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Hibernation behavior of *Rana lessonae* and *R. esculenta* in their natural habitat

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Abstract We studied the hibernation behavior of the water frog *Rana lessonae* and its hybridogenetic associate *R. esculenta* in their natural habitat during three successive winters. Animals caught in pitfall traps at a fenced pond were individually marked with PIT tags and some ($n=36$) were additionally equipped with radio transmitters. Of the animals caught, 85% left the fenced pond for hibernation. More *R. esculenta* remained inside the fenced area compared to *R. lessonae*. *R. lessonae* emigrated earlier in autumn and came back later in spring than *R. esculenta*, but the distance to their hibernation sites did not differ. Both species left the fenced pond earlier in the year when ambient temperatures were lower. All radio-tracked animals hibernated in woodland, 3–7 cm below the surface in soil, under moss, fallen leaves or small branches. Soil temperatures at the actual hibernation sites were significantly higher than at randomly chosen control sites. A surprising finding was that most frogs changed their hibernation sites during winter, and often more than once. Movements were more frequent in the warmer first half of the winter than in the cooler second half, but some animals were active even on days with mean temperatures below 1°C. These results show that both species do not spend the whole winter torpid in one particular hibernation site but move around, especially at higher temperatures. Most of the animals lost weight during the winter, and the weight loss was greater in females than in males and higher in warm than in cold winters. To what extent weight loss and survival is influenced by the chosen hibernation sites and the amount of movement during winter, and whether this contributes to the differences in species and sex ratios found in mixed populations, needs more investigation.

Key words Hibernation · Temperature · Winter habitat · Hybridogenesis · Anuran

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Introduction

In the temperate zone, most vertebrate ectotherms face potential problems in dealing with low ambient temperatures during winter. Possible strategies for surviving such temperatures include freeze-tolerance and hibernation in places that are buffered against extreme air temperatures. Hibernating in water, digging deep into the soil or using tunnels and burrows reaching below the frost line all serve to avoid extremes, but these strategies require certain environmental conditions, which may not always exist. Digging deep needs a soft or sandy soil, and empty refuges below the frostline are not always available. Among amphibians, only terrestrial toads (*Bufo calamita*, *B. bufo*, *B. americanus*) seem to be able to burrow down as far as 1 m (Froom 1982; Sinsch 1989; Denton and Beebee 1993) while salamanders and newts are described as using existing insulated refuges below the frostline (Froom 1982; Kwet 1996). Most frogs seem either to tolerate freezing or overwinter in water (Berger 1982; Licht 1991; Sinsch 1991; Pasanen and Sorjonen 1994; Stinner et al. 1994). However, both strategies require some specific adaptations. Hibernation under water poses potentially lethal physiological problems because of the scanty amount of oxygen in the water (Lutschinger 1988; Tattersall and Boutilier 1997). On the other hand, tolerance to freezing depends on the ability to increase blood glucose levels as a cryoprotectant (Schmid 1982; Storey and Storey 1986; Costanzo et al. 1992, 1993; Storey et al. 1996; Storey 1997). These physiological aspects have been investigated for some frog species, and it seems that freeze-tolerance is associated with a pre-existing mechanism for dealing with water stress (Lee et al. 1992; Costanzo et al. 1993; Churchill and Storey 1995; Storey 1997). Hence, species that are tolerant to desiccation are probably also tolerant to freezing.

While physiological mechanisms have been studied in great detail, field data on hibernation in anurans are scarce, and most information is anecdotal and often the result of accidental discoveries. In this study, we investigate the hibernation behavior of two types (here termed

species) of water frogs, *Rana lessonae* and its hybridogenetic associate *R. esculenta* (Berger 1968; Günther 1973; Graf and Polls-Pelaz 1989). Both species occur sympatrically in many areas of Europe and breed in the same ponds during spring and summer, but their relative numbers and the sex ratios differ markedly among ponds and slightly between years (Blankenhorn 1974, 1977; Berger 1977, 1983; Binkert 1981; Neveu 1991; Holenweg 1999). Since *R. lessonae* has been reported to spend the winter mainly on land, whereas *R. esculenta* seems to hibernate on land or under water (Berger 1970, 1982; Heym 1974; Lutschinger 1988; Günther 1990; but see Sinsch 1991), a likely hypothesis was that differences in hibernation behavior, through their effect on survival, affect species and sex ratios. To test this hypothesis, we searched for species and sex differences in time and duration of hibernation and in conditions at the hibernation sites.

