



## Freezing tolerance of the European water frogs

Yann Voituron, Pierre Joly, Michel Eugène, Hervé Barré

### ► To cite this version:

Yann Voituron, Pierre Joly, Michel Eugène, Hervé Barré. Freezing tolerance of the European water frogs. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 2005, 288 (6), pp.1563-1570. <10.1152/ajpregu.00711.2004>. <halsde-00340380>

**HAL Id: halsde-00340380**

**<https://hal.science/halsde-00340380v1>**

Submitted on 29 Sep 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

# Freezing tolerance of the European water frogs: the good, the bad, and the ugly

Yann Voituron,<sup>1</sup> Pierre Joly,<sup>2</sup> Michel Eugène,<sup>3</sup> and Hervé Barré<sup>1</sup>

<sup>1</sup>Physiologie des régulations énergétiques, cellulaires, et moléculaires (UMR CNRS 5123), Université Claude Bernard Lyon1, Villeurbanne

<sup>2</sup>Ecologie des hydrosystème fluviaux (UMR CNRS 5023), Université Claude Bernard Lyon1, Villeurbanne

<sup>3</sup>Laboratoire de RMN, Hôpital St-Louis, Paris, France

Freezing tolerance of the European water frogs: the good, the bad and the ugly. *Am J Physiol Regul Integr Comp Physiol* 288: R1563–R1570, 2005; doi:10.1152/ajpregu.00711.2004.—Survival and some physiological responses to freezing were investigated in three European water frogs (*Rana lessonae*, *Rana ridibunda*, and their hybridogen *Rana esculenta*). The three species exhibited different survival times during freezing (from 10 h for *R. lessonae* to 20 h for *R. ridibunda*). The time courses of percent water frozen were similar; however, because of the huge differences in body mass among species (from 10 g for *Rana lessonae* to nearly 100 g for *Rana ridibunda*), the ice mass accumulation rate varied markedly (from  $0.75 \pm 0.12$  to  $1.43 \pm 0.11$  g ice/h, respectively) and was lowest in the terrestrial hibernator *Rana lessonae*. The hybrid *Rana esculenta* exhibited an intermediate response between the two parental species; furthermore, within-species correlation existed between body mass and ice mass accumulation rates, suggesting the occurrence of subpopulations in this species ( $0.84 \pm 0.08$  g ice/h for small *R. esculenta* and  $1.78 \pm 0.09$  g ice/h for large ones). Biochemical analyses showed accumulation of blood glucose and lactate, liver glucose (originating from glycogen), and liver alanine in *Rana lessonae* and *Rana esculenta* but not in *Rana ridibunda* in response to freezing. The variation of freeze tolerance between these three closely related species could bring understanding to the physiological processes involved in the evolution of freeze tolerance in vertebrates.

cold hardiness; ice content; osmolality; glucose

DURING WINTER, THE VERTEBRATE ectotherms living in temperate and arctic regions have to face low ambient temperatures and the risks of freezing. Because migration capacities of these animals are restricted, survival depends on adaptations to local cold conditions. Some species use deep overwintering sites (whether digging down like toads or taking advantage of preexisting cavities like newts), thus providing reliable buffers against extremely low air temperatures. Aquatic habitats also provide protective thermal conditions to overwintering animals but require specific aptitudes to endure possible hypoxia because of the scanty amount of oxygen available under water, especially when the surface is frozen (49). Others, principally frogs, overwinter in superficial sites under bark and trees. Such little-buffered locations require cold hardiness strategies that often result from freeze tolerance (12, 44). This tolerance to freezing depends on the ability to restrict ice formation to the extracellular spaces, which is principally achieved by the control of 1) the site of ice formation, 2) the crystallization

temperature (Tc), and 3) the amount of ice formed (44). Cold-adapted ectotherms have achieved this control by evolving a number of physiological and biochemical mechanisms, including production of a high level of cryoprotectants such as glucose and/or glycerol, as well as ice nucleating agents (44).

Although terrestrial anurans have been largely studied, data on cold tolerance of aquatic *Ranidae* are scarce. Schmid (38) showed that *Rana septentrionalis* and *Rana pipiens* die after being frozen for a period of 5 days at  $-6^{\circ}\text{C}$ . However, further studies demonstrated that the latter species can survive 8 h of freezing at  $-2^{\circ}\text{C}$  (25) and that this species accumulates glucose in response to freezing (13). Recently, it was shown that the marsh frog *R. ridibunda* can tolerate more than 24 h of freezing at  $-2^{\circ}\text{C}$  without any cryoprotective system, the survival time being strongly dependent on body mass (52). In the present study, we investigate the cold tolerance of the *Rana esculenta* hybridization complex, which is characterized by a widespread and abundant natural occurrence of hybrid frogs (reviewed in Ref. 17). The *Rana esculenta* complex, involving the parental species *R. ridibunda* Pallas 1971 and *R. lessonae* Camerano 1882 and the hybridogen *R. esculenta* Linnaeus 1758 (3, 5), occurs in Central and Eastern Europe (17). The originality of this complex lies in the fact that hybrid individuals (*esculenta*) transmit only one of their parental genomes (*ridibunda*) to their progeny, the other one (*lessonae*) being discarded before meiosis. The maintenance of the complex supposes something like a “sexual parasitism” by hybrid females that preferentially mate with parental *lessonae* males, thus restoring hybridity (21). Within this assemblage, the habitat preference of each species has been well studied (23, 30, 32). *R. ridibunda* preferentially inhabits well-oxygenated waters (often lakes, rivers, and dead arms near rivers), whereas the two other species are found in shallow ponds. Such differences could be linked to the hypoxia tolerance of the tadpole, which is significantly lower in *R. ridibunda* than in *R. lessonae* and *R. esculenta* (36, 37). Furthermore, the species differ by their wintering behavior. *R. ridibunda* usually hibernates under water (4), *R. lessonae* prefers terrestrial wintering sites, and *R. esculenta* can exhibit both wintering behaviors (4, 19). A likely hypothesis is that cold hardiness capacity probably differs among these taxa, with a greater freeze tolerance in *R. lessonae*, which uses superficial overwintering sites. We tested this hypothesis by investigating 1) Tc on wet substrata, 2) survival in the frozen state, 3) ice content tolerated and time course of ice accumulation, 4) organ dehydration induced by freezing,

and finally 5) whether freeze tolerance was linked to cryoprotectant accumulation.

## MATERIALS AND METHODS

### Animals

The present investigation was carried out according to the ethical principles of the French (Ministère de l'Agriculture) and European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Council of Europe, no. 123, Strasbourg, 1985).

