To Freeze or Not to Freeze? An Evolutionary Perspective on the Cold-Hardiness Strategies of Overwintering Ectotherms

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ABSTRACT: We address the question of whether freeze-tolerance, freeze-avoidance, or mixed strategy represents the best adaptation for overwintering ectotherms to endure severe winter. To this end, we develop an optimization fitness model that takes into account different physiological parameters such as energetic level, the physiological stress associated with each strategy, and climatic variables. The results show that the freeze-tolerance strategy is strongly dependent on a low sensitivity to the number of freezing days and on a capacity to reduce stress associated with freezing. This strategy is also favored when the initial energetic level is low compared to the freeze-avoidance strategy, which is favored by a high initial energetic level, a low stress associated with the supercooling, and a low sensitivity of this strategy to climatic conditions. From a theoretical point of view, the mixed strategy permits survival in harsher environments but requires the optimization of all parameters involved in both cold-hardiness strategies. However, the mixed strategy shows energetic advantages in variable environments allowing animals to resist the harshest periods. From the model results, it appears that the phys-

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iological processes developed by ectotherms to reduce these stresses might be a key to understanding the evolution of the cold-hardiness strategies.

Environmental temperature determines the body temperature of ectotherms and thereby affects many of their biological processes. Therefore, the duration and the intensity of the temperate and Arctic winters represent a serious challenge to all overwintering terrestrial ectotherms. Various adaptations have evolved in order to ensure ectotherm survival during periods of subzero temperatures. Most overwintering animals are completely aphagic and thus must rely on stored energy reserves to meet metabolic demands, particularly for their cold-hardiness strategy. Cold-hardiness strategies are commonly divided into two main groups: freeze tolerance and freeze avoidance via an extensive supercooling capacity. The freeze-tolerance strategy, where animals endure the conversion of a fraction of body water into ice, is characterized by mechanisms such as the production of ice nucleators that allow the initiation of freezing at high subzero temperatures and cryoprotectant substances that allow controlled propagation of ice within the body (Storey and Storey 1988). In contrast, the freeze-avoidance strategy is characterized by various metabolic adaptations involving the release/masking of potent ice nucleators and the accumulation of low molecular weight carbohydrates and antifreeze proteins that provide freezing-point depression (Zachariassen 1985).

In terms of energetic costs and benefits, the two strategies differ strongly (Block 1991; Joanisse and Storey 1996a). For instance, the maintenance of metabolic functions is more costly in a supercooled state than in living frozen tissues (Salt 1958; Asahina 1969). However, the freezing strategy can involve ice damage to tissues, anoxia, ischemia stress, and end-product accumulation that can entail a long-term survival cost (Storey and Storey 1988). To maximize benefits and minimize costs, a mixed strategy (including both freeze avoidance and freeze tolerance)

would represent an alternative solution and is, indeed, found in a few organisms such as insects and reptiles (Ring and Tesar 1980; Horwath and Duman 1984; Duman et al. 1991; Costanzo et al. 1995).

The various physiological mechanisms involved in cold hardiness have received increasing attention over the last three decades. However, to understand the evolution of cold-hardiness strategies, one needs to put them into an evolutionary context where the costs and benefits associated with each strategy are identified and where the characteristics of the environment that determine the life cycle of the species are known (Danks 1996). Although some elements of comparison have already been presented (Zachariassen 1985; Block 1991, 1995; Duman et al. 1991; Somme 1995), until now, no general scenario for the evolution of cold-hardiness strategies has been proposed.

In this article, we examine, from a theoretical point of view, how animals maximize their winter survival together while confronted with the imperative of minimizing the energy expenditure during this period where resource exploitation is impossible. To do so, we present a predictive model where the major physiological characteristics of hibernation have been integrated in order to determine which strategy is better in different environments and why. We choose an energetic definition of fitness (Levins 1968; Brown et al. 1993) because the costs and the relative benefits of overwintering strategies might be expressed either as winter survival or in terms of subsequent reproductive success (Bale 1987; Kozlowski 1991; Irwin and Lee 2000). We therefore assume that fitness is directly correlated with the amount of energy available at the end of winter. This amount will depend on the initial prewinter energy level and the metabolic demands of the particular cold-hardiness strategy. Furthermore, we examine the effect of the environment on the fitness of the cold-hardiness strategies. To do this, we investigate the influence of temperature (T) and the number of freezing days (N). This is because the physiological mechanisms (activated metabolic pathways, final energy status, accumulation of end product) involved in the two cold-hardiness strategies when facing the same environment (constant or fluctuating) are different (Churchill and Storey 1989; Block 1991; Storey and Storey 1992).

Our approach is initially analytical. To obtain more detailed results, we then follow it with numerical simulations. Finally, model predictions are compared with what is currently known from the literature.

The Model

We model variation in the energy level of an animal, W, as a function of different physiological constraints imposed by each strategy and the environmental conditions (number of freezing days, N, and cold intensity, T).

Even if overwintering ectotherms must primarily survive winter, they must also emerge in good condition because energy demands (reproduction and growth/metamorphosis) are high just after emergence (Fast 1964; Fitzpatrick 1976). The equations have been defined to model crucial elements of the cold-hardiness strategies (energy used during subzero temperature and injuries associated to each strategy) that underlie the survival of the overwintering animal. Using survival as fitness index would not induce significant changes to our energy-based model since similar shape of curves should then be defined for the two strategies.

Assumptions

Let us define W_T, W_A, and W_M, respectively, as the fitness of the freeze-tolerance, freeze-avoidance, and mixed strategies at the end of the winter. These variables depend on the species initial energetic level (at the onset of winter), W_0, winter environmental conditions, and the physiological stresses associated with each strategy.

Physiological Stresses. Each strategy has a physiological stress associated with the onset of the subzero temperature (S_T for the freeze-tolerant strategy and S_A for the freeze-avoidance strategy) that includes the metabolic cost of activating and using a strategy.

For freeze tolerance, such stresses include physical disruptions of extracellular structures induced by ice crystal growth and all the structural and metabolic consequences of freezing in terms of osmotic shock, anoxia, and ischemia (Storey and Storey 1988; Storey 1996). In addition to these factors, a few physiological phenomena, such as cryoprotectant synthesis at the onset of the freezing process (i.e., in frogs) can represent an additional energetic cost at the beginning of the freezing period, which has to be taken into account.

For freeze avoidance, various laboratory and field studies have shown the existence of a cold stress in supercooled insects that can lead to the death of the animal before they attain their supercooling point (SCP; Turnock et al. 1983, 1985; Bale 1996). Such stress, S_A, may be induced by the damage caused by cold to some structural components, such as membranes and proteins (Storey and Storey 1988 and references therein). However, it is commonly assumed that a freeze-avoidance strategy induces little physiological stress since the supercooling state is compatible with life processes. In fact, it is tolerated by a number of species, even subtropical ones (Lowe et al. 1971). Thus, in our model, we assume that $S_T > S_A$. The animals using the

Table 1: Definitions of symbols used in the text

Variables	Definition	Units
W_0	Energetic reserves metabolizable at the beginning of winter: lipids and carbohydrates (principally glycogen)	Calories/mass unit
W_A	Energy quantity available at the end of the winter period for the freeze-avoidance strategy	Calories/mass unit
W_T	Energy quantity available at the end of the winter period for the freeze-tolerant strategy	Calories/mass unit
W_M	Energy quantity available at the end of the winter period for the mixed strategy	Calories/mass unit
N	Number of freezing days	d
T	Cold intensity	°C
S_A	Energetic cost associated with the freeze-avoidance strategy	Calories/mass unit
S_T	Energetic cost associated with the freezing strategy	Calories/mass unit
a	Sensitivity of the freeze-avoidance strategy to the climatic conditions, which can also be considered the energy required to produce reliable cryoprotection for a defined NT	None
N_{max}	Maximum number of freezing days before death	d
T_{lim}	Cold intensity limit beyond which the freeze-tolerant strategy has a greater fitness than the freeze-avoidance strategy	°C
N_b	Number of days after which the mixed strategy switches from supercooling to freezing	d
$N_{threshold}$	Sensitivity limit for each strategy to variations in N	d
N_{var}	Number of days after which the temperature changes in a variable environment	d

mixed strategy are confronted with combined stresses associated with the two strategies ($S_M = S_T + S_A$).

Freeze-Tolerance Strategy and the Environment. A freeze-tolerant animal can survive a maximum number of freezing days, defined as N_{max}, after which it dies. This limit depends on the physiological capacities of the species, such as the use of endogenous energy and the tolerance of end-product accumulation and anoxia (K. B. Storey and J. M. Storey 1984, 1985; J. M. Storey and K. B. Storey 1985). Within 0 and N_{max}, the sensitivity of the freeze-tolerance strategy to the number of days frozen is dependent on a parameter called "θ." This parameter allows an examination of different patterns of fitness that decrease with time.

The majority of freeze-tolerant animals synthesize ice nucleating agents (INAs) and a large amount of cryoprotectant (before or after the onset of freezing) that promote safe extracellular freezing at high subzero temperatures. But once frozen, animals meet their energetic demands through anaerobic pathways since oxygen is not available. Hence, we assume that the energy cost incurred by frozen animals, in terms of energy, is independent of temperature. This assumption is supported by empirical data for insects (Zachariassen and Hammel 1976; Bale 1996).

Freeze-Avoidance Strategy and the Environment. The freeze-avoidance strategy is dependent on polyols, carbohydrates, and thermal hysteresis protein synthesis (Zachariassen 1985). The amounts of these compounds animals need to stay in a supercooled state depend on the cold intensity (T; Turnock et al. 1985; Nedved et al. 1998). Thus, the fitness of the freeze-avoidance strategy depends on NT (the product of the number of freezing days and cold intensity), which may be considered to be a variable describing the climatic conditions. The parameter a is defined as the sensitivity of the freeze-avoidance strategy to NT and thus can be considered to be the energy required to produce reliable cryoprotection for a defined NT. It is important to note that some ectotherms (principally insects) go into winter with a preset level of cryoprotectant, but this synthesis occurs with late summer feeding (Rickards et al. 1987). With the definition of W_0 in our model, we postulate that all the physiological preadjustments needed for a successful overwintering are made (preparation of the overwintering site, accumulation of reserves, enzyme conformation changes, gut clearance, and so forth). The cost of the synthesis of a preset amount of low molecular weight substances is therefore not incorporated in our model.

Equations

We now present the equations that define the fitness (energy) for each strategy. The meaning of each parameter is given in table 1.

The Freeze-Tolerance Strategy.

$$W_T(N) = (W_0 - S_T)\left[1 - \left(\frac{N}{N_{max}}\right)^\theta\right]. \qquad (1)$$

When an animal freezes, it pays a stress cost S_T, that is subtracted from the initial energetic level, W_0. Once frozen, it can survive until N_{max} d as defined above. The parameter θ allows us to generate a number of different situations that are represented in figure 1. When $\theta > 1$, the fitness is nearly constant but declines abruptly near N_{max}. In contrast, when $\theta < 1$, a sharp drop of fitness occurs just after freezing, and then it remains relatively constant. If $\theta = 1$, the decline is linear.

The Freeze-Avoidance Strategy.

$$W_A(N, T) = (W_0 - S_A) - aTN. \quad (2)$$

When the animal enters a supercooled state, it pays a stress cost, S_A, that is subtracted from the initial energetic level, W_0. Because the freeze-avoidance strategy is penalized both by the number of cold days, N, and the cold intensity, T, we use the global climatic variable NT. This variable is weighted by the parameter a, which reflects the sensitivity of W_A to that climatic variable. Such an NT variable represents a limitation of our model because it assumes that the temperature overwintering site of supercooled animals is constant.

The Mixed Strategy. By definition, the mixed strategy uses both the freeze-tolerance and the freeze-avoidance strategies. Because of physical constraints, the animal always starts with supercooling before it freezes (even if the supercooling capacity is very poor). The energetic cost associated with switching from supercooling to freezing is defined as S_T. We also define N_b as the number of days after which the switch occurs. The fitness equation then takes a different form before and after N_b:

$$N < N_b \Rightarrow Wm = (W_0 - S_A) - aTN,$$

$$N \geq N_b \Rightarrow Wm = [(W_0 - S_A - aTN_b) - S_T]$$

$$\times \left[1 - \left(\frac{N - N_b}{N_{max}} \right)^\theta \right]. \quad (3)$$

It is important to note that overwintering strategies involve adaptations to cold, but the maintenance of their water balance during the winter is also crucial. During subzero temperature periods, water is present only in the form of ice and snow, therefore reducing the availability of fluid water, which may induce a desiccation. However, many studies have shown that, in frozen ectotherms, the body fluids are in vapor pressure equilibrium with the ice within and outside the body, regardless of temperature. Thus, no detectable water loss occurs (Baust and Nishino
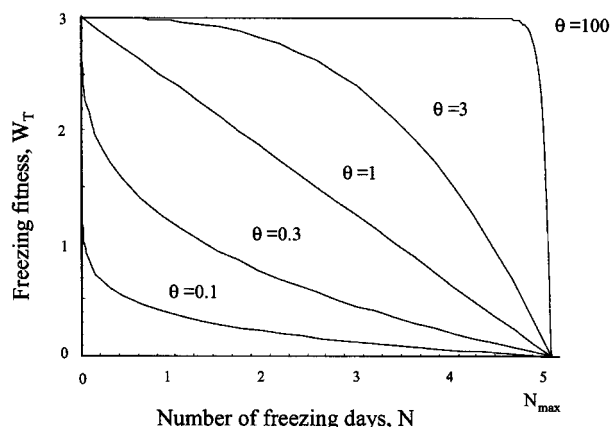

Figure 1: Fitness of the freeze-tolerance strategy as a function of the number of days at subzero temperatures for different values of θ.

1991; Lundheim and Zachariassen 1993). The situation is different for supercooled ectotherms because the water vapor pressure in equilibrium with the supercooled water is higher than that in equilibrium with ice, which induces a gradual water loss by evaporation. However, the accumulation of polyols and other low molecular weight substances, which are considered to be cryoprotectants, reduces water losses (Somme 1995). Thus, cryoprotection and desiccation resistance functionally overlap because the adaptations that evolved to counter desiccation also aid cold hardiness (Ring and Danks 1994; Block 1996).

We thus conclude that the integration of the water balance into the model does not result in qualitative modifications to our results since no additional cost of energy will be incurred.

Results

We first compare the freeze-tolerance and the freeze-avoidance strategies, and then we examine the mixed strategy. In all cases, we have used an analytical and a numerical approach.

Freezing Tolerance versus Freezing Avoidance

Analytical Results. Let us define Ψ as the difference between the fitness of the freeze-tolerance and supercooling strategies:

$$\Psi = (W_0 - S_T) \left[1 - \left(\frac{N}{N_{max}} \right)^\theta \right] - W_0 + S_A + aTN. \quad (4)$$

Solving this equation with respect to Ψ determines the

optimal conditions for one or the other strategy. It is equivalent to the result of a competition process between two species that exhibit different cold-hardiness strategies. If $\Psi > 0$, then the freezing tolerance will outperform the freeze avoidance. If $\Psi < 0$, then the opposite will occur.

Conditions favoring a freeze-tolerance strategy ($\Psi > 0$). A freeze-tolerance strategy is favored by high values of θ because, while $N < N_{max}$, the ratio between N and N_{max} raised to the power θ tends toward 0 when θ tends toward infinity. Furthermore, the physiological stress associated with freezing, S_T, has to be low (meaning that the physiological mechanisms responsible for reducing freezing stresses and injuries have to be reliable), and the maximum number of freezing days, N_{max}, must be high. A low initial energetic level, W_0, also favors this strategy. If we consider two animals with the same low W_0 using each strategy, at $N = 0$, the one that supercools has a higher fitness than the one that adopts freeze tolerance, which pays the cost of freezing. When N increases, the freeze-tolerance strategy becomes advantageous because of the concavity of its fitness curve ($W_0 = 6$; see fig. 2). However, W_0 must be higher than S_T, otherwise W_T will be below 0, and the animal will die from the physiological stress of freezing. Thus, we can then define upper and lower limits of W_0, where the freeze-tolerance strategy has a greater fitness than the supercooling strategy:

$$S_T < W_0 < \left(\frac{N}{N_{max}}\right)^{-\theta}\left[\left(\frac{N}{N_{max}}\right)^{\theta} S_T + S_A + aNT\right]. \quad (5)$$

Conditions favoring a freeze-avoidance strategy ($\Psi < 0$). The conditions favoring the freeze-avoidance strategy are easily obtained from equation (4). We should have short exposure to subzero temperature (low N value) and low values for the stress associated with supercooling, S_A, and the sensitivity to climatic conditions, a. As a corollary to the above, W_0 has to be high (i.e., there must be abundant energy reserves at the onset of winter; $W_0 = 12$; fig. 2).

The influence of the environment. Because the freeze-tolerance strategy is energetically independent of cold intensity (see the model assumptions), one can easily predict that it will be more efficient than the freeze-avoidance strategy for high values of cold intensity, T. From equation (4), we define a limit for T, T_{lim}, beyond which the fitness of the freeze-tolerance strategy is greater than for the freeze avoidance ($\Psi > 0$):

$$T_{lim} = \frac{(W_0 - S_T)(N/N_{max})^{\theta} + S_T - S_A}{aN}. \quad (6)$$

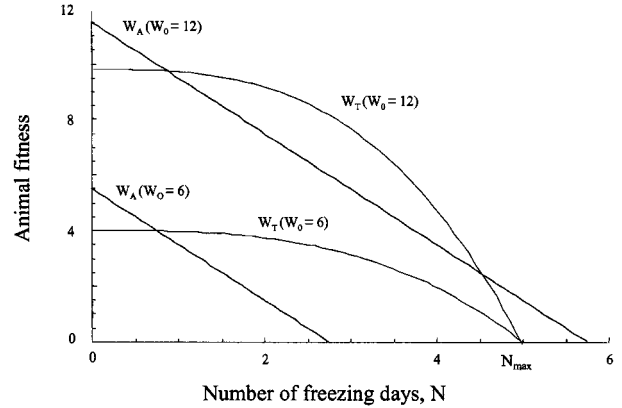A low value of T_{lim} favors the freeze-tolerant strategy.

Figure 2: Fitness of freeze-tolerance, W_T, and supercooling, W_A, strategies as a function of the number of days at subzero temperatures for two different values of W_0. The other parameters are $S_T = 2$, $S_A = 0.5$, $T = 4$, $a = 0.5$, $\theta = 3$, and $N_{max} = 5$. The fitness function, for W_T, is flat deviations from the optimality solution, and this will have little impact in many cases.

Note that the fitness of the supercooling strategy is always greater than that for the freezing strategy when the number of freezing days, N, is low because, when $N \to 0$, $T_{lim} \to +\infty$.

From equations (4) and (6), the number of freezing days, N, appears to have two opposite effects on the relative fitness of each strategy. From equation (6), it appears that the numerator of T_{lim} decreases with N, but at the same time, N, being in the denominator, must also be high to favor a low value of T_{lim}. To understand this antagonistic effect, we have calculated the sensitivity of T_{lim} to variations in N:

$$\Phi = \frac{\partial T_{lim}}{\partial N} = \frac{W_0 - S_T}{aN_{max}^{\theta}}(\theta - 1)N^{\theta-2} - \frac{S_T - S_A}{aN^2}. \quad (7)$$

When Φ is positive or negative, T_{lim} will increase or decrease, respectively, as N increases. The absolute value of Φ gives the shape of that variation.

With special values of θ, and considering that $W_0 > S_T$ and $S_T > S_A$, one can find general tendencies for Φ: If $\theta \leq 1$, then $\Phi < 0$. If $\theta = 1$, then

$$\Phi = -\frac{S_T - S_A}{aN^2} < 0. \quad (8)$$

If $\theta > 1$, then

$$\Phi = \frac{(W_0 - S_T)(\theta - 1)(N/N_{max})^{\theta} - (S_T - S_A)}{aN^2}, \quad (9)$$

and

$$\left|\begin{array}{l} N \rightarrow 0 \Rightarrow \Phi \rightarrow -\infty, \\ N = N_{\text{threshold}} = N_{\max}\left[\dfrac{S_{\text{T}} - S_{\text{A}}}{(W_0 - S_{\text{T}})(\theta - 1)}\right]^{1/\theta} \Rightarrow \Phi = 0, \\ N \rightarrow \infty \Rightarrow \Phi \rightarrow 0^+. \end{array}\right.$$

(10)

When $\theta \leq 1$, Φ is always negative, and T_{lim} decreases as N increases; this means that the freeze-tolerance strategy is penalized less than the freeze-avoidance strategy by an increasing number of freezing days, N.

When $\theta > 1$, the analysis becomes more complicated. We define $N_{\text{threshold}}$, which determines the sensitivity limit for each strategy to variations in N. When $N < N_{\text{threshold}}$, T_{lim} decreases as N increases, and in contrast, T_{lim} increases with N when $N > N_{\text{threshold}}$. Solving $N_{\text{threshold}} - N_{\max} = 0$, one can determine whether $N_{\text{threshold}}$ is lower than $N_{\max}$ in the range of possible existence for the freeze-tolerance strategy when $N \in [0, N_{\max}[$:

$$\left[\frac{S_{\text{T}} - S_{\text{A}}}{(W_0 - S_{\text{T}})(\theta - 1)}\right]^{1/\theta} < 1.$$

(11)

This condition depends on the model parameters; however, note that when

$$\theta \rightarrow \infty \Rightarrow \left[\frac{S_{\text{T}} - S_{\text{A}}}{(W_0 - S_{\text{T}})(\theta - 1)}\right]^{1/\theta} \rightarrow 1.$$

(12)

When $\theta \rightarrow +\infty$, $N_{\text{threshold}} \rightarrow N_{\max}$, and the sensitivity limit disappears, which entails that T_{lim} always decreases as N increases. The parameter θ then has a positive effect on W_{T} when it increases, and the fitness of the freezing strategy increases. In the same way, decreasing W_0 moves $N_{\text{threshold}}$ to the right (fig. 3). These results will now be more fully explored through numerical simulations.

Numerical Results. We conducted a numerical study to improve our understanding of the model and to illustrate the influence of the parameters and variables such as θ, W_0, T, and N. By giving particular values to those parameters, we define different kinds of species (determining the combination of parameters that correspond physiologically to different classes of animals; for instance, a high value of W_0 for insects [Fast 1964], an intermediate one for reptiles [Derickson 1976], and a low one for amphibians [Fitzpatrick 1976]). We also examined the influence of variation in N and T in order to define for a given environment type the most fit combination of parameters. Results are presented in figure 3.

The shape of the existence zone for the freeze-tolerance strategy is strongly dependent on a high value of θ. These types of curves indicate a phenomenon of energy reserves depletion and/or accumulation of injuries over time. The limit between the freezing and supercooling strategy is defined by T_{lim} (eq. [6]). When $\theta < 1$, T_{lim} decreases, which indicates that the freeze-avoidance strategy is more heavily penalized when the number of freezing days increases than is the freeze-tolerance strategy (fig. 3*d*, 3*g*). However, when $\theta > 1$, a threshold, at $N_{\text{threshold}}$, is found, beyond which T_{lim} increases (fig. 3*b*, 3*e*). For high values of θ, $N_{\text{threshold}} = N_{\max}$, and the threshold disappears (fig. 3*c*, 3*f*, 3*i*).

In figure 3, we also illustrate the effect of W_0. As W_0 increases, the range of environmental conditions (aTN) that promote adoption of the freeze-avoidance strategy increases. Note that at low values of W_0, the threshold $N_{\text{threshold}}$ also disappears.

Mixed Strategy versus Freeze-Tolerance and Freeze-Avoidance Strategies

In this section, we consider that only one switch from supercooling to freezing tolerance is possible during the subzero temperature period at N_b.

Constant Environment. We first define the conditions that allow the mixed strategy to have a greater fitness than the freezing strategy. When $N < N_b$, the mixed strategy is equivalent to the supercooling strategy, and thus, the constraints are the same for producing a higher fitness than the freezing strategy. When $N \geq N_b$, W_{M} has to be >0 after the switch from supercooling to freezing:

$$W_{\text{M}}(N = N_b) > 0,$$

$$N_b < \frac{W_0 - S_{\text{A}} - S_{\text{T}}}{aT} \rightarrow W_0 > S_{\text{A}} + S_{\text{T}} + aTN_b. \quad (13)$$

Thus, a high W_0 appears to be an important condition for the mixed strategy to be successful because it permits a high number of days to occur at subzero temperatures before switching. In this case, one can thus see that, to maximize its fitness, an animal should leave the switch as late as possible.

Compared to the supercooling strategy, the mixed one will have a better fitness if the following inequality is met after the switch: $W_{\text{M}}(N) > W_{\text{A}}(N)$.

Just after initiation of freezing, W_{M} is lower than W_{A} due to the cost S_{T}. However, W_{M} is not dependent on temperature and is therefore less dependent on a large number of freezing days (with high values of θ). Furthermore, W_{M} decreases at a slower rate than W_{A} and will progressively inverse the tendency. Thus, given a sufficiently low S_{T} and a high θ, W_{M} reaches 0 later than W_{A}
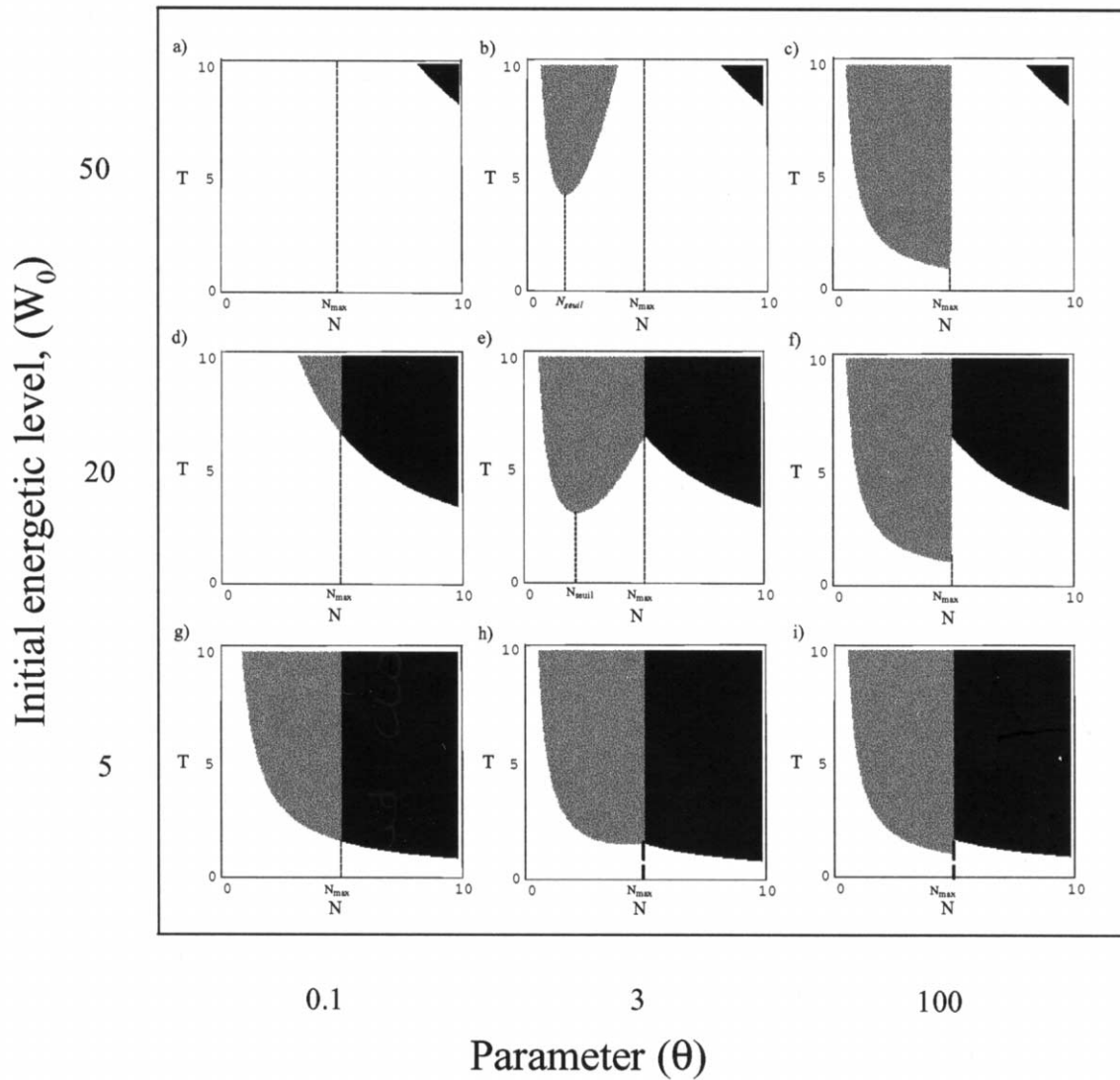
Figure 3: Domains of parameters where the freeze-tolerance and supercooling strategies perform best as a function of environmental variations (N and T) for different combination of W_0 and θ. *White*, the freeze-avoidance strategy is more efficient than freeze-tolerance strategy ($W_A > W_T$); *grey*, freeze-tolerance strategy is more efficient than freeze-avoidance strategy ($W_T > W_A$); *black*, both fitnesses are <0. The variables N and T vary from 0 to 10. The other parameters are $S_T = 2$, $S_A = 0.5$, $N_{max} = 5$, and $a = 1$.

(fig. 4). However, this is only true if it happens before $N = N_b + N_{max}$, where $W_M = 0$. It is equivalent to asking whether $W_M > W_A$ for $N = N_b + N_{max}$. At this time, $W_M = 0$, and thus, one has only to solve $W_A(N = N_b + N_{max}) < 0$:

$$W_0 < S_A + aT(N_b + N_{max}). \tag{14}$$

From equations (13) and (14), it is now possible to define the limits for W_0:

$$aTN_b + S_A + S_T < W_0 < S_A + aT(N_b + N_{max}). \tag{15}$$

The mixed strategy has the best performance for intermediate values of W_0. If W_0 is too low, the mixed strategy cannot withstand the cost of the switch, and if W_0 is too high, the supercooling strategy will always have a greater fitness after the switch. Note that S_A is present in both parts of equation (15) and thus does not influence the mixed strategy. In general, the conditions for the existence of the mixed strategy are close to those for the freeze-tolerance strategy (see above):
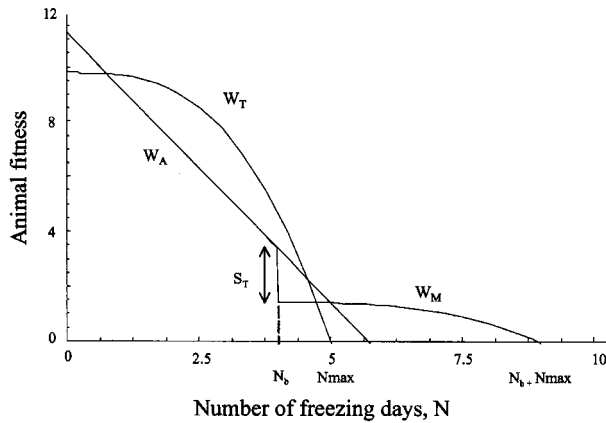
Figure 4: Fitness of freeze-tolerance strategy, W_T, freeze-avoidance strategy, W_A, and mixed strategies in function of the number of freezing days. Parameters are $W_0 = 12$, $S_T = 2$, $S_A = 0.5$, $T = 4$, $a = 0.5$, $\theta = 3$, $N_{max} = 5$, and $N_b = 4$.

maximization of S_T and N_{max}. Therefore, the mixed strategy is strongly penalized because it has to pay the costs of the two stresses.

Numerical Results. We now examine, for the three strategies, the influence of the parameters and variables θ, W_0, T, and N. We study variation in N and T to determine in which environment a particular combination of parameters is more adaptive. Results are presented in figure 5.

As shown above, the shape of the existence zone of the mixed strategy is strongly dependent on W_0 and is optimal for intermediate values of W_0 (fig. 5d–5f).

Because of the cost S_T paid by the mixed strategy when the switch occurs, the supercooling strategy alone (*dark grey*) has a greater fitness for a short time window. This is particularly evident at low θ and W_0 (fig. 5g), because, once S_T has been paid, it takes a longer time for W_M to become $>W_A$.

The mixed strategy allows animals to endure a higher number of freezing days and lower temperatures than the others strategies. However, its domain of existence is not very extensive because it requires the optimization of all parameters associated with each strategy.

Variable Environments. It is important to discuss the conditions in which animals could survive using the mixed strategy with regard to the physiology of the animals. The mixed strategy involves the optimization of parameters of both strategies, which is highly improbable within one species. For instance, the lizard *Lacerta vivipara* has a mixed cold-hardiness strategy (Costanzo et al. 1995), but even if this species has a high energy level at the beginning

of the winter (Avery 1970, 1974), it has a low N_{max} (Costanzo et al. 1995). According to our model, such a strategy should not be selected in a constant environment.

However, if one considers the environment to be variable, one can see that the mixed strategy has the obvious advantage of using the freezing as a "refuge" during harsh subzero temperature periods.

Let us consider that after N_{var} d at subzero temperatures, the temperature decreases from T to xT. In this case, the supercooling strategy will be highly penalized. However, if the animal switches to the freeze-tolerance strategy before N_{var}, it will minimize the energetic costs during this period. The corresponding equations are

$$N < N_{var} \Rightarrow W_M = W_A = W_0 - S_A - aTN,$$

$$N \geq N_{var} - 0.5 \Rightarrow W_M = [(W_0 - S_A - aTN_b) - S_T]$$

$$\times \left[1 - \left(\frac{N - N_b}{N_{max}}\right)^\theta\right],$$

$$N \geq N_{var} \Rightarrow W_A = (W_0 - S_A - aTN_{var}) - axTN.$$

(16)

We illustrate this case in figure 6a, with $x = 3$. In this example, the mixed strategy never exhibits a better fitness if temperature stays constant (fig. 6b), while this strategy yields a higher fitness when T shifts to $3T$ for some period of time. Thus, it is temperature variation that enhances the competitiveness of the mixed strategy.

Discussion

Winter is a powerful selective force that has resulted in substantial adaptations in the life cycles of all temperate and arctic animals (Levins 1969). In particular, ectotherms, because of their restricted ability to endure severe winters, have evolved adaptive strategies in order to ensure winter survival. Two main strategies have emerged: freeze tolerance and freeze avoidance. In this study, we have shown that the freeze-tolerance strategy is advantageous when animals are capable of surviving long periods of freezing (high θ and N_{max}), when the stress associated with freezing (S_T) is low, and when the initial energy reserves (W_0) are low but higher than a minimal threshold represented by S_T. However, the freeze-avoidance strategy is favored when the stress associated with supercooling (S_A) is low, when initial energy reserves (W_0) are high, and when the energy required to produce cryoprotection (or to resort to other tactics; a) is low. The domain of existence of mixed strategies is restricted in a constant environment; however, its existence is strongly linked to a variable environment.
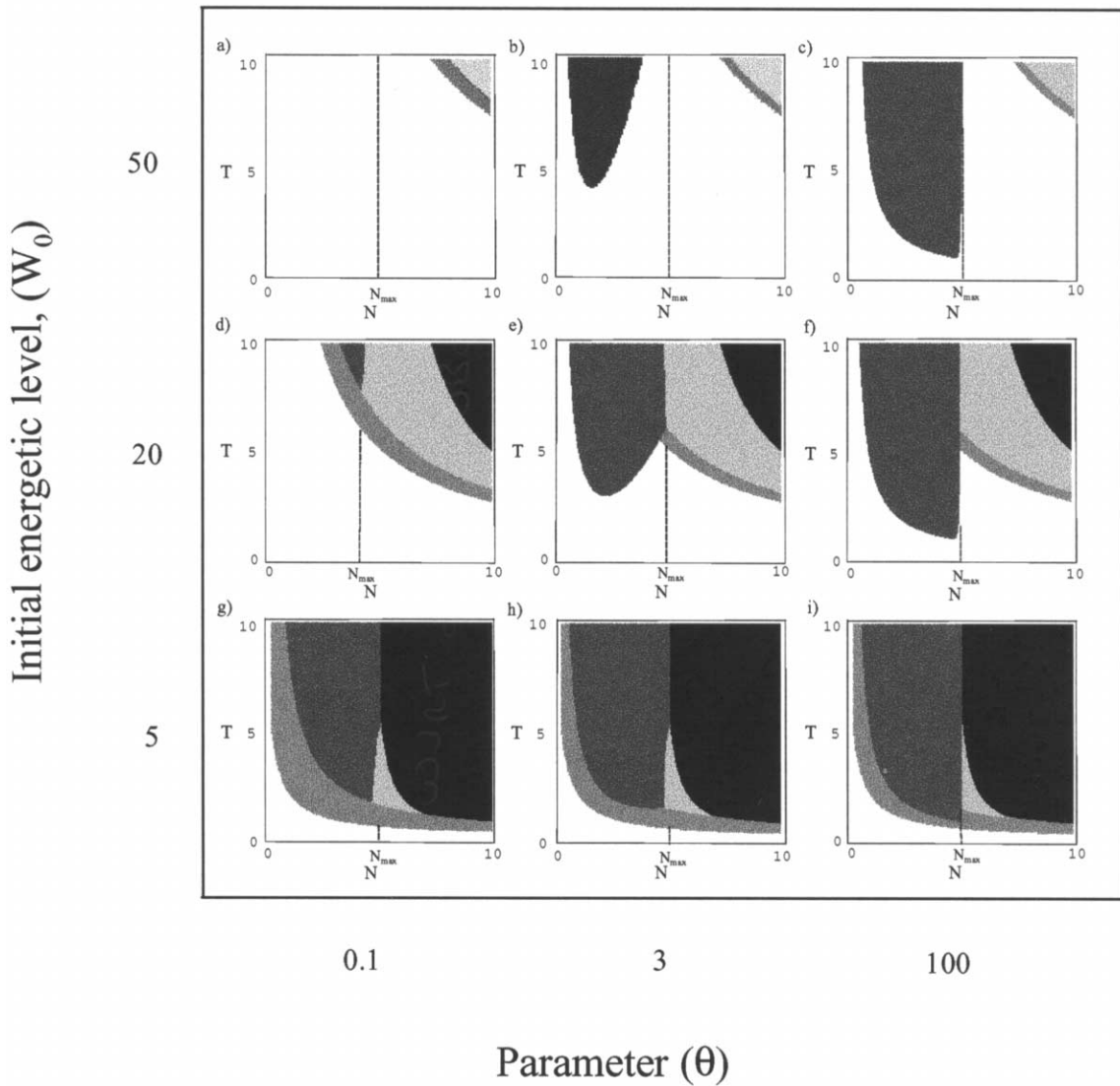
Figure 5: Domains of parameters where the freeze-tolerance strategy, freeze-avoidance strategy, and mixed strategies perform best as a function of environmental variations (N and T) for different combination of W_0 and θ. *White,* the freeze-avoidance strategy and mixed are equal and more efficient than freezing tolerance; *grey,* freeze-avoidance strategy is more efficient than other strategies; *dark grey,* freeze-tolerance strategy is more efficient; *light grey,* mixed strategy is more efficient; *black,* the three fitnesses are <0. The variables N and T vary from 0 to 10. The other parameters are $S_T = 2$, $S_A = 0.5$, $N_{max} = 5$, and $a = 1$. For the mixed strategy, N_b is function of environmental variable (eq. [13]). Thus, we use $N_b = [(W_0 - S_A - S_T)/(W_0 - S_A - S_T)aT] - x$, with $x = 0.5$; in other words, the mixed strategy switches 0.5 d before N_b. The switch is delaying as long as possible.

The Freeze-Tolerance Strategy

Not surprisingly, the freeze-tolerance strategy is favored in cases where a species can sustain long periods of freezing (i.e., have a high N_{max}). In ectotherms, N_{max} has rarely been assessed. Instead, survival for different freezing durations has been measured, generally stopping when 50% mortality (or less) occurs (Storey and Storey 1984). Using this approach, it has been demonstrated that invertebrates may endure months of freezing while vertebrates may survive, at most, several weeks in a frozen state (Storey and Storey 1988). Although the reasons for this difference are unclear, it is thought to be linked to the capacity of insects to metabolize/excrete the anaerobic end products, or the energetic costs associated with the physiological "architecture" itself. This low ability of vertebrate ectotherms to
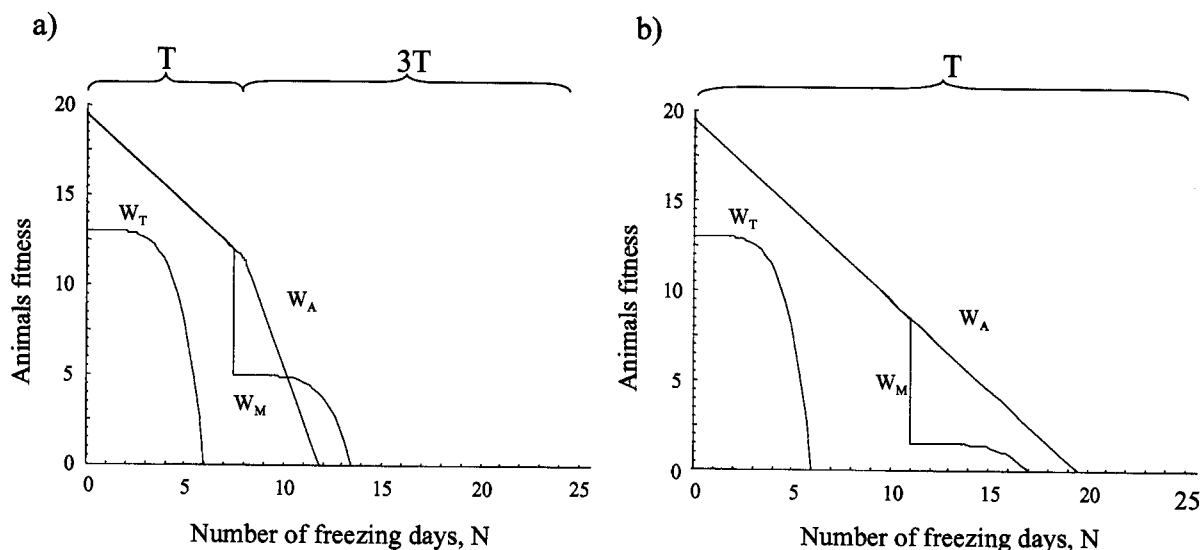
Figure 6: Fitness of freeze-tolerance strategy, W_T, freeze-avoidance strategy, W_A, and mixed strategies as a function of the number of freezing days. *a,* The temperature switches from T to $3T$ after N_{var} d of freezing. Parameters are $W_0 = 20$, $T = 2$, $x = 3$, $S_T = 2$, $S_A = 0.5$, $N_{max} = 6$, $a = 0.6$, $\theta = 3$, $N_{var} = 6$, and $N_b = 7.5$. *b,* T is constant during all the freezing period. Parameters are identical, except $N_b = 11$.

sustain long periods of freezing might partly explain their restricted colonization of the Arctic areas (for a broader discussion of stress and geographic distribution, see Chow and Clarke 2000).

The capacity to resist a freezing episode is dependent not only on N_{max} but also on the capacity of the organism to fully preserve its future survival and fecundity (θ or fitness value). The freeze-tolerance strategy is critically dependent on this capacity. In other words, for a fixed N_{max}, this is only when θ is high (i.e., when the accumulated damage remains low as N_{max} is approached). If we consider winter survival rate as a relevant indicator of fitness, data from previous laboratories studies have confirmed that most species that are freeze tolerant exhibit very high survival rates (near 100% postfreezing), at least within the first several days (for vertebrates) or months (for invertebrates; Asahina 1969; Somme and Conradi-Larsen 1979; Costanzo et al. 1995; Layne et al. 1998). From an energetic point of view, Storey and Storey (1986*b*, 1988) have shown that the energy charge of two freeze-tolerant animals does not decrease significantly during at least 3 d for an amphibian (the wood frog *Rana sylvatica*) and over 12 wk for an insect (the gall fly *Eurosta solidaginis*). Interestingly, the tolerance to freezing is strongly season dependent. If animals are not acclimated, all the biochemical/physiological processes required to survive at subzero temperatures are unreliable, and θ has a lower value (i.e., Wharton et al. 2000). This suggests that some control mechanisms

have developed to prepare animals to sustain as well as to enter into the frozen state.

