Frequencies of the cold-induced +H and −H on the long arm of the 5th chromosomes in *Vicia faba* karyotype ACB

	Cells observed	% of 5th chromosomes showing			
		+H and −H	Only +H	Only −H	Neither +H nor −H
Late prophase	22	52	25	9	14
Metaphase	40	21	22	34	22

Late prophases and metaphases in which 12 chromosomes of the complement were interdistinguishable and well spread were chosen and photographed. Count and judgement of +H and of −H were made on the positive prints with magnification × 2600.

Results and discussion. The cold treatment revealed that the 5th chromosome possessed one +H and one −H on its long arm (Figure). The frequencies of the +H and those of the −H at late prophase and at metaphase are summarized in the Table. At late prophase, the +H and the −H appeared with similar frequencies (+H, 77%; −H, 61%). The chromosomes showing neither +H nor −H increased in number at metaphase more than at late prophase. This implies that both +H and −H become less recognizable when the cell cycle approaches metaphase. From late prophase to metaphase, the +H frequencies decreased more rapidly than the −H frequencies (+H, from 77% to 43%; −H, from 61% to 55%). This explains, at least in part, why many researchers who studied cold-treated *Vicia faba* have described only −H. Through chromosome condensation from late prophase to metaphase, the relative position of the +H and that of the −H along the chromosome did not change as shown in the Figure. If the tightly-coiled +H occurs at the expense of loosely-coiled −H, the chromosomes showing only +H are not to be observed. This, however, was not the case, as shown in the Table. The occurrence of the chromosomes showing only +H supports the view that the occurrences of +H and −H are mutually independent matters, but not that the occurrence of +H is −H occurrence-dependent as OCKEY suggested.

In *Vicia faba* the chromocenters, i.e., heterochromatin regions at interphase, decondense transiently to a euchromatic state twice during mitotic prophase[13]. The H disappears first at the beginning of prophase ('Zerstäubungsstadium'[13]), and reappears at the stage subsequent to the 'Zerstäubungsstadium'. As prophase proceeds, chromosomes undergoing spiralization become recognizable separately as elongated threads (spiral-prophase[13]). At an early stage of this spiral-prophase, the distinction between H and E becomes impossible again; i.e., H disintegrates to E. In this experiment, the prophases which corresponded to the early stage of spiral-prophase did not show any H differentiation. As chromosome condensation proceeds, H reappears (late prophase in this paper). It was known that without the cold treatment both +H and −H became apparent at this late prophase[10, 14]. Afterwards, in the case of non-cold-treatment, this H differentiation vanishes up to metaphase. It is considered that cold treatment disturbs the vanishing of the H differentiation from late prophase on and eventually brings about the +H and −H revelation on metaphase chromosomes.

The cold-induced −H has been shown in *Trillium*[4, 15] and in *Vicia faba*[16] to be a differentially darker stained segment by Giemsa banding methods. The present results showed that +H and −H were independent phenomena. In Chinese hamster, the darker Giemsa bands were the tightly-coiled, i.e., +H chromosome segments[17]. In *Vicia faba*, too, some of the Giemsa-positive segments revealed by Giemsa banding methods[12, 15, 16, 18, 19] may correspond to cold-induced +H segments. This, however, is at present an open question.

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Genetic Control of LDH Isozymes in the *Rana esculenta* Complex

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Summary. Studies of LDH isozymes in the European green frogs showed that the synthesis of the B subunits is controlled by 3 alleles at a single genetic locus. The genetic evidence supports the hypothesis that *Rana esculenta* is the hybrid of *R. lessonae* × *R. ridibunda*.

Hybridization experiments and biometric studies of the 3 types of European green frogs indicate that *Rana lessonae* and *Rana ridibunda* are 2 distinct species, while *Rana esculenta* represents their hybrid[2–6]. Cytological analysis of the karyotypes[7], as well as electrophoretic examination of the serum proteins[8–11], support this conclusion. In order to obtain further information about the taxonomic relationships of these 3 frog types, we carried out a detailed study of the genetic control of the lactate dehydrogenase (LDH) isozyme. The present paper is a brief report of this study. The detailed results will be published later.