Materials and methods

Study area and animals

The study area, near Zurich airport, Switzerland, included several permanent ponds of different sizes, two ditches, woodland, and grassland (Fig. 1). One pond was surrounded by a fence enclosing an area of 570 m². Depending on the water level, and hence the surface area, of the pond (c. 75–190 m²), the fence was 3–15 m from the shoreline. There were small patches of grassland, and some bushes and trees within the fenced area. On both sides the fence was equipped with pitfall traps that were checked daily between 15 March and 25 November and – depending on air temperature – every 2–7 days during the rest of the year. All caught animals ≥ 40 mm snout-vent length were identified by species, sexed, weighed, marked with PIT tags (Trovan ID 100, Pameda AG, Switzerland), and released on the opposite side of the fence. These tags could be identified afterwards by a hand reader (Trovan LID 500). The mark-recapture study took place between April 1994 and December 1996. Hence, the time and the number of animals that left the pond area for hibernation and returned the following spring, and their mass loss, are known for the two winters 1994/1995 and 1995/1996. The first animals emigrated early in September and came back by the end of May. Since the animals were still feeding in September and probably at the beginning of October, and again in April and May, we restricted the mass loss calculations to animals that were caught after 15 October and before 15 April.

We also equipped a total of 36 individuals with radio transmitters (BD-2A, Bd-2 and BD-2G, Holohil Systems Ltd., Ontario) during the two winters 1995/1996 and 1996/1997. In 1995, 21 of them were caught at the fence after 25 September and in 1996, 15 were caught after 15 October. The transmitter was tied around the frog's waist with a synthetic thread, lined with a silicon tube. On average, the weight of the transmitter and the belt was around 6% of the animal's weight and never exceeded 10%. We checked the animal's state 1 day after release. One animal had a slightly swollen leg, and we removed the transmitter; three animals lost their transmitters during the first few days. For the remaining 32 frogs, we recorded their positions three times a week until early December and at least once a week until March, depending on the air temperatures. A portable receiver (LA12, AVM Instrument Co. Ltd., Livermore) with hand-held antenna (HB9CV, Andreas Wagener, Köln) was used for locating the frogs. The range of detection was about 200 m, even if the animals were hidden in soil or undergrowth. For determining the exact position we used the Trovan hand reader which was able to identify the PIT tag through different kinds of substrate down to 8 cm. Once during winter we dug the animals out, changed the transmitters before their batteries became low and then buried the frogs in the same location.

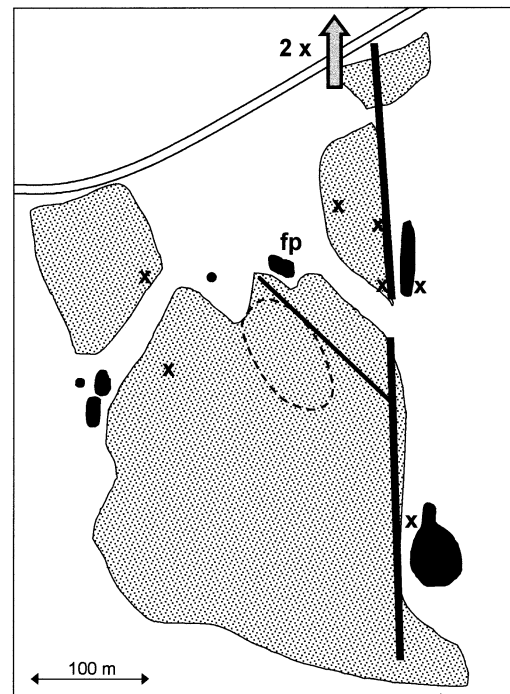


Fig. 1 Map of the study area showing the location of the breeding ponds (filled areas), ditches (solid lines), grassland (unfilled areas) and of the forest areas (stippled). One of the ponds (fp) was surrounded by a drift fence. The vast majority of the frogs hibernated in the area marked by the dashed ellipse, a few individuals in places marked by a cross. Two frogs crossed the road (arrow, double line) to hibernate in the forest beyond it