In order for the freeze-tolerance strategy to be more efficient than the freeze-avoidance strategy, the physiological stress associated with the freezing process (S_T) must be low. Very few experiments have assessed the value of S_T in terms of survival. When the stress induced by a change of state was assessed by quantification of the antioxidant defenses, there was no cost associated with the entrance into freezing (Storey et al. 1981; Joanisse and Storey 1996*b* for invertebrates and vertebrates, respectively). It is important to notice that these results were obtained for species that are considered model systems (*R. sylvatica* and *E. solidaginis*) for the study of cold hardiness because they exhibit strong capacities to resist cold. However, there are a number of species that show a weaker tolerance to freezing and are not capable of surviving if ice formation reaches equilibrium (Claussen et al. 1990; Sinclair 1999), and the measure of S_T, which is not a current variable used in the study of cold-hardiness strategies, may help in the classification of the main strategies used by species that show potential freeze resistance (Sinclair 1999) within the same evolutionary and energetic contexts. However, measuring this variable independently of θ and N_{max} may prove to be difficult if these variables have strongly coevolved.

The freeze-tolerance strategy is also favored when energy reserves at the onset of hibernation are low. This is not

to say that low fat content decreases the costs associated with freeze tolerance but that low energy reserves preclude adoption of the freeze-avoidance strategy. In the model, W_0 represents not only the energy reserves necessary to ensure winter survival but also the energy required to initiate growth and reproduction after emergence from hibernation (Fitzpatrick 1976; Irwin and Lee 2000). Energy reserves take three different forms: lipids, carbohydrates, and, marginally, proteins. Lipids are considered to be the major energy source during hibernation because they are the most economical form of energy storage, although glycogen offers the advantage of being more easily and rapidly metabolized (Storey and Storey 1988). The freezing state induces the unavailability of oxygen for metabolism, and although metabolic activity is much reduced during freezing, it is still present. Lipids cannot be metabolized without an oxygen supply, while the glucose (from glycogen) can be catabolized anaerobically. It is therefore not surprising that freeze-tolerant species (at least for vertebrates) use glycogen reserves during a freezing episode, while freeze-avoidance species preferentially use lipids (Storey and Storey 1987; Costanzo and Lee 1993; Joanisse and Storey 1996a) since oxygen is available to supercooled animals. The energy required by physiological processes associated with the freeze-tolerance strategy must be low because glycogen is energetically less efficient than lipids and needs more space/weight to be stored (Stryer 1988), which is likely to represent an important morphological constraints. However, the ability to survive freezing depends on the glucose level at the onset of freezing, which is itself dependent on the amount of glycogen stored in the animal (Duman et al. 1991; Costanzo and Lee 1993; Layne 1999). It follows that a positive relationship between glycogen levels and the survival time in a frozen state (N_{max}) is expected, although not with amount of lipid stored. Freeze-tolerant species have, indeed, developed physiological mechanisms that avoid the loss of glycogen, such as gluconeogenesis and bladder glucose reabsorption following thawing (Costanzo et al. 1997). However, it is important to keep in mind that survival is not only dependent on the amount of glycogen but also on the capacity of the organism to mobilize glycogen into glucose and to transport the glucose to the organs.

What can we say about body fat content from the data presented in literature? Once more, data are scarce and scattered over many different orders. For example, if we compare the energy reserves in amphibians, all terrestrial anurans exhibit high glycogen content (especially in winter), but toads exhibit higher lipid content than frogs (3% and 1%, respectively, of the body mass). Interestingly, no toads have been found to be freeze tolerant (Storey and Storey 1986a), in contrast to many temperate frogs (Schmid 1982), which also overwinter in the soil. Because glycogen is used in the reproduction of all anurans (Fitzpatrick 1976), we can make the hypothesis that toads have not evolved toward freeze tolerance because this higher lipid content is usable in a supercooled state. In addition, many toads exhibit the behavioral strategy of avoiding harsh subzero temperatures by burying themselves deeply into the ground. Among invertebrates, freeze-tolerant species are found among insects, gastropods, annelids, and nematoda (Storey and Storey 1988), but the relationship to energy resource levels is complicated by great differences in physiological structures, the diversity of lifestyles, and the lack of comparative data. Furthermore, freeze-tolerant species may still be found to have high lipid reserves that may be allocated to other functions such as growth and reproduction following hibernation. In other words, except for the role of W_0 in shaping the cold-hardiness strategies, there is no reason to expect a trade-off between glycogen and lipid reserves.

The Freeze-Avoidance Strategy

For species that are killed by freezing, dependence on supercooling is the only way to survive subzero temperatures. Supercooling is a physical phenomenon describing the unfrozen state of aqueous solutions (or other liquids) below the melting point. For instance, small volumes of purified water can supercool to temperatures close to $-40°C$ (MacKenzie 1977). Because of this physical characteristic, all living organisms (even tropical ones) exhibit greater or lesser supercooling capacities. However, a thermal environment leading to a supercooled state imposes physiological stress that may reduce winter survival (e.g., overwintering aphids are killed within a few minutes at temperatures of $-5°$ to $-15°C$, although their SCP is $-25°C$; Pullin and Bale 1988) as well as future life-history traits (e.g., lower postdiapause development rate for dipteran and lepidopteran larvae exposed to subzero temperatures; Turnock et al. 1985; Turnock and Bodnaryk 1991). In our model, such stress is represented by S_A, and, not surprisingly, the freeze-avoidance strategy is favored by a low value of this parameter. Unfortunately, very few experiments have assessed the physiological stress of supercooling (Turnock et al. 1983, 1985; Turnock and Bodnaryk 1991). Quantification of the stresses associated with supercooling (within and between species) would greatly help our understanding of the evolution of this cold-hardiness strategy. Furthermore, it would explain the differences in freeze-avoiding insects observed by Bale (1993) that led him to propose a reclassification of the freeze-avoidance strategy into three subgroups (the chill susceptible, the moderately chill tolerant, and the highly chill tolerant). It would be particularly interesting to quantify the stresses incurred by partially freeze-tolerant species.

According to the Sinclair's (1999) classification, these are species that "are fascinating from an evolutionary point of view, because it is suggestive of an intermediate position between freeze avoidance and freeze tolerance" (p. 158). Such an analysis would answer a crucial question in the evolution of freeze tolerance, which involves various adaptations coming from fundamental responses to stress such as glucose production (Costanzo et al. 1993).

Organisms adopting a freeze-avoidance strategy in both temperate and arctic climates have developed physiological adaptations allowing them to increase their supercooling capacities (via, e.g., low molecular weight cryoprotectant accumulation) and reduce stress associated with supercooling. On this basis, in our model, the S_A parameter, which describes the stress associated with the supercooling state, could explain the differences highlighted by Bale (1993). For instance, a high value of S_A induces a rapid drop of fitness leading to a negative W_0 (see eq. [3]), biologically meaning the death of animals, even if the supercooling point, often considered the lowest temperature endurable by a freeze-avoiding animal (Bale 1993), has not been attained. Thus, if we follow the model's predictions, the highly chill-tolerant species should have a low stress associated with supercooling. Unfortunately, no experiments have been conducted that examine the link between physiological stress in a supercooled state and supercooling capacity.