Materials and methods. Adult frogs were collected from the vicinity of Zürich and kept in running water in the laboratory. The different crosses, which were partly made by artificial fertilization, are summarized in Table I.

Table I. Parental phenotypes of adult female and male frogs used in different crosses and the resulting F_1 phenotypes

Parental phenotypes			No. of crosses	F_1 phenotypes
Female		Male		
les[a]	×	*les*	16	*les*
les	×	*esc*	3	*esc*
les	×	*rid*	5	*esc*
esc	×	*les*	13	*esc*
esc	×	*esc*	10	(*rid*)[b]
esc	×	*rid*	5	*rid*
rid	×	*rid*	1	*rid*

[a] Abbreviations refer to the first 3 letters of the corresponding frog types. [b] Offspring from a single cross. Larvae from the other 9 crosses were all lethal.

Embryos and larvae from each cross were reared at room temperature (about 22–24 °C) and fed powdered nettle leaves. On the basis of both the external features and the serum albumin patterns of the metamorphosed individuals, the morphological phenotypes of the F_1 generation were identified[11].

For analysis of the LDH isozyme patterns in the F_1 offspring, either a whole larva aged about 4 weeks, or a tail piece of such a larva, was homogenized in a buffered saline solution. Following centrifugation an aliquot of the supernatant containing 20–30 µg protein was used for polyacrylamide gel electrophoresis, employing a 2.5% sample gel and a 5.5% separation gel (MAURER[12]). The isozyme bands were stained by a modified method given by DIETZ and LUBRANO[13]. For comparison various tissues (liver, skin, heart, ovary, testis) of the adult female and male frog used in each cross were dissected out and their patterns of LDH isozymes were analyzed in the same way.

Results and discussion. As can be seen in Table I, all hybrids of the crosses *lessonae* ♀ × *ridibunda* ♂, *lessonae* ♀ × *esculenta* ♂ and *esculenta* ♀ × *lessonae* ♂ were of the *esculenta* phenotype. Out of a total of 10 *esculenta* ♀ × *esculenta* ♂ crosses, only in one cross did the larvae complete metamorphosis; the progeny of this cross were identified as *ridibunda*. Larvae of the remaining 9 crosses all stopped development 1 week after hatching and died 1 month later. These results are in agreement with those reported previously by BERGER[5].

We found 3 LDH patterns (I, II, III) in *lessonae*, 3 (III, IV, V) in *ridibunda* and 4 (II, III, IV, VI) in *esculenta* (Figure 1). The zymograms I in *lessonae* and III in *lessonae* and *esculenta* each have 5 isozymes. The zymograms III and V in *ridibunda* showed only 4 isozymes, although 5 bands could have been expected. The missing band may either be not formed, or be formed but have no enzyme activity. In any case, from the spacing of the enzyme bands in each zymogram, it can be concluded that LDH isozymes in the green frogs, as in mammals and other vertebrates[14,15], are tetramers formed by random assembly of the A and B subunits. Since in all these patterns the least anodal band has the same mobility, it may further be concluded that the genetic locus coding for the A subunit is the same in the 3 types of green frogs. On the other hand, the most anodal bands and the intermediate isozymes differ in their electrophoretic mobilities, suggesting that there are 3 allelic genes controlling the synthesis of 3 different kinds of B subunits. Following the nomenclature of WRIGHT and MOYER[16,17], these allelic genes will be designated LDB^a, LDB^b and LDB^c. The corresponding subunits coded by them will be called B^a, B^b and B^c in the order of increasing negative charge. The allele LDB^a is characteristic for *lessonae*, LDB^b for *ridibunda*, while LDB^c is present in both species. Thus, animals which show the pattern I, V or

