TECHNICAL NOTE

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Enzyme Phenotyping of Alaskan Bears for Wildlife Law Enforcement

REFERENCE: Wolfe, J. R., **"Enzyme Phenotyping of Alaskan Bears for Wildlife Law Enforcement,"** *Journal of Forensic Sciences*, JFSCA, Vol. 29, No. 3, July 1984, pp. 910-914.

ABSTRACT: Two enzymes in Alaska brown and black bears were examined using cellulose acetate electrophoresis. Both species exhibited a two-allele polymorphism at the lactate dehydrogenase (LDH)-2 (M) locus (three phenotypes) with brown bear also showing a three-allele polymorphism at the 6-phosphogluconate dehydrogenase (6PGD) locus (six phenotypes). Allele frequencies for the LDH-2 locus were 0.65 and 0.35 for black bears, and 0.96 and 0.04 for brown bears. 6PGD allele frequencies for brown bears were 0.19, 0.72, and 0.09. Phenotyping bear meat and blood can be of great value in investigating poaching cases when used for the comparison of meat or blood samples.

KEYWORDS: pathology and biology, bears, electrophoresis, enzymes, isozymes, black bear, brown bear, phenotyping, *Ursus arctos, Ursus americanus*

Enzyme phenotyping of big game meat and blood for law enforcement purposes is not a common practice in crime laboratories that analyze wildlife evidence [*1,2*]. Screenings for polymorphic enzymes in Scandinavian moose (*Alces alces*), polar bears (*Ursus maritimus*), black bears (*Ursus americanus*), Yellowstone elk (*Cervus canadensis*), white-tailed deer (*Odocoileus virginianus*), and large grazing mammals in general have been reported [*3-8*], but the majority of these studies have not been detailed enough to be applied directly to forensic science analysis. Recent studies addressing the forensic science aspects of big game meat and blood analysis have centered about differentiating closely related species [*9,10*] and have not investigated differences within species which could be used for individualization.

This paper describes phenotypic variation found in lactate dehydrogenase (LDH; EC 1.1.1.27) from Alaskan brown bears (*Ursus arctos*) and black bears and variation found in 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) from brown bears by means of electrophoresis on cellulose acetate membranes. Proteins from meat (6PