate and colony identity,
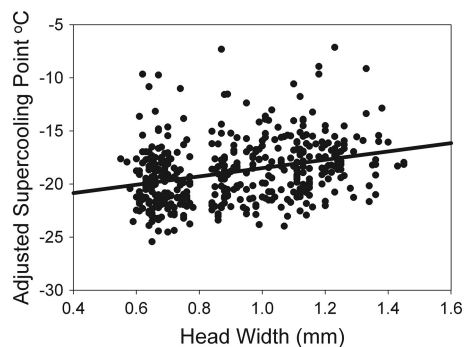

Fig. 1. There was a positive relationship between body size and supercooling point when all other factors were held constant. Larger workers had higher supercooling points than smaller workers across colonies and cooling rate treatments. Points represent the ANCOVA-adjusted least square means.
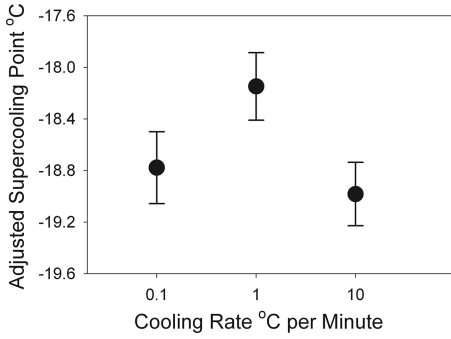
Fig. 2. There was a marginally significant effect of cooling rate on supercooling point when all other factors were held constant, but the differences among the means for the three cooling rate treatments was <1°C. Points represent the ANCOVA-adjusted least square means, and the bars represent SE.

indicating that the colonies differed in their supercooling response to cooling rate (Fig. 3).

Discussion

As expected, body size had an effect on supercooling point, with large workers freezing at higher temperatures than small workers. The regression equation from adjusted residual values predicted a supercooling point difference of ≈3°C from the smallest to the largest workers. This is in agreement with previous studies in fire ants that have also shown that small workers have lower supercooling points (Francke et al. 1986, James et al. 2002). We saw no evidence for an interaction between body size and cooling rate. Therefore, the allometry of supercooling point was constant across cooling rates, and large workers were not more susceptible to differences in cooling rate than small workers, as we had predicted they would be. There was a small effect of cooling rate on supercooling point that approached statistical significance ($P = 0.059$) wherein supercooling points were highest in the intermediate cooling rate regimen of 1°C/min.
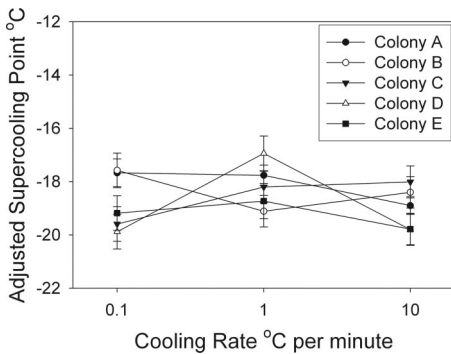

Fig. 3. Some of the colonies differed in their responses to cooling rate, leading to a significant colony X cooling rate interaction. Points represent the ANCOVA-adjusted least square means, and the bars represent SE.

However, the difference in mean supercooling point across the cooling rate treatment groups was <1°C even though the rates of cooling varied over two orders of magnitude (0.1, 1, and 10°C/min). Therefore, we feel that the difference in supercooling point across cooling rate treatments observed in our study is likely not biologically meaningful even though it approaches statistical significance. Other studies have shown that there is little effect of cooling rate on supercooling point in the cooling rate range of −1 to −5°C/min, but the effects of cooling rate on supercooling point increase as rates slow down below 1°C/min (Salt 1966a, b, Miller 1978, Shimada and Riihimaa 1988). We did not find a difference between our slowest cooling regimen of −0.1°C/min and either of our two faster regimens of −1 or −10°C/min, although even slower cooling regimens may have shown an effect.

We did find a large effect of colony of origin on supercooling rate and a significant two-way interaction between cooling rate and colony of origin on supercooling point. Despite the fact that all of these colonies had been reared from queens under uniform conditions in the laboratory and were of the same approximate size and ontogenetic stage, colonies differed in their mean supercooling point within each of the treatments and in their response across treatments (Fig. 3). Among-colony variation in physiological, behavioral, and life history traits is a well-known feature of fire ants and other social insects that can be caused by numerous factors including colony genotype, maternal, environmental, and colony-size related effects (Hölldobler and Wilson 1990, Porter and Tschinkel 1993, Bourke and Franks 1995, Hahn 2006). Because environment and size were kept the same among colonies in this study, the strong observed colony-level effects could have been caused by genetic effects, maternal effects, or perhaps demographic stochasticity. Clearly the basis for among-colony variation in stress physiology merits further study in social insects.

A long-term goal of our work is to better parameterize cold-tolerance in fire ants, extending our knowledge about causes of overwinter mortality and improving current models used to predict the spread of this invasive species. Because of the large economic and ecological impacts of colonization by *S. invicta*, several ecological models have been developed to predict the spread of this species in the United States (Pimm and Bartell 1980, Stoker et al. 1994, Killion and Grant 1995, Korzukhin et al. 2001), and one model has even been expanded to include the potential range of this ant worldwide (Morrison et al. 2004). All of these models rely on published data to project ranges based on estimated effects of temperature on colony growth and overwintering mortality. The effects of temperature on colony growth and sexual production have been relatively well studied and provide an adequate basis for parameterizing ecological models of thermal effects on range limitation (Porter 1988, Calabi and Porter 1989, Porter and Tschinkel 1993, Macom and Porter

1995). Unfortunately, cold tolerance and overwintering mortality have received much less detailed attention, limiting the efficacy of models predicting range limitations based on winter temperatures.

Periodic exposure to cold is a critical source of mortality for fire ant colonies, and colony mortality during a cold event is influenced by both the absolute lowest temperature experienced and the length of time that air temperature at a site spent <0°C (Morrill et al. 1978, Callcott et al. 2000). It is clear that fire ant workers are not freeze-tolerant, because none survive freezing. Although supercooling points vary considerably among studies, all of the literature including our study estimates fire ant supercooling points to be below −6°C and most are between −12 and −20°C (Francke et al. 1986, Taber et al. 1987, Diffie and Sheppard 1989, Landry and Phillips 1996, James et al. 2002, Quarles et al. 2005). Fire ants dwell in complex mounds in the soil and have a well-developed capability for sensing thermal conditions and moving within the mound to optimize their temperatures and the temperature of their brood during development (Porter and Tschinkel 1993, Tschinkel 2005). Fire ants are known to retreat further into their mounds during cold weather, and mounds provide significant thermal insulation, making it unlikely that temperatures within the top of the mound decline below −2 to −4°C in even the coldest locales where fire ants are present (Markin et al. 1984, Porter and Tschinkel 1987, Vogt et al. 2003). One aim of our study was to determine whether a slower cooling rate (0.1°C/min) would push the supercooling points of workers up just below zero, so that freezing could be a potential source of mortality in light of the high subzero temperatures that ants in mounds in the field might experience. However, we found little effect of cooling rate on supercooling points. Even cooling at our slowest rate yielded supercooling points well below temperatures that ants would likely experience in their mounds in the field. Interestingly, all fire ant supercooling studies to date have worked with isolated individuals, not ants in their natural mounds where they would be in contact with a variety of nucleating agents, including ice crystals that may form in damp soil, that could cause freezing at temperatures just below 0°C (Costanzo et al. 1995). Therefore, understanding the importance of freezing at relatively high subzero temperatures to overwintering mortality will require greater study in a field-based, ecologically relevant context.

Acknowledgments

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