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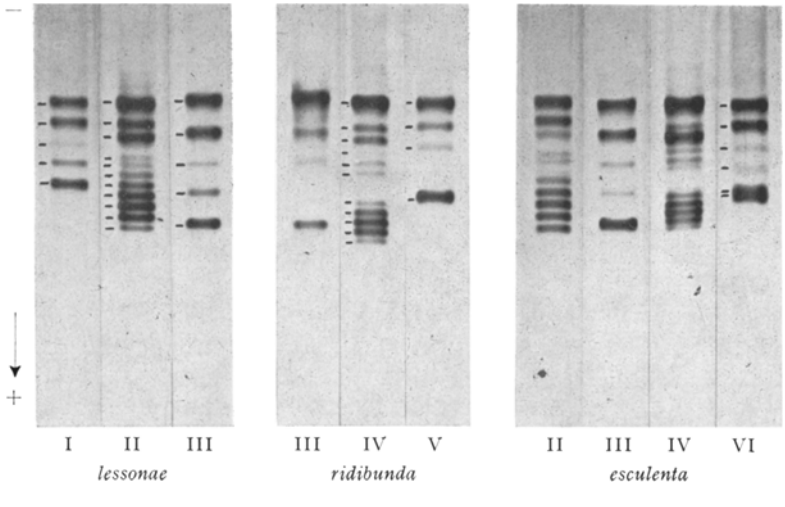

Fig. 1. Zymograms of LDH isozymes in the 3 types of green frogs *Rana lessonae*, *R. ridibunda* and *R. esculenta*.

Table II. Morphological phenotypes, the genotypes for the B subunits and the LDH patterns of the parental female and male frogs, and the corresponding data for the F_1 *esculenta* generation

Female			×	Male		No. of crosses	F_1 Offspring		
Phenotype	Genotype B subunit	LDH pattern	Phenotype	Genotype B subunit	LDH pattern		Phenotype	Genotype B subunit	LDH pattern
les	LDB^a/LDB^a	I	*rid*	LDB^c/LDB^c	III	1	*esc*	LDB^a/LDB^c	II
les	LDB^a/LDB^c	II	*rid*	LDB^c/LDB^c	III	1	*esc*	LDB^a/LDB^c	II
								LDB^c/LDB^c	III
les	LDB^a/LDB^c	II	*rid*	LDB^b/LDB^c	IV	3	*esc*	LDB^a/LDB^c	II
								LDB^c/LDB^c	III
								LDB^b/LDB^c	IV
								LDB^a/LDB^b	VI
les	LDB^a/LDB^a	I	*esc*	LDB^b/LDB^c	IV	1	*esc*	LDB^a/LDB^b	VI
les	LDB^a/LDB^c	II	*esc*	LDB^a/LDB^b	VI	1	*esc*	LDB^b/LDB^c	IV
								LDB^a/LDB^b	VI
les	LDB^a/LDB^c	II	*esc*	LDB^b/LDB^c	IV	1	*esc*	LDB^b/LDB^c	IV
								LDB^a/LDB^b	VI
esc	LDB^a/LDB^b	VI	*les*	LDB^a/LDB^a	I	6	*esc*	LDB^a/LDB^b	VI
esc	LDB^a/LDB^b	VI	*les*	LDB^a/LDB^c	II	2	*esc*	LDB^b/LDB^c	IV
								LDB^a/LDB^b	VI
esc	LDB^a/LDB^c	II	*les*	LDB^a/LDB^a	I	1	*esc*	LDB^a/LDB^c	II
esc	LDB^b/LDB^c	IV	*les*	LDB^a/LDB^a	I	3	*esc*	LDB^a/LDB^b	VI
esc	LDB^b/LDB^c	IV	*les*	LDB^c/LDB^c	III	1	*esc*	LDB^b/LDB^c	IV

III have the homozygous combination LDB^a/LDB^a, LDB^b/LDB^b or LDB^c/LDB^c respectively in regard to the B subunit (Figure 2).

According to the subunit theory, the A and B subunits are under the control of separate loci. Consequently frogs of the heterozygous combination LDB^a/LDB^c,LDB^b/LDB^c or LDB^a/LDB^b produce a total of 3 different subunits (one A subunit + 2 B subunits), and zymograms exhibiting 15 LDH isozymes would be expected. We found that pattern II in *lessonae* and *esculenta* and pattern IV in *ridibunda* and *esculenta* each have 11 isozymes (Figure 1). Several possibilities may account for the 4 missing bands. As mentioned above, these heteropolymers are either not formed at all, or are formed but enzymatically inactive. In addition, some isozyme bands may overlap and thus be indistinguishable, as demonstrated in Figure 2 for the bands 4, 2 and 5 in zymograms II, III and IV respectively. We do not yet have sufficient information to decide between these possibilities. Since the allele LDB^a is characteristic of *lessonae* and LDB^b of *ridibunda*, the heterozygous combination LDB^a/LDB^b should occur only in *esculenta* assuming the hybrid hypothesis is true. Our data indicate that this is indeed the case.

Frogs of the F_1 generation with the phenotype *esculenta* were obtained from a total of 21 crosses, namely 5 *lessonae* ♀ × *ridibunda* ♂, 3 *lessonae* ♀ × *esculenta* ♂ and 13

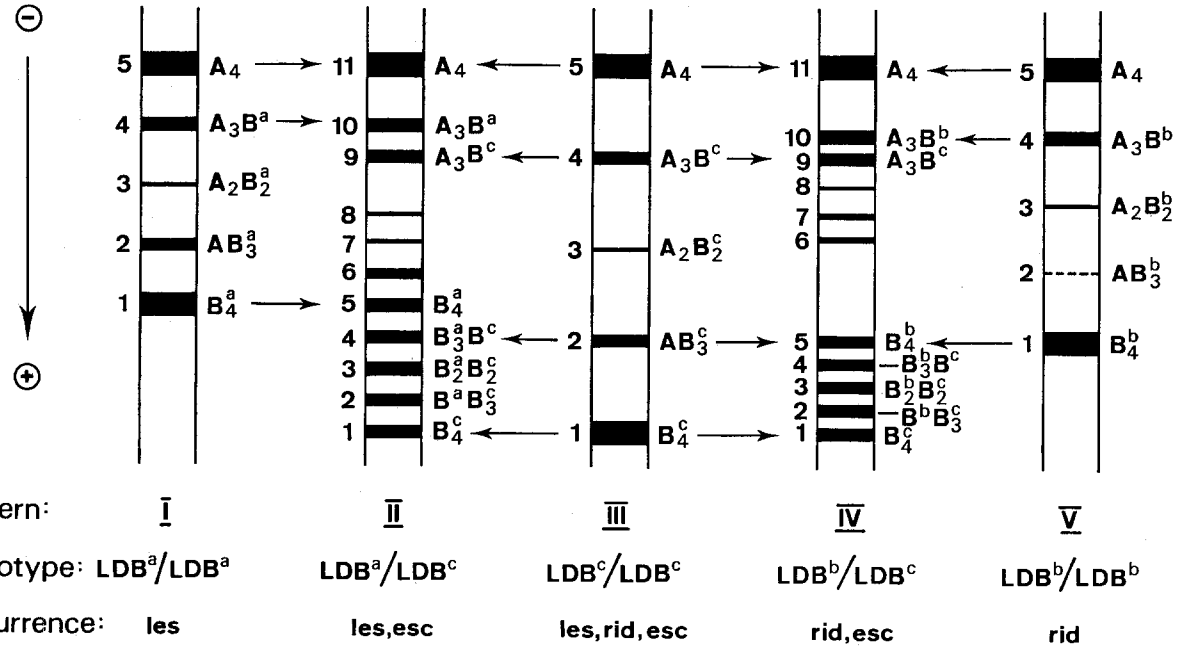

Fig. 2. Diagrams to explain the LDH isozyme patterns shown in Figure 1, on the basis of the genotypes at the locus specifying the B subunits. The reason for excluding pattern VI in this figure is given in the text.

esculenta ♀×*lessonae* ♂. Table II summarizes the morphological phenotypes, the genotypes for the B subunit, and the isozyme patterns of the parental female and male frogs, and those of the F_1 offspring in each of these crosses. The 5 *lessonae* ♀×*ridibunda* ♂ crosses provide the most clear-cut evidence for the hybrid nature of *esculenta*, since the experimental data agree perfectly with the prediction. However, a closer examination of the results of those crosses involving *esculenta* females or males showed that not all genotypes and isozyme patterns which were expected on the basis that *esculenta* consist of both *lessonae* and *ridibunda* genomes were observed. This can be explained by our finding that, in contrast to *lessonae* and *ridibunda*, eggs of the LDB^a/LDB^c *esculenta* female with pattern II showed only isozymes of pattern III, suggesting the presence of only the B^c subunit in the egg cytoplasm. Similarly, eggs of those LDB^a/LDB^b *esculenta* females with pattern VI showed only pattern V, indicating the occurrence only of the B^b subunit. The consistent absence of the B^a subunit, which originates from *lessonae*, demonstrates clearly that in the *esculenta* eggs only the *ridibunda* genome is retained. Analyses of the LDH isozymes in ovaries and testes suggest that the same event takes place during both oogenesis and spermatogenesis. In other words, in the female and male germ cells of *esculenta*, the genetic information from *lessonae* is always eliminated. With such a hypothesis, all observed isozyme patterns of the F_1 *esculenta* individuals can be explained.