Frogs were caught from late August to mid-September from ponds and rivers around Lyon (France). Because the use of morphological characters is not sufficient to allow a reliable identification of the different taxa of the *R. esculenta* complex (33), we used a specific allozymic marker, lactate dehydrogenase, detected by protein electrophoresis on somatic tissues (fingertip). After capture, the frogs were cold acclimated at 4°C in total darkness without food for at least 6 wk before experimentation. To mimic natural hibernating conditions, the housing conditions differed among the three species. *R. ridibunda* were placed in slanting boxes where the water level was delineated so as to provide choice between being totally immersed or remaining dry, and *R. lessonae* were maintained in humid moss. Because *R. esculenta* is known to hibernate in either aquatic or terrestrial habitats (4), individuals of this species were given the choice between water and humid moss. The *R. ridibunda* ( $n = 25$ ), *R. lessonae* ( $n = 24$ ), and *R. esculenta* ( $n = 50$ ) used in these experiments had mean body masses of  $37.5 \pm 25.3$  g,  $10.3 \pm 3.3$  g, and  $20.9 \pm 12.7$  g, respectively (means  $\pm$  SD).

### Freezing Protocol

Freezing exposures were performed as previously reported (52). Briefly, animals kept at 4°C were rapidly placed on a pad of wet paper toweling and outfitted with a thermocouple probe in contact with their abdomen and connected to an EIRELEC MT 120 KC. A band of masking tape was used to secure the thermocouple onto the animal. The animal was then placed in a Precision low-temperature incubator (model 815) set at  $-2.5^\circ\text{C}$ , and the course of cooling was monitored. The initiation of freezing was detected by the jump in body temperature associated with the release of crystallization heat (the exotherm). If freezing did not begin before the body temperature equilibrated with the chosen incubator temperature, then the temperature was adjusted downward at  $0.5^\circ\text{C}$  intervals until the exotherm was observed. Incubator temperature was then immediately returned to  $-2.5^\circ\text{C}$  and maintained constant for the duration of the freezing. This protocol induces a cooling rate of  $\sim 0.19 \pm 0.07^\circ\text{C/h}$ . The frogs were sampled after different intervals of freezing ranging from 1 to 40 h.

### Ice Content

To determine the ice content of frozen frogs, we used the whole-body calorimetry technique described by Layne and Lee (27, 28). The calorimeter consisted of an insulated flask that was imbedded in a block of Styrofoam insulation and fitted with a Styrofoam plug that fitted down into the flask, leaving a space of only  $\sim 200$  ml at the bottom. A thermocouple was placed below the water surface and connected to a digital thermometer. Stirring was performed with a magnetic stirrer. We recorded the change in water temperature caused by frog thawing. We used experimentally determined values to calculate body ice content: factor for the calorimeter (F), percentage of body mass for each species (from measurements of wet and dry masses of 6 frogs), specific heat of the dry mass measured by calorimetry, and melting point of body fluids estimated from osmolality determination. These values are shown in Table 1. Body water content was determined for five frogs per species by drying carcasses at  $105^\circ\text{C}$  to constant mass. Ice content was expressed in terms of both

Table 1. Water contents and the different parameters used in the calculation of the ice content in water frogs, *Rana lessonae*, *R. ridibunda*, and *R. esculenta*

	Water Content, %	F Value	Sd Value	Mp Value, $^\circ\text{C}$
<i>R. ridibunda</i>	$81.02 \pm 1.26$	$1.01 \pm 0.01$	$0.25 \pm 0.02$	$-0.44$
<i>R. esculenta</i>	$80.20 \pm 1.66$	$1.01 \pm 0.01$	$0.24 \pm 0.03$	$-0.43$
<i>R. lessonae</i>	$80.62 \pm 0.43$	$1.01 \pm 0.01$	$0.23 \pm 0.02$	$-0.45$

Values are means  $\pm$  SD. Sd, specific heat of the dry mass; Mp, melting point of body fluids.

absolute mass and fraction of total body water. After completion of the rapid thawing in water (close to  $20^\circ\text{C}$ ) necessary for the empirical determination of ice content, survival was assessed during 2 wk after the freezing trial.

### Dynamics of Bulk Water in Organs During Freezing

The water contents of organs from unfrozen and frozen frogs at  $-2.5^\circ\text{C}$  were compared to determine whether tissues of the different species dehydrated during freezing. Frogs (5 controls and 5 frozen for each species) were double-pithed and dissected. The heart, liver, gut, and muscle were excised and weighed to the nearest 0.001 g. The tissues were reweighed after 2 days at  $105^\circ\text{C}$ . Water content was calculated from mass loss.

### Tissue Analyses

We aimed to determine the metabolic responses of the frogs to freezing. To obtain comparable physiological states between species, frozen frogs of the three species were allowed to reach between 40 and 45% ice within their bodies before being double-pithed and dissected. The blood, liver, and muscle of 10 frogs (5 frozen frogs and 5 control frogs) per species were removed. Blood samples were immediately separated by centrifugation at  $5^\circ\text{C}$  (10,000 rpm, 5 min), and plasma was collected and stored at  $-80^\circ\text{C}$  until assay.

**Osmolality.** The plasma samples were analyzed using a Wescor INC 51 vapor pressure osmometer.

**NMR spectroscopy.** Tissue samples were treated by perchloric acid extraction and analyzed with NMR spectroscopy, which can assay all of the molecules with mobile proton. Proton NMR spectra were recorded on a Bruker AM400WB spectrometer (Wissembourg, France) at 400 MHz. The signal from residual water was suppressed by the presaturation technique with an irradiation of 0.08 W for 2 s. Chemical shift resonances were expressed in parts per million (ppm), with reference to sodium trimethylsilylpropionate (external) assigned to 0 ppm. One-dimension (1D) experiments were performed at  $20^\circ\text{C}$  with a  $60^\circ$  flip angle, and 128 transients were accumulated. Acquisition time was 0.68 s on 8,000 data points corresponding to a sweep width of 6,000 Hz. The Fourier transformation was performed after a zero filling to 16,000 data points, and exponential multiplication corresponding to 1-Hz line broadening. Two-dimension (2D)-correlated spectroscopy experiments were performed with 1,000 data points in the F2 direction and 256 data points in the F1 direction. The sweep width was reduced to 2,700 Hz, eliminating the aromatic region of the spectrum where no resonance was detected in 1D acquisitions. The 2D-Fourier transformation was applied after zero filling to 512 data points in the F1 direction and a sine-bell function in both directions. Each experiment consisted of three 1D acquisitions and three 2D-correlated spectroscopy spectra. Peak assignments were based on data from the literature and from spectra obtained on standards.