In addition to the low S_A value, the freeze-avoidance strategy is also favored by a low value of the parameter a, which means that variation in climatic conditions must have a minimal effect on animal fitness. This implies that physiological mechanisms are only slightly dependent on the duration and intensity of the subzero temperatures. Some adaptations that are less dependent on climatic conditions allow some ectotherms to be cold hardy without synthesizing a large amount of cryoprotectant, which is highly costly. For instance, first, a higher supercooling capacity is obtained if all ice-nucleator agents (INAs), such as gut contents, bacteria, or lipoprotein, are removed (Somme 1982); second, in various soil invertebrates, a "protective dehydration mechanism" provides a significant SCP decrease (Somme and Birkemoe 1997; Holmstrup and Somme 1998); and third, supercooling capacities could be enhanced by the synthesis of specific proteins such as thermal hysteresis proteins, which stabilize the supercooling state along the whole range of subzero temperatures encountered by the animals and that act at very low concentrations (Zachariassen and Husby 1982; Duman et al. 1993).

The model assumes a linear decrease in fitness for supercooled animals (see eq. [2]). However, it has been previously suggested that the survival rate of supercooled insects exposed to different durations of subzero temperatures (N) and temperature (T) decreases in a nonlinear manner (Nedved et al. 1998). In this study, when a supercooled animal reexperiences a nonfreezing period, the fitness decrease rate returns to its level at the beginning of the supercooling period. Thus, for a fixed N value, the survival rate is higher for animals that endure subzero temperatures interrupted by nonfreezing periods than for animals continuously exposed to subzero temperatures. This nonlinearity of the fitness decrease does not significantly change our model's predictions and simply reduces the existence zone of the freeze-avoidance strategy, thus increasing the existence domain of the freeze-tolerance strategy.

The Mixed Strategy

From a theoretical point of view, the mixed strategy allows the endurance of a greater number of freezing days and lower temperatures (fig. 5), but its region of existence is not very extensive. This is explainable by the fact that all parameters related to both strategies have to be optimized to be more efficient than the other strategies. In the field, only a few animal species exhibit a mixed strategy, and furthermore, the manner in which these strategies are used differs significantly.

For instance, a few Arctic and alpine coleoptera and diptera species, such as *Pytho deplanatus* and *Mordellistena unicolor*, are tolerant to freezing and exhibit a very low SCP of around −40° or −50°C (Baust et al. 1979; Ring 1982). However, the lower lethal temperature of these animals is not far below the SCP. Thus, data from the literature confirm that the frozen state allows them to endure a longer period of subzero temperatures, but the data do not confirm that the frozen state allows endurance of significantly lower temperatures. According to Ring (1982), this kind of mixed strategy is especially relevant during spring or summer periods. During these times, insects may again encounter subzero temperatures at night, and their gut contains nutriments that are very efficient nucleators (Salt 1966). Because of their ability to survive freezing, mortality is reduced.

Among Old World vertebrates, the lizard *Lacerta vivipara* is known to exhibit the two strategies during winter. At the same overwintering site (and thus encountering the same microenvironmental conditions), individuals can be found in the two physiological states (C. Grenot, personal communication). Laboratory experiments have shown that this lizard can remain in a supercooled state for at least 3 wk (the longest record for vertebrates) and in a frozen state for 3 d (Costanzo et al. 1995). Because of the damp substrate encountered in their natural hibernacula (Grenot and Heulin 1988), animals are regularly exposed to seed crystals. So, the freeze-tolerance capacity of this species,

even if it is not highly developed (N_{max} around 3 d), allows a nondeleterious ice nucleation and thereby reduces winter mortality. This low N_{max} does not allow *Lacerta vivipara* to survive in a very harsh constant environment, such as an Arctic winter. However, in a variable environment, such a mixed strategy becomes advantageous, even if the parameters are not optimal, since harsh environmental periods can be avoided by freezing (fig. 6).

A third type of mixed strategy can be found in overwintering larvae of two beetle species, *Dendroides canadensis* and *Cucujus clavipes*. These beetles exhibit different strategies depending on the year (Horwath and Duman 1984; Duman et al. 1991), alternating annually between a freeze-tolerant and a freeze-avoidance strategy. Even if our model is not well suited for this case, we hypothesize that such a switch is directly dependent on the initial energy level at the onset of the winter and/or on variations of the winter environment. This hypothesis may be tested experimentally by a prewinter manipulation of the food intake of animals and direct measurement of the resulting influence of the adopted cold-hardiness strategies on the reproductive success in the following season (Baust and Rojas 1985; Bale 1987).

This model represents the first theoretical analysis of the evolution of cold-hardiness strategies and shows that the optimal cold-hardiness strategy depends on the environmental conditions and the physiological state of the animals. One predicts that freeze tolerance will be more successful in regions of extremely low temperatures if the freezing-associated physiological parameters are optimized (see fig. 3*i*). This conclusion is supported by the fact that numerous Arctic species are freeze tolerant (Miller 1982). In contrast, the freeze-avoidance strategy is favored by mild winters and is strongly dependent on the initial energy level. The mixed strategy, which does not improve survival in harsh winters, seems to be particularly useful in variable environments by allowing organisms the effects of the very cold periods by freezing (see fig. 6).

A number of experimental results support the model's predictions and give some credence to using energy as a relevant approach for evolution of cold-hardiness strategies. From a theoretical point of view, it would now be of interest to include other physiological costs that occur after the periods of subzero temperatures, such as the cost of recovery or repair following freezing, which is greater for animals exposed to greater cold (Layne and First 1991; Layne et al. 1998). Furthermore, to give realistic values to the parameters, it would also be interesting to focus on the study of a few species such as *Eurosta solidaginis* and *Rana sylvatica*, where a number of characteristics of the overwintering ecology and physiology are already known. In parallel, from an experimental point of view, all results involving the initial energy level (W_0) will require confir-

mation because data in the literature are scarce, and no study has been designed to link energy reserves and cold-hardiness strategies. In this framework, species exhibiting changes in cold-hardiness strategy with latitude would represent a group of relevant ecophysiological model species with which to test the statement "whether freeze tolerance or freeze avoidance is the better strategy must be applied not only to the species concerned, but also to a specific population in a particular environment at a defined time" (Block 1995, p. 363). With the aim of better inserting cold-hardiness biology into an ecophysiological framework, we need to design experiments aimed at showing a direct relationship between cold-hardiness strategies and different life-history traits such as reproduction capacity (including number and fitness of offspring).

Furthermore, species using both strategies in temporal succession are important for examining the existence of a continuum between freeze avoidance and freeze tolerance. Some elements already indicate that such a continuum should exist: a number of physiological adaptations to cold are common to both strategies (i.e., carbohydrates and polyols used as antifreeze and/or as cryoprotectants; thermal hysteresis proteins that stabilize the supercooled state and also help animals to avoid recrystallization), and partially freeze-tolerant species (Sinclair 1999) may represent a group evolving toward full freeze tolerance. The extent of the stresses associated with both strategies for different durations and intensities of cold are probably at the basis of the different cold-hardiness capacities inside freeze avoidance and/or freeze tolerance (see Bale 1993; Sinclair 1999). An understanding of the physiological processes developed by ectotherms to reduce these stresses could be a key to understanding the ecology and the evolution of cold-hardiness strategies.

Acknowledgments

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