It should be pointed out that the isozyme pattern VI in *esculenta* with the genotype LDB^a/LDB^b seems to be an exception, since inspite of the presence of 3 kinds of subunits (A, B^a, B^b) the zymograms showed only 5 bands (Figure 1). One line of evidence for the heterozygous condition of the B subunit in these frogs is that their most anodal isozyme occurs as 2 closely located subbands with an intermediate mobility between the most anodal band of pattern I (LDB^a/LDB^a) in *lessonae* and that of pattern V (LDB^b/LDB^b) in *ridibunda*. Owing to the small charge difference between the B^a and B^b subunits, it may be that the 15 isozyme bands expected were not resolved by our electrophoretic procedure. More extensive evidence is needed to clarify this point.

As to the morphogenetic changes of the LDH isozymes, our results can be summarized as follows: When eggs with the homozygous pattern I, III or V developed into larvae with the heterozygous pattern II or IV, the isozyme bands of such a heterozygous pattern became first detectable in embryos showing heart beat and at hatching (Shumway stage 19–20). This means that the paternal gene is activated only about 4–5 days after the beginning of development. Alternatively, when eggs showing heterozygous patterns developed into larvae with homozygous patterns, the maternal LDH isozymes persisted until about 14–20 days after hatching. This could be due either to a low turnover of the enzyme protein, or to the occurrence of stable mRNA. The same results have been reported by WRIGHT and MOYER[16,17] from their studies of parental influences on the LDH isozymes in various hybrid frogs.

Chromosomal Variation and Heterochromatin Polymorphisms in *Peromyscus maniculatus*

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Summary. Evidence is presented that chromosomal variation in *Peromyscus* results from 1. addition of heterochromatic short arms to acrocentric chromosomes, and 2. pericentric inversions. Constitutive heterochromatin polymorphisms contribute to variation in the amount of heterochromatin in *Peromyscus* populations.

Chromosome studies on *Peromyscus* have shown that although the diploid chromosome number is always 48, the total number of chromosome arms, or fundamental number (FN)[4], may vary greatly both between and within different subspecies populations[5–8]. Variation in the FN in *Peromyscus* originally was attributed to pericentric inversions[5,6]. However, based upon recent studies HSU has proposed that variation in the FN in *Peromyscus* results from the addition of heterochromatic short arms to acrocentric chromosomes[7,9]. The present study used both Giemsa banding and heterochromatin staining techniques to determine the basis for variation in both the FN and the amount of heterochromatin within a population of *Peromyscus maniculatus*.

Materials and methods. All *Peromyscus maniculatus* ssp. *nebrascensis* were trapped within 12 miles of Laramie, Wyoming. Animals were colchicinized 2 h prior to sacrifice. Bone marrow was flushed from the femur and tibia, incubated 20 min in 0.075 *M* KCl at 37 °C, and fixed 45 min in 3:1 methanol-glacial acetic acid. Cells were placed onto cold, wet slides and air dried. Chromosomes were stained with a urea Giemsa banding technique[10], photographed and destained. Constitutive heterochromatin was identified in the same cells from 10 individuals by the C-banding technique[11]. Homologous chromosomes were identified by the Giemsa banding pattern of their long arms. Chromosomes were classified according to the Giemsa banding pattern described for *P. m. nebrascensis*[12].

Results and discussion. The diploid chromosome number in *Peromyscus maniculatus nebrascensis* was 48 and the FN varied from 86 to 89 in 10 individuals studied

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