**Glycogen assay.** Some tissue was homogenized in 1 M KOH at  $80^\circ\text{C}$  for 3 h, and the resulting solution was centrifuged (15 min at 2,500 g,  $4^\circ\text{C}$ ). Glycogen was then precipitated by 70% ethanol (15 h at  $4^\circ\text{C}$ ) and centrifuged (20 min at 2,500 g,  $4^\circ\text{C}$ ), and the pellet was

rinsed twice with 70% ethanol. After desiccation, the pellet was dissolved in 0.05 mol/l NaF (pH 4). This solution was used for glycogen assay, using a standard enzymatic method (6).

#### Statistical Analysis

Values are presented as means  $\pm$  SD where appropriate. Statistical analyses were performed with Statview computer statistical package. Comparisons between means were made through nonparametric one-way ANOVA and Mann-Whitney U-test. Body mass of frogs was a covariate in analyses of data for accumulation of ice in grams per hours to remove the effect of body size. Spearman's rank correlation test was used for the allometry of ice accumulation rate of the three taxa. A 5% ( $P < 0.05$ ) level of significance was used in all tests.

## RESULTS

### Freezing Survival and Ice Content

*The pool frog (R. lessonae).* The mean Tc of the pool frogs cooled on a wet substratum was  $-1.3 \pm 0.2^\circ\text{C}$  (ranging from  $-0.9$  to  $-1.6^\circ\text{C}$ ;  $n = 13$ ). All frogs with an ice content below 54% survived for a maximum time of  $\sim 9$  h at  $-2.5^\circ\text{C}$ . The time course of ice accumulation was analyzed in animals subjected to freezing at  $-2.5^\circ\text{C}$  (Fig. 1A). The lethal content of ice for this species was between 54 and 61% of the total body water. If we took into account only values of individuals that survived freezing, we found that the ice accumulation produced a close fit to a second-order hyperbolic curve ( $r^2 = 0.77$ ). However, Fig. 1B shows that a correlation between the ice accumulation rate and mass of the individuals was significant at  $-2.5^\circ\text{C}$  ( $z = -1.80$ ;  $P = 0.05$ ). Equilibrium ice content (estimated from frozen frogs exhibiting a body temperature equal to the envrioning temperature) was  $79.4 \pm 4.4\%$  ( $n = 4$ ).

*The marsh frog (R. ridibunda).* This species exhibited a mean Tc on a wet substratum of about  $-1.1 \pm 0.2^\circ\text{C}$  (ranging from  $-0.7$  to  $-1.5$ ;  $n = 13$ ). As Voituron et al. (52) previously reported, *R. ridibunda* tolerated more than 50% ice within its body (Fig. 1C). This ice content was attained after a few hours (between 10 and 20 h, depending on the mass of the individual). Indeed, Fig. 1D shows that a correlation between the ice accumulation rate and the mass of the individuals was significant at  $-2.5^\circ\text{C}$  ( $z = -2.58$ ;  $P = 0.01$ ). Similar to findings for *R. lessonae*, we found that the ice accumulation in *R. ridibunda* produced a close fit to a second-order hyperbolic curve, yielding a  $r^2$  of 0.88. Equilibrium ice content in this species was  $81.0 \pm 2.7\%$  ( $n = 3$ ).

*The edible frog (R. esculenta).* Similarly to the two other species, *R. esculenta* exhibited only modest supercooling, with Tc ranging from  $-0.8$  to  $-1.4^\circ\text{C}$  (mean value of  $-1.0 \pm 0.2$ ;  $n = 18$ ). The time course of ice accumulation was analyzed in animals subjected to freezing at  $-2.5^\circ\text{C}$  (Fig. 1E). The lethal content of ice for this species was between 57 and 67% of the total body water. All frogs with an ice content below 57% survived for a maximum time of  $\sim 13$  h at  $-2.5^\circ\text{C}$ . Similarly to the two other species, Fig. 1F shows that the correlation between ice accumulation rate and the mass of the individuals was significant at  $-2.5^\circ\text{C}$  ( $z = -2.40$ ;  $P = 0.01$ ). Moreover, the edible frog also exhibited an allometric relationship between body mass and "absolute" ice accumulation rate, expressed in grams of ice produced per hour (comparison of the two groups via Mann-Whitney U-test,  $P = 0.02$ ). However,

although the smallest *R. esculenta* accumulated  $0.84 \pm 0.08$  g ice/h, the largest ones accumulated  $1.78 \pm 0.09$  g ice/h. Such a relationship was not detected in *R. lessonae* and *R. ridibunda*. Equilibrium ice content in this species was  $81.2 \pm 2.7\%$  ( $n = 6$ ).

The three taxa exhibited no significant differences regarding Tc (Kruskal-Wallis = 0.15;  $P = 0.93$ ) and equilibrium ice content (Kruskal-Wallis = 0.68;  $P = 0.74$ ) and tolerated similar maximal ice content (between 54 and 57% for the three species). The three time courses of percent water frozen were also close but not comparable because of large differences in body mass among species, which ranged from 9 g for *Rana lessonae* to nearly 100 g for *Rana ridibunda* ( $P < 0.0001$ ). If we express the amount of ice in grams per hour, the values varied markedly (from  $0.75 \pm 0.12$  to  $1.78 \pm 0.09$  g ice/h) and were lowest in the terrestrial hibernator *Rana lessonae* (see Table 2 for all values). Upon removal from the cooling chamber, all of the frogs with high ice content ( $>30\%$ ) showed clear evidence of internal freezing, such as dark skin, rigid limbs, opaque eyes, and lack of responsiveness to mechanical stimuli. Two weeks later, no mortality had occurred, even in individuals with hematoma. However, the freezing injuries persisted over the 2 wk and did not show any significant remission.

### Dynamics of Bulk Water in Organs During Freezing

Freezing of the three taxa to  $-2.5^\circ\text{C}$  did not significantly reduce the water content of heart, liver, muscle, and gut. However, some differences among organs were detected. In fact, whatever the species and the treatment (control or frozen), the tissues can be ranked from the most to the least hydrated as follows: heart, gut, muscle, and liver (Table 3).