Temperature measurements

Mean soil temperatures during the winter 1995/1996 (measured 5 cm below the surface) showed no significant differences between positions in open grassland, at the edge of, or within the woodland. In the following winter, we therefore recorded minimum and maximum temperatures within the three habitat types. We buried 24 mini-max thermometers 5 cm deep into the ground, 8 in open grassland, 8 along the edge of the woodland, and 8 in the woodland. The thermometers were exposed to soil temperatures from 5 December 1996 to 26 March 1997. Within each of the three habitats distance between adjacent thermometers was 25 m. In addition, we measured the actual temperatures 5 cm below ground at ten randomly chosen positions and close to the radio-tracked animals three times in each habitat (23 December 1996, 28 January, and 7 March 1997). Air temperatures were measured during all three years by a data logger installed close to the fenced pond.

Statistics

Statistics were calculated using Systat 6.0 for Windows (SPSS 1996) for analyses of variance (ANOVA) and using SPSS for Windows Release 6.1.3. (Norusis 1995) for logistic regression. All stepwise analyses proceeded backwards, starting with the full model and then gradually eliminating independent variables and interactions with $P > 0.10$. To approximate normal distribution, all proportions were arcsin-square-root transformed. In a few cases we caught the same animals at the fence in both winters. These animals were randomly assigned to one of the two winters to avoid dependent data in the statistical analysis.

Results

Time of hibernation

Most animals left the fenced pond in autumn and returned the next spring. Two stepwise backward regressions were performed, one with time of emigration after 1 September ($n=313$), the other with immigration before 31 May ($n=370$) as the dependent variable, and species, sex, mean snout-vent-length, year, and their interactions as independent variables (Table 1). On average, *R. lessonae* left the pond about 3 weeks earlier and arrived 5 days later than *R. esculenta* (Fig. 2). The males of both species arrived 1 week earlier than the females, but the sexes did not differ in departure time. In 1995, with an average daily temperature of 8.4 ± 0.5 (SE) $^{\circ}\text{C}$ from 1 September to 2 December, both species left earlier than in 1994, with a corresponding average of $9.7 \pm 0.4^{\circ}\text{C}$ (Fig. 2). In terms of arrival, this year effect interacted with a species effect. After the cooler second winter, with an average temperature of $5.2 \pm 0.4^{\circ}\text{C}$ between 1 September and 31 May, *R. lessonae* arrival was delayed by an average of 4 days, compared to the warmer first winter ($6.7 \pm 0.3^{\circ}\text{C}$), whereas *R. esculenta* arrival was advanced by 4 days (Fig. 2).

Place of hibernation

Pooled over both winters, 166 animals left the fenced pond after 1 September and came back before 31 May of the following year, while 30 animals hibernated within the fenced area. A backward stepwise logistic regression showed a significant species effect, with *R. lessonae* hibernating

outside the fence more often than *R. esculenta*, and a year effect with more animals hibernating within the fenced area during the first winter than the second one (Table 2, Fig. 3). Of the 19 individuals that were followed over two winters, 16 maintained their overwinter-

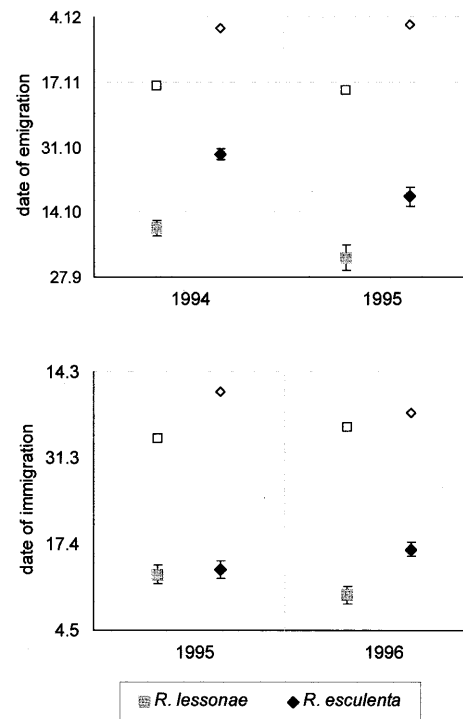


Fig. 2 Mean dates (with SEs) **a** of emigration in 1994 and 1995 and **b** immigration in 1995 and 1996. Open symbols represent latest and earliest dates, respectively

Table 1 Factors affecting the time of emigration in **a** autumn (after 1 September) and **b** immigration in spring (before 31 May) of the frog species *Rana lessonae* and *R. esculenta*. Shown are results from

Source	a Emigration in autumn				b Immigration in spring			
	df	MS	F	P	df	MS	F	P
Species	1	23425	70.55	<0.001	1	3622	13.84	<0.001
Year	1	5180	15.60	<0.001	1	2	0.01	0.932
Sex					1	4906	18.74	<0.000
Species×Year					1	1249	4.77	0.030
Error	310	332			365	262		

two stepwise backward analyses of variance with date as the dependent variable, and species, sex, mean snout-vent length, year, and their interactions as independent variables ($R^2_a=0.220$, $R^2_b=0.083$)

Table 2 Factors determining whether *R. lessonae* and *R. esculenta* hibernated within or outside the fenced pond area. Results from a logistic regression with hibernation within or outside the fenced pond area as the dependent variable, and species, sex, year, and their interactions as independent variables ($R=0.847$)

Source	Estimated coefficient	SE	Wald statistics	df	P
Species	0.641	0.286	5.01	1	0.025
Year	0.780	0.244	10.24	1	0.001
Constant	-2.291	0.318	52.08	1	<0.000
Variables not in equation	Score				
Sex	1.650			1	0.199
Species×Sex	0.209			1	0.648
Species×Year	0.240			1	0.625
Year×Sex	0.306			1	0.650
Species×Sex×Year	0.247			1	0.619

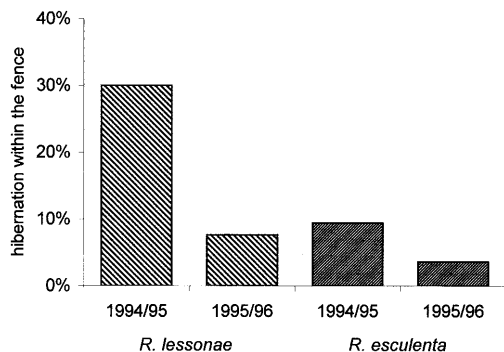


Fig. 3 Proportion of *Rana lessonae* ($n=60$) and *R. esculenta* ($n=136$) that hibernated within the fenced pond area

ing behavior in both winters (15 outside, 1 inside the fenced area), while 3 animals changed it (2 inside the fence in the first and outside in the second winter, and 1 vice versa).

A total of 9 *R. lessonae* and 27 *R. esculenta* were equipped with radio transmitters during the winters 1995/1996 and 1996/1997. After a few days, 3 animals had lost their transmitters and 1 transmitter had to be removed (see Methods). Before the end of November, 4 transmitters stopped working and 4 animals were probably eaten; we found both the transponders and the radio transmitters, the latter often damaged. By the end of January the sample size was further reduced by 5, because 3 more transmitters were lost, 1 stopped working, and 1 animal was eaten. This left us with 7 *R. lessonae* (4 females, 3 males) and 17 *R. esculenta* (10 females, 7 males) that could be radio-tracked until mid-December, while 5 *R. lessonae* (2 females, 3 males) and 14 *R. esculenta* (9 females, 5 males) remained at least until the end of January. The 24 animals followed until mid-December hibernated in the woodland and spent most of the time on land. They were found 3–7 cm below the surface in soil, under moss, fallen leaves or small branches. Two males (1 of each species) were mostly at the edge of the woodland but still under trees; 4 animals, 1 *R. lessonae* and 3 *R. esculenta* (2 females, 1 male), were in water for part of the time, due to changing water levels in a nearby ditch or to flooding of the area (Fig. 1).

Apart from water, the animals had three habitat types available for hibernation: grassland, edge and interior of the woodland. Minimum and maximum soil temperatures 5 cm below the surface differed significantly between these three habitat types in a multivariate analysis (Wilks' $F_{4,32}=3.259$, $P=0.024$), but in univariate tests only maximum temperatures differed ($F_{2,17}=5.732$, $P=0.013$; Fig. 4a). It was significantly higher in the grassland than at the edge and in the interior of the woodland ($P<0.05$ for both pairwise comparisons, Scheffé test). Actual temperatures, measured three times in the three habitat types and close to the radio-tracked animals, also differed between sites, and were related to time with lowest temperatures at date 2 (Table 3, Fig. 4b). The significant time×site effect requires more

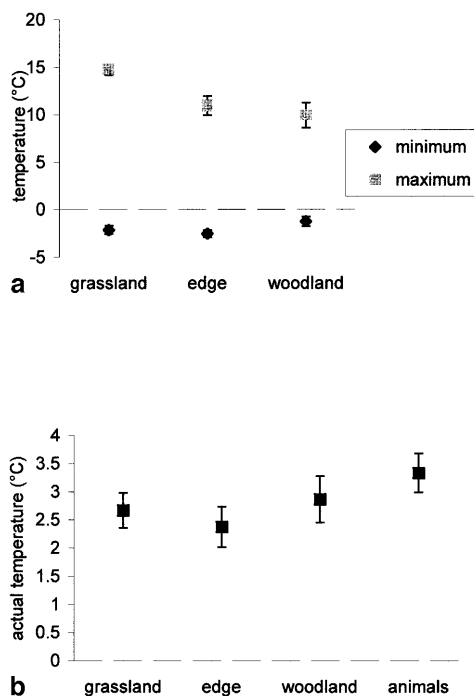


Fig. 4 Average **a** maximum and minimum soil temperatures and **b** actual soil temperatures, with SEs, during the winter of 1996/1997. All temperatures were measured 5 cm below the surface, **a** in three different habitats (*grassland*, *edge* of woodland, inside the *woodland*), and **b** additionally within a few centimeters of the sites where the radio-tracked animals actually hibernated (*animals*). **a** Minima and maxima were averaged over the whole winter, **b** actual temperatures were measured at three times

Table 3 Spatial and temporal effects on soil temperature. Results from an analysis of variance with soil temperature as the dependent variable and time (repeated measurements), and site (*grassland*, *edge*, and interior of woodland, as well as actual hibernation sites) as independent variables

Source	SS	df	MS	F	P
Between subjects					
Site	8.43	3	2.81	3.73	0.020
Error	25.55	34	0.75		
Within subjects					
Time	357.11	2	178.56	391.75	<0.000
Time×Site	9.33	6	1.56	3.41	0.005
Error	30.99	86	0.46		

investigation; it may simply result from the fact that gradual loss of radio-equipped animals changed the relative numbers and types of hibernating animals. For the site effect, pairwise comparisons revealed higher temperatures at sites close to the animals than at all other sites (all $P<0.003$; Scheffé tests), but also between the woodland and the edge of the woodland ($P=0.003$). Comparisons between the temperatures measured close to the animals [ANOVA with soil temperature as dependent variable, time (repeated measures), species and sex as independent variables] showed that there was a near-significant tendency for *R. lessonae* to hibernate at warmer

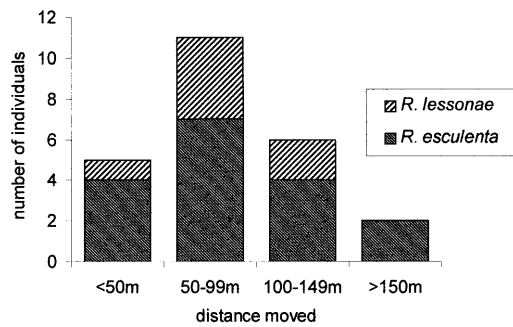


Fig. 5 Numbers of individuals and distances moved from the fenced pond to hibernation sites

sites than *R. esculenta* ($F_{1,7}=4.036$, $P=0.077$), while there were no differences between the sexes ($F_{1,7}=0.029$, $P=0.960$).

Distance to the hibernation sites and movements during winter

Most hibernation sites were close to the pond (Figs. 1, 5). The average distance was 98.5 m, the longest distance – moved by a *R. esculenta* male – was 470 m. The distance moved was not significantly related to species, sex, year or any of their interactions (all $P>0.3$; all $F_{1,17}<0.87$; ANOVA).

However, the animals not only moved from the breeding pond to a hibernation site, they also moved during winter and often more than once. In doing so, they covered distances ranging from a few meters to up to 100 m. The ratio of moving to total investigated frogs did not differ between species, sexes or years (all $P>0.12$; ANOVA with repeated measures), but – independent of these potential effects – it was higher during the first than during the second half of the winter (Table 4). From

1 November to 15 December, 22 of 24 animals changed hibernation sites (91.6%) whereas from 16 December to 31 January only 11 of 19 frogs did so (57.9%). This significant difference ($P<0.01$, $\chi^2=6.985$, $df=1$) is paralleled by corresponding differences in ambient temperatures. Pooled over both years, average temperatures were higher during the first than during the second period (2.49 versus -1.39°C). Fifteen animals changed hibernation sites during days with a mean air temperature below 4°C and three animals moved even during days with a mean temperature below 1°C .

Mass loss during winter

A total of 40 animals left the fenced area after 15 October and came back before 15 April in 1994/1995 and 1995/1996; 37 of them were *R. esculenta* (22 females, 15 males) and only 3 *R. lessonae* (1 female, 2 males). A backward stepwise analysis of variance was performed with mass loss per day (mg day^{-1}) as the dependent factor, species, sex, weight (measured in autumn), their interactions, and year as independent variables (Table 5). The sex of the animals, and their weight influenced their mass loss, and year had a near-significant effect. Females lost significantly more weight than males and heavier animals lost more than lighter ones. The mass loss was slightly larger in the first, warmer, winter than in the second.

Discussion

The timing of hibernation was clearly influenced by ambient temperature. Both species left the breeding pond earlier in 1995, with low September-December temperatures, than in 1994, when temperatures in these months were higher. Mean date of return to the breeding pond

Table 4 Number of animals that moved during the specified time period (**bold**) compared to the total number of animals that were radio-tracked in this period

	1 November–15 December		16 December–31 January	
	1995	1996	1995/1996	1996/1997
<i>R. lessonae</i>				
Females	3/3	1/1	1/2	1/1
Males	2/3		0/2	
<i>R. esculenta</i>				
Females	4/4	6/6	3/3	4/6
Males	3/4	3/3	2/2	0/3
Total	12/14	10/10	6/9	5/10

Table 5 Determinants of mass loss between 15 October and 15 April. Results from stepwise analysis of variance with mass loss (mg day^{-1}) as the dependent variable and species, sex, weight, their interactions, and year as independent variables ($R^2=0.331$)

Source	SS	df	MS	F	P
Sex	275.17	1	275.17	8.307	0.007
Weight	165.85	1	165.85	5.007	0.032
Year	128.74	1	128.74	3.886	0.056
Error	1192.53	36	33.13		

was earlier after a warm than after a cold winter, but only in *R. lessonae*. The most pronounced difference in hibernation behavior between the two species was in the length of hibernation time. In both years, *R. lessonae* left the fenced breeding pond earlier and came back later than *R. esculenta*. The reason for this difference in behavior is not clear. It is unlikely to be related to distance from hibernation sites, because these distances did not differ between the species, and both species were able to move even at very low temperatures, as shown by the radio-tracking data. The date of return to the breeding pond is probably influenced by sexual activity, which seems to start earlier in *R. esculenta* than in *R. lessonae* (Heym 1974). The fact that high spring temperatures advance arrival of the later-returning *R. lessonae*, but not of the early-returning *R. esculenta*, suggests that the latter may already be returning as early as possible. It should be remembered, however, that the R^2 values in Table 1 are very low. Hence, other factors than those measured are likely to determine the timing of hibernation and affect the immigration dates in spring.