### Osmolality, Glycogen, and NMR Spectroscopy

Table 4 allows comparisons between controls and frozen frogs with 40–45% ice in their bodies by giving the concentrations of different metabolites in liver and muscles. Although glucose increased significantly in liver and muscles in both *R. lessonae* and *R. esculenta*, its level remained constant in *R. ridibunda*. Factorial increments of glucose concentration in the frozen state relative to controls (frozen concentration/control concentration) and associated  $P$  values were as follows: *R. lessonae* liver = 2.3 ( $P < 0.002$ ), *R. lessonae* muscle = 4.0 ( $P < 0.001$ ), *R. esculenta* liver = 2.4 ( $P < 0.002$ ), and *R. esculenta* muscle = 3.2 ( $P < 0.001$ ). Control values significantly differed between the species, with the concentration in *R. lessonae* liver threefold higher than that in *R. ridibunda*.

At the same time, liver glycogen before freezing was significantly higher than after 8 h of freezing in *R. lessonae* and *R. esculenta* ( $P < 0.001$  and  $< 0.002$ , respectively). The portion of glycogen reserves consumed during freezing in *R. lessonae* was 43% in liver and 65% in muscles. For *R. esculenta*, values were 30% in liver and 40% in muscles. Liver and muscle glycogen contents did not vary significantly in *R. ridibunda*.

Lactate accumulation in tissues caused by progressive ischemia during freezing differed among taxa. Although the lactate levels were high in liver and muscles of 8-h frozen *R. lessonae* (twofold for both tissues), increases reached significant levels only in liver of both *R. esculenta* and *R. ridibunda*. The concentrations of alanine significantly increased only in liver of *R. lessonae* and *R. esculenta* (threefold and twofold, respectively).

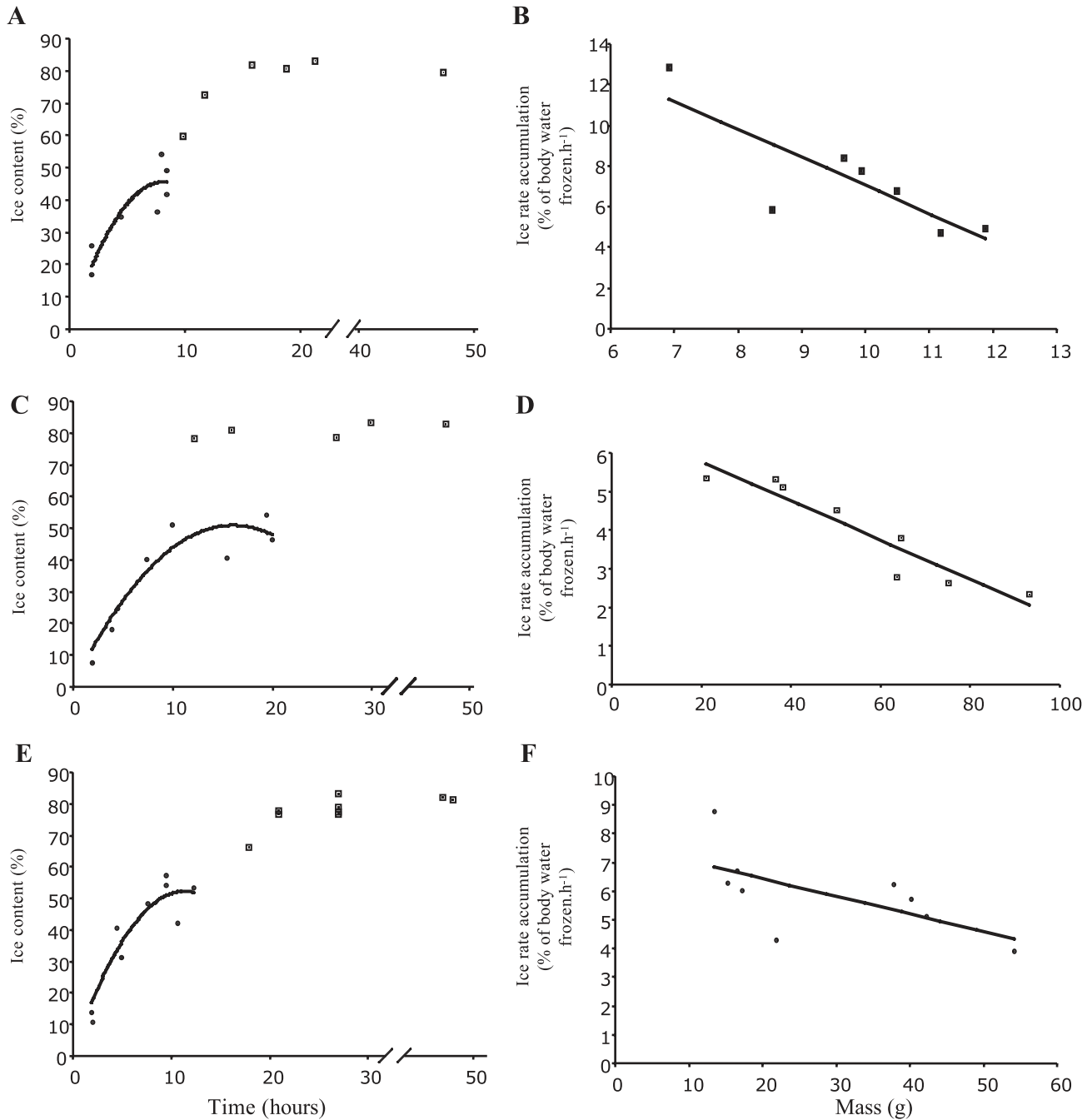


Fig. 1. Time course of ice accumulation in *R. lessonae* (A), *R. ridibunda* (C), and *R. esculenta* (E) subjected to freezing at  $-2.5^{\circ}\text{C}$ . The plots  $y = -0.66x^2 + 10.97x$  ( $R^2 = 0.77$ );  $y = -0.2x^2 + 6.29x$  ( $R^2 = 0.88$ ); and  $y = -0.4x^2 + 9.13x$  ( $R^2 = 0.88$ ) represent the best fits to the percentage of body water frozen in *R. lessonae*, *R. ridibunda* and *R. esculenta*, respectively, over time. □, Animals that died during freezing trials. Allometry of ice accumulation rate in *R. lessonae* (B), *R. ridibunda* (D), and *R. esculenta* (F) maintained at  $-2.5^{\circ}\text{C}$  after the onset of freezing. The plots  $y = -1.38x + 20.79$  ( $R^2 = 0.68$ );  $y = -0.05x + 6.78$  ( $R^2 = 0.88$ ); and  $y = -0.06x + 7.65$  ( $R^2 = 0.41$ ) represent the best fits to the ice accumulation rate vs. the body mass in *R. lessonae*, *R. ridibunda*, and *R. esculenta*, respectively.

Freezing exposure also induced changes in the plasma composition of frogs (Table 5). Plasma osmolality was 13% higher in frozen *R. lessonae* than in unfrozen ones (U-test = 15.5;  $P = 0.03$ ). In this species, plasma glucose and lactate concentrations were 3.4- and 3.5-fold, respectively, higher in the frozen than in the unfrozen frogs, but increase of lactate was not quite significant ( $P = 0.057$ ). The pattern was quite similar in *R. esculenta*, with 14% osmolality increase (U-test = 16,

$P = 0.03$ ), 2.9-fold glycemia increase, and no significant lactate accumulation. In contrast, no significant variation of osmolality, glycemia, or lactate was detected in *R. ridibunda*.

## DISCUSSION

The taxa that compose the hybridization complex of the water frogs differ in their habitat use. In western Europe as in

Table 2. Ice accumulation rate for *Rana lessonae*, *R. ridibunda*, and *R. esculenta* during the freezing episode at  $-2.5^{\circ}\text{C}$

	<i>Rana lessonae</i>	<i>Rana esculenta</i>		<i>Rana ridibunda</i>
		Small	Large	
Ice accumulation rate, g ice/h	$0.75 \pm 0.12$	$0.84 \pm 0.08$	$1.78 \pm 0.09$	$1.43 \pm 0.11$

Values are means  $\pm$  SD ( $n = 7$  *R. lessonae*, 8 *R. esculenta*, and 9 *R. ridibunda*) adjusted by ANCOVA to the grand mean of 33.7 g for body mass of the frogs.

the region where the frogs were caught, mixed assemblages of *R. esculenta* and *R. lessonae* occupy shallow eutrophicated ponds, whereas *R. ridibunda* is mainly found in rivers and lakes (32). As a correlate, these taxa are also considered as differing in their wintering habits. Whereas *lessonae* frogs usually leave the ponds by the end of August and the beginning of September to reach forested grounds, *ridibunda* frogs remain under water (4). The hybrids *esculenta* exhibit both overwintering behaviors. Terrestrial overwintering is suspected to allow the frogs to avoid the anoxic conditions caused by high proportions of organic matter in the substratum of most still waters (34). In contrast, aquatic wintering is made possible in rivers because of the continuous renewal of water in substrata, mainly composed of porous mineral materials. However, if terrestrial wintering may be an adaptive response to anoxia, it exposes the frogs to higher risks of freezing. As a matter of fact, winter survival varies according to hibernation site even at a small scale in *R. esculenta* and *R. lessonae*. In populations near Zurich, a negative correlation was detected between survival and winter harshness, with considerable year-to-year variation ranging from 6 to 98% (1). Such results suggest that cold resistance does not make it possible to avoid mortality in these two species. However, we hypothesized that the taxa that hibernate on land exhibit higher freeze tolerance than those that hibernate under water. This hypothesis is supported by several studies. Indeed Berger (4) found some *R. lessonae* still alive in an ice block, whereas Voituron et al. (52) showed that *R. ridibunda* have no cryoprotective system. Surprisingly, we first showed similar survival times under freezing and similar time courses of ice accumulation among the three taxa (Fig. 1, A, C, and E). These results suggest that freezing tolerance cannot be considered as a key element of winter survival (survival times range from 8 to 20 h), and they do not support the hypothesis of better protection against freezing in the taxa that hibernate on land.

However, some differences were detected among the studied taxa, at least in the physiological responses to freezing. First, the three likely time courses of ice accumulation mask a significant difference in the rates of ice accumulation in terms of mass of ice, mainly because of high differences in body mass among the taxa (from 7.5 to 54.3 g in this study). Interestingly, if we express ice accumulation rate in grams of ice per hour, the values then differ markedly among the three taxa, ranging from  $0.75 \pm 0.13$  g ice/h in *R. lessonae* to  $1.43 \pm 0.11$  g ice/h in *R. ridibunda*. We are therefore led to conclude that physiological responses during freezing vary within the *R. esculenta* complex.

## Metabolic Responses

Ice accumulation rate was lowest in the pool frog (*R. lessonae*), which hibernates on land. This species probably possesses a physiological mechanism that restrains ice formation during freezing. Lower ice accumulation cannot be explained by the accumulation of a cryoprotectant. Even if osmolality increases by  $\sim 31$  mosmol/kgH<sub>2</sub>O during freezing in this species (which represents the highest increase among the three taxa), no large accumulation of carbohydrates was detected. The NMR spectroscopy analyses detected variations only for blood glucose and lactate. Freezing induces similar responses in *esculenta* frogs except for lactate, for which the response was made nonsignificant by high variation among control individuals (Table 5). In these two taxa, glycemia was close to 1.5  $\mu\text{mol/ml}$  in controls (value commonly observed in anurans) (7, 16, 24, 26), although a threefold increase was detected in frozen individuals. Lactate followed a similar pattern. However, our results reveal that such increases accounted for only 14 and 10% of the total osmotic increase in *lessonae* and *esculenta* frogs, respectively, with the rest being possibly due to variations in the balance of Na<sup>+</sup> and K<sup>+</sup> or other osmolytes not detected in this study (11).

All freeze-tolerant frogs mobilize glucose from hepatic stores as a direct nonanticipatory response to tissue freezing (15, 43, 46, 47). A similar mechanism may occur in both *R. lessonae* and *R. esculenta* since these taxa showed a significant increase of glucose in liver and a decrease of glycogen content during freezing (see Table 4). However, the highest concentrations observed in water frogs were markedly lower than those achieved by a freeze-tolerant frog such as *R. sylvatica*. This species accumulated 140  $\mu\text{mol/g}$  of liver glucose during the first 7 h of freezing (data calculated from Ref. 45), whereas *R. lessonae* and *R. esculenta* only accumulated 74 and 42  $\mu\text{mol/g}$ , respectively. Glucose synthesis from glycogen requires only three enzymes (phosphorylase, phosphoglucotomutase, and the glucose-6-phosphatase), and it is known that cold exposure increases the hepatic gluconeogenic capacity of water frogs at least through glucose-6-phosphatase (16). The

Table 3. Water contents of organs from the water frogs (*R. esculenta*, *R. lessonae*, and *R. ridibunda*) unfrozen and after freezing at  $-2.5^{\circ}\text{C}$

	Unfrozen	Frozen at $-2.5^{\circ}\text{C}$
<i>Rana lessonae</i>		
Muscle	$80.9 \pm 1.2$	$80.1 \pm 0.4$
Liver	$74.9 \pm 1.0$	$74.5 \pm 3.7$
Heart	$85.5 \pm 2.0$	$87.1 \pm 3.3$
Gut	$83.0 \pm 1.2$	$79.7 \pm 4.1$
<i>Rana esculenta</i>		
Muscle	$79.9 \pm 0.8$	$80.2 \pm 2.6$
Liver	$73.0 \pm 1.6$	$75.0 \pm 0.8$
Heart	$85.5 \pm 1.6$	$86.6 \pm 2.1$
Gut	$82.3 \pm 1.8$	$81.9 \pm 1.5$
<i>Rana ridibunda</i>		
Muscle	$80.9 \pm 0.8$	$80.3 \pm 0.7$
Liver	$73.4 \pm 2.2$	$76.3 \pm 3.3$
Heart	$87.3 \pm 1.5$	$84.9 \pm 2.4$
Gut	$83.1 \pm 1.0$	$83.4 \pm 0.8$

Values are means  $\pm$  SD;  $n = 5$  frogs for each group.



Table 4. *Effect of freezing on metabolite levels in the water frogs, Rana lessonae, R. ridibunda, and R. esculenta*

	<i>Rana lessonae</i>		<i>Rana esculenta</i>		<i>Rana ridibunda</i>	
	Control	Frozen	Control	Frozen	Control	Frozen
Glycogen						
Liver	143±8.5	82±3.9*	207±20.4	144±15.4*	190±15.9	170±12.3
Muscle	2.0±0.1	0.7±0.1*	1.5±0.1	0.9±0.1*	1.3±0.2	1.1±0.2
Glucose						
Liver	32.6±6.1	73.7±9.7*	17.6±0.6	41.9±0.2*	9.2±1.0	8.1±0.7
Muscle	1.5±0.03	6.0±0.1*	1.0±0.05	3.2±0.2*	1.2±0.1	1.4±0.1
Lactate						
Liver	15.5±1.9	38.9±3.7*	15.8±1.1	41.7±5.5*	30.9±3.9	60.0±4.4*
Muscle	12.6±0.8	24.7±1.8*	17.6±1.0	17.5±2.4	29.5±4.3	26.7±0.7
Alanine						
Liver	5.6±1.1	17.9±3.2*	6.9±0.1	14.1±1.2*	6.9±2.2	6.4±0.6
Muscle	1.2±0.1	1.6±0.4	0.9±0.2	1.6±0.4	0.6±0.1	0.6±0.1

Values are means ± SD, expressed as µmol/g fresh weight;  $n = 4$  or 5 frogs for each group. \*Significantly different from control values ( $P < 0.05$ ).

low capability to synthesize glucose could be related to a low liver phosphorylase activity that determines the conversion rate from glycogen to glucose (47). Furthermore, the low level of hyperglycemia in frozen water frogs may tentatively be explained by a small number and/or low activities of plasma membrane glucose transporters (22). These results are consistent with data observed in other freeze-intolerant anurans such as *R. pipiens* and *Bufo americanus* (13), but they are not sufficient to ensure freezing survival and cannot explain low ice accumulation rate. However, if we compute survival under freezing of a 10-g frog that responds like a *R. ridibunda*, the survival time under freezing would be 2.9 h, although survival of a *lessonae* frog of similar mass would be actually ~9 h. This result highlights how the physiological mechanisms that we detected alleviate the consequence of small body size in this species. Further studies are thus needed to better understand this phenomenon.

The lack of any cryoprotective system in the marsh frog (*R. ridibunda*) is consistent with a previous study of individuals from other portions of its geographical range (52). Except for liver lactate concentration, which is multiplied by two during freezing, no quantified molecule shows significant variation (see Tables 4 and 5). Osmolality did not differ between frozen and control frogs, which was already shown with frogs from Turkey (52). Similarly, no accumulation of glucose was detected in each sample. Also, glycogen contents were similar to those measured in other studies (51). They remained stable over freezing exposures, once again suggesting the lack of any physiological response to freezing. Whereas several studies have detected geographic variations of cold tolerance in various ectotherms (2, 48, 53), the similarities observed in our study between French and Turkish populations can be explained by the recent introduction of *R. ridibunda* into western

Europe from populations of eastern Europe and Anatolia (18). It is noteworthy that none of our results provide any reason why *R. ridibunda* could not overwinter on land. Hence, further investigations are needed to better understand why this species seems to be a specific aquatic hibernator (4).

#### Ecological and Evolutionary Aspects

Although water frogs do not survive extensive freezing, they are also unable to rely on supercooling capacities to survive subzero temperatures. In dry environments, the water frogs supercool to  $-2^{\circ}\text{C}$  (unpublished data). No significant differences occurred between species, and  $T_c$  was not correlated to body mass. In the presence of ice, the water frogs do not resist inoculation and freeze at high subzero temperatures (greater than  $-1^{\circ}\text{C}$ ). Thus, under field conditions, both *R. lessonae* and *R. esculenta* may be inoculated by ice and would die. Therefore, these species may select microhabitats that remain above the freezing point of their tissues and the relative prominence of their metatarsal tubercle may provide some burrowing capabilities. Indeed, Holenweg and Reyer (20) found that soil temperatures at the hibernation sites were significantly higher than at randomly sampled control sites. Their data also showed that most frogs were able to change their overwintering site during winter and often more than once. Thus, even if *lessonae* and *esculenta* frogs did not evolve toward long-term cold tolerance, the physiological mechanisms that we identified sufficiently show how these frogs could survive an overnight freezing (between 8 and 12 h) and subsequently find a more insulated microenvironment. Such physiological and behavioral responses to cold could explain high survival rates, which range from 85 to 90% for *R. lessonae* and from 70 to 80% for *R. esculenta* (19) during mild winters. Regarding the similar

Table 5. *Plasma osmolality, glucose content, and lactate content in control and frozen water frogs Rana lessonae, R. ridibunda, and R. esculenta*

	<i>Rana lessonae</i>		<i>Rana esculenta</i>		<i>Rana ridibunda</i>	
	Control	Frozen	Control	Frozen	Control	Frozen
Osmolality, mosmol/kgH <sub>2</sub> O	240±17	271±5*	231±11	264±10*	239±9	238±12
Glucose, µmol/ml	1.4±0.7	4.8±0.4*	1.6±0.7	4.6±0.6*	0.9±0.2	1.3±0.3
Lactate, µmol/ml	0.4±0.1	1.3±0.1*	0.8±0.5	1.2±0.1	0.7±0.3	1.8±0.1*

Values are means ± SD;  $n = 4$  frogs for each group. \*Significantly different from control values ( $P < 0.05$ ).

cold hardiness between these two species, the differences in winter survival rates may be explained by the tendency for *R. lessonae* to hibernate at warmer sites than *R. esculenta* (20).

In other respects, the data show that body mass influences freezing tolerance. A number of biological parameters such as longevity, fecundity, metabolic rate, desiccation endurance, and winter survival are related to size (8, 35, 39). As far as we know, only one study previously examined allometry of freezing tolerance in vertebrate ectotherms, demonstrating that ice content and prefreezing and postfreezing cooling rates scale with body mass (9). Our results are in complete agreement with these findings, but we cannot conclude that body mass has a selective value regarding freezing.

The ecological success of *R. esculenta* has been suspected to be due to heterotic effects because its genome is similar to that of F1 hybrids. High genetic diversity resulting from hybridization could provide high tolerance capacity [general-purpose genotype hypothesis (28)]. Although this hypothesis has been supported by some studies (40–42, 50), others have shown that the hybrids exhibit intermediate performances (36, 37), thus suggesting that the causes of the success of hybrid frogs is to be found in processes other than heterotic advantages. The present study converges with the latter ones in revealing intermediate performance of the hybrids with regard to parental ones. The threshold-like response of this taxon could be related to its greater genetic heterogeneity since the population may be composed of two main hemiclones, as suggested by an analysis of its genetic structure (10).

### Perspectives

This study provides some elements on freeze tolerance among water frogs and raises several questions that must be elucidated by further studies. First, in western Europe, winters are characterized by mild temperatures; thus high cold tolerance is perhaps not necessary for successful overwintering. However, *R. lessonae* and *R. esculenta* can also be found in Poland and Russia, where long-lasting subzero temperatures occur. Thus it should be very interesting to test eastern populations to detect possible interpopulational variations in the freeze tolerance (for discussion, see Refs. 14, 31).

Second, the hybrid *R. esculenta* exhibits an intermediate response between the parental species, but it is surprisingly the only taxon that expresses a relationship between mass and ice mass accumulation rate ( $0.84 \pm 0.10$  g ice/h for large *R. esculenta* and  $1.78 \pm 0.09$  g ice/h for small ones). This suggests the occurrence of subpopulations of *R. esculenta* related to the body mass. As previously mentioned, we have to be aware that geographic distribution of *R. ridibunda* in western Europe is due to introductions by humans, especially in Switzerland and France (18). Although this “invasion” provides interesting situations to study behavioral and physiological adaptations to cold environments, it renders the case of *R. esculenta* highly complex. Yet, *R. ridibunda* from many regions of eastern Europe can mate with native *R. esculenta* and *R. lessonae*. This has resulted in primary hybrids of *R. esculenta* carrying novel *R. ridibunda* genomes (54). These “new” *R. esculenta* could thus be less adapted to temperate climates than ancient lineages present in western Europe for more than 20,000 yr. Further studies are needed to discriminate different

types of hybrids and detect potential differences between them regarding cold tolerance.

### ACKNOWLEDGMENTS

We express our gratitude to Dr. Plenet for assistance with collecting frogs and to Pr. Pattee for critically reading the manuscript.

### GRANTS

This work was supported by a research grant from Centre National de la Recherche Scientifique France.

### REFERENCES

1. Anholt BR, Hotz H, Guex GD, and Semlitsch RD. Annual variation in over-winter survival of the water frog *Rana lessonae* and its hemiclinal associate *Rana esculenta*. *Ecology* 84: 391–397, 2003.
2. Baust JB and Lee RE. Population differences in antifreeze/cryoprotectant accumulation patterns in an Antarctic insect. *Oikos* 40: 120–124, 1983.
3. Berger L. Embryonal and larval development of F1 generation of green frogs different combinations. *Acta Zool* 12: 123–124, 1967.
4. Berger L. Hibernation of the European water frogs (*Rana esculenta* complex). *Zool Poloniae* 29: 57–72, 1982.
5. Berger L. Morphology of the F1 generation of various crosses within *Rana esculenta* complex. *Acta Zool* 12: 321–324, 1968.
6. Bergmeyer HU. *Methods of Enzymatic Analysis*. Weinheim: VCH, 1985.
7. Byrne JJ and White RJ. Cyclic changes in liver and muscle glycogen tissue lipid and blood glucose in a naturally occurring population of *Rana catesbeiana*. *Comp Biochem Physiol* 50A: 709–715, 1967.
8. Civantos E, Salvador A, and Veiga JP. Body size and microhabitat affect winter survival of hatchling *Psammotromus algirus* lizards. *Copeia* 1999: 1112–1117, 1999.
9. Claussen DL and Zani PA. Allometry of cooling, supercooling, and freezing in the freeze-tolerant turtle *Chrysemys picta*. *Am J Physiol Regul Integr Comp Physiol* 261: R626–R632, 1991.
10. Colon L. *L'hybridogénèse dans le Complexe Rana klepton esculenta*. *Apport des Marqueurs Génétiques* (PhD thesis). Lyon, France: Lyon1 Univ., 2004.
11. Costanzo JP and Lee RE. Cryoprotectant production capacity of the freeze-tolerant wood frog, *Rana sylvatica*. *Can J Zool* 71: 71–75, 1993.
12. Costanzo JP and Lee RE. Supercooling and ice nucleation in vertebrate ectotherms. In: *Biological Ice Nucleation and Its Applications*, edited by Lee RE, Warren GJ, and Gusta LV. St. Paul, MN: Am. Phytopathol. Soc. Press, 1994.
13. Costanzo JP, Lee RE, and Lortz PH. Physiological responses of freeze-tolerant and intolerant frogs: clues to evolution of anuran freeze tolerance. *Am J Physiol Regul Integr Comp Physiol* 265: R721–R725, 1993.
14. Costanzo JP, Litzgus JD, Larson JL, Iverson JB, and Lee RE. Characteristics of nest soil, but not geographic origin, influence cold hardiness of hatchling painted turtles. *J Therm Biol* 26: 65–73, 2001.
15. Costanzo JP, Wright MF, and Lee RE. Freeze-tolerance as an overwintering adaptation in cope's gry treefrog (*Hyla chrysoscelis*). *Copeia* 2: 565–569, 1992.
16. Dzięwulska-Szwajkowska D, Lozinzka-Gabska M, and Dzugaj A. *Rana esculenta* L. liver Fru-P2ase and G-6-Pase activity and Fru-2,6-P2 concentration after acclimation at 5 and 25°C. *Comp Biochem Physiol A* 118A: 745–751, 1997.
17. Graf JD and Polls Pelaz M. Evolutionary genetics of the *Rana esculenta* complex. In: *Evolution and Ecology of Unisexual Vertebrates*, edited by Dawley R and Bogart J. New York: New York State Museum, 1989, p. 289–301.
18. Grossenbacher K. *Atlas de Distribution des Amphibiens de Suisse*. Neuchâtel, Switzerland: Musée d'Histoire Naturelle de Neuchâtel, 1988.
19. Holenweg AK. Survival in adults of the water frog *Rana lessonae* and its hybridogenetic associate *Rana esculenta*. *Can J Zool* 79: 652–661, 2001.
20. Holenweg AK and Reyer HU. Hibernation behavior of *Rana lessonae* and *R. esculenta* in their natural habitat. *Oecologia (Berl)* 123: 41–47, 2000.
21. Joly P. The future of the selfish hemiclone: a neodarwinian approach of waterfrog evolution. *Mitt Mus Natur Berlin* 77: 31–38, 2001.
22. King PA, Rosholt MN, and Storey KB. Adaptations of plasma membrane glucose transport facilitate cryoprotectant distribution in freeze-tolerant frogs. *Am J Physiol Regul Integr Comp Physiol* 265: R1036–R1042, 1993.



23. Lada GA, Borkin LJ, and Vinogradov AE. Distribution, population systems and reproductive behavior of green frogs (hybridogenetic *Rana esculenta* complex) in the central Chernozem territory of Russia. *Russian J Herpetol* 2: 46–57, 1995.
24. Layne JR. Freeze tolerance and cryoprotectant mobilization in the gray treefrog (*Hyla versicolor*). *J Exp Zool* 283: 221–225, 1999.
25. Layne JR. Postfreeze survival and muscle function in the leopard frog (*Rana pipiens*) and the wood frog (*Rana sylvatica*). *J Therm Biol* 17: 121–124, 1992.
26. Layne JR and Jones AL. Freeze tolerance in the gray treefrog: cryoprotectant mobilization and organ dehydration. *J Exp Zool* 290: 1–5, 2001.
27. Layne JR and Lee RE. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can J Zool* 65: 2062–2065, 1987.
28. Layne JR and Lee RE. The importance of body tissue composition to calorimetric assessments of ice content in whole animals. *J Therm Biol* 16: 61–64, 1991.
29. Lynch M. Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Q Rev Biol* 59: 257–285, 1984.
30. Morand A and Joly P. Habitat variability and space utilization by the amphibian communities of the French Upper-Rhône floodplain. *Hydrobiologia* 300–301: 249–257, 1995.
31. Packard GC and Janzen FJ. Interpopulational variation in the cold-tolerance of hatchling painted turtles. *J Therm Biol* 21: 183–190, 1996.
32. Pagano A, Crochet PA, Graf JD, Joly P, and Lodé T. Distribution and habitat use of water frog hybrid complexes in France. *Global Ecol Biogeogr* 10: 433–441, 2001.
33. Pagano A, Joly P, and Hotz H. Taxon composition and genetic variation of water frogs in the Mid-Rhône floodplain. *C R Acad Sci Serie III* 320: 759–766, 1997.
34. Pagano A, Joly P, Plenet S, Lehman A, and Grolet O. Breeding habitat partitioning in the *Rana esculenta* complex: The intermediate niche hypothesis supported. *Ecoscience* 8: 294–300, 2001.
35. Peters RH. *The Ecological Implications of Body Size*. Cambridge, UK: Cambridge Univ. Press, 1983.
36. Plenet S, Hervant F, and Joly P. Ecology of the hybridogenetic *Rana esculenta* complex: differential oxygen requirements of tadpoles. *Evol Ecol* 14: 13–23, 2000.
37. Plenet S, Pagano A, Joly P, and Fouillet P. Variation of plastic responses to oxygen availability within the hybridogenetic *Rana esculenta* complex. *J Evol Biol* 13: 20–28, 2000.
38. Schmid W. Survival of frogs in low temperature. *Science* 215: 697–698, 1982.
39. Schmidt-Nielsen K. *Animal Physiology: Adaptation and Environment*. Cambridge, UK: Cambridge Univ. Press, 1979.
40. Semlitsch RD. Adaptive genetic variation in growth and development of tadpoles of the hybridogenetic *Rana esculenta* complex. *Evolution* 47: 1805–1818, 1993.
41. Semlitsch RD. Effects of different predators on the survival and development of tadpoles from the hybridogenetic *Rana esculenta* complex. *Oikos* 67: 40–46, 1993.
42. Semlitsch RD and Reyer HU. Performance of tadpoles from the hybridogenetic *Rana esculenta* complex: interactions with pond drying and interspecific competition. *Evolution* 46: 665–676, 1992.
43. Storey KB and Storey JM. Freeze tolerance and intolerance as strategies of winter survival in terrestrially-hibernating amphibians. *Comp Biochem Physiol* 83A: 613–617, 1986.
44. Storey KB and Storey JM. Freeze tolerance in animals. *Physiol Rev* 68: 27–84, 1988.
45. Storey KB and Storey JM. Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze-thaw cycles. *Can J Zool* 64: 49–56, 1986.
46. Storey KB and Storey JM. Persistence of freeze tolerance in terrestrially hibernating frogs after spring emergence. *Copeia*: 720–726, 1987.
47. Swanson DL, Graves BM, and Koster KL. Freezing tolerance/intolerance and cryoprotectant synthesis in terrestrially overwintering anurans in the great plains, USA. *J Comp Physiol [B]* 166: 110–119, 1996.
48. Tanaka K. Seasonal and latitudinal variation in supercooling ability of the house spider, *Achaearanea tepidariorum* (Araneae: Theridiidae). *Funct Ecol* 10: 185–192, 1996.
49. Tattersall GJ. Balancing hypoxia and hypothermia in cold-submerged frogs. *J Exp Biol* 200: 1031–1038, 1997.
50. Turner HG and Nopp H. Heterosis in the common European water frog. *Naturwissenschaften* 66: 268–269, 1979.
51. Vogiatzis AK and Loumbourdis NS. Exposure of *Rana ridibunda* to lead. II. Impact of lead on various parameters of liver metabolism of the frog *Rana ridibunda*. *J Appl Toxicol* 21: 269–274, 2001.
52. Voituron Y, Eugène M, and Barré H. Survival and metabolic responses to freezing by the water frog (*Rana ridibunda*). *J Exp Zool* 299A: 118–126, 2003.
53. Voituron Y, Heulin B, and Surget-Groba Y. Comparison of the cold hardiness capacities of the oviparous and viviparous forms of *Lacerta vivipara*. *J Exp Zool* 301A: 367–373, 2004.
54. Vorburger C. *Consequence of Clonal Inheritance in the Hybridogenetic Water Frog Rana esculenta*. Zurich: Univ. of Zurich, 2001.