There are several possible hibernation sites in our study area, including ponds and ditches that never froze throughout, and relatively undisturbed grassland and woodland. Of the 196 recorded individuals, 15% hibernated inside the fenced area; the others left it in autumn. This does not necessarily mean that the 15% hibernated under water, while the other 85% hibernated on land. There was terrestrial habitat inside the fenced area and aquatic habitat outside. Nevertheless, the radio-tracking data showed that the frogs that left the pond did, indeed, hibernate on land, with only a few being occasionally under water. Conversely, investigations of the bottoms of two ponds with a dip-net and the hand reader detected only one *R. esculenta* in each (A.-K. Holenweg, unpublished work). This indicates that most frogs remaining within the fenced area also may have hibernated on land. The suggestion is further supported by the finding that during the warmer winter of 1994/1995 more animals remained within the fenced area than in the colder winter of 1995/1996. If staying close to the pond were equivalent to aquatic hibernation, we would expect the reverse pattern because prolonged periods of frost may cause greater mortality in frogs overwintering terrestrially than aquatically.

On the other hand, in both winters more *R. esculenta* than *R. lessonae* hibernated within the fenced area, i.e., close to the pond. This is at least consistent with the idea that *R. esculenta*, which is more tolerant of oxygen deficiency, is better adapted to aquatic hibernation (Tunner and Nopp 1979; Lutschinger 1988), while *R. lessonae*, which is more tolerant of freezing, better fulfil the requirements for terrestrial hibernation (Berger 1982). However, hibernation both on land and in water has been described for both species (Berger 1970, 1982; Heym 1974; Lutschinger 1988; Sinsch 1991). At present it remains unclear whether physiological adaptations differ among animals from different geographic regions (Storey and Storey 1986; Costanzo et al. 1993) or wheth-

er individual frogs change their strategies depending on ambient temperature (Sinsch 1991).

The importance of ambient temperature for selecting hibernation sites is obvious from the temperature measurements in the terrestrial habitat. Temperatures were significantly higher close to the radio-tagged frogs than in randomly chosen places. This indicates the animals' ability to thermoregulate behaviorally by selecting warmer sites (Brattstrom 1979). A very surprising finding was that most animals changed hibernation sites during winter. Some of them were even active on days with a mean temperature below 1°C. Terrestrial movements at such ambient temperatures have been described for *Rana temporaria* in nature (e.g., Pasanen and Sorjonen 1994) and for *R. lessonae* hibernating in aquaria (Sinsch 1991). It is also known that aquatically hibernating ranid species are able to swim at low temperatures (Licht 1991; Stinner et al. 1994), but such activities have never previously been reported for naturally hibernating *R. lessonae* and *R. esculenta*. Both species interrupted their hibernation and changed sites, not only in late autumn but also in winter, although less often. Two *R. esculenta* even changed between aquatic and terrestrial hibernation. These results show two things: both species are apparently not torpid throughout the winter, and they are not restricted to one particular hibernation site. An obvious question is: why do they move around? Moving around costs energy and, consequently, it has to yield some benefit. Although food intake cannot totally be ruled out, it is unlikely that many invertebrates are available. Moreover, feeding seems to decrease freeze-tolerance for physiological reasons (Storey and Storey 1987). Perhaps the most likely explanation is that movements serve to escape from unfavorable conditions, such as changing water levels or sites with extremely low temperatures which can be survived only for a limited amount of time (cf. Layne and Kefauver 1997).

Out of the 36 frogs originally equipped with transmitters in autumn, we only radio-tracked 6 animals until the end of March of the following year. Some radios failed, some animals lost them, some animals were eaten, and some died. It certainly needs more investigation, i.e., more equipped animals and more than two winters, before survival rates can be related to thermal quality of hibernation sites. The transpondered frogs captured at the fence in autumn (after 15 October) and recaptured the next spring (before 15 April) provide data on weight loss during winter, but this is not linked to the hibernation sites. According to these data, females lost more weight than males. This is paralleled by lower female than male annual survival rates in both species (Sjögren 1988, 1991). The explanation for higher weight loss or mortality in females probably lies in egg production, which occurs during fall and winter and seems to be quite costly (Hasumi 1996; Loumbourdis and Kyriakopoulousklavounou 1996). The animals lost more weight in the warmer first winter than the second. Since in ectotherms metabolic rate (and hence energy expenditure) is

a function of ambient temperature, this is to be expected. However, the low R^2 values in Table 5 suggest that factors other than the independent variables used in the statistical model must also be important for mass loss. One such factor could be the thermal quality of the hibernation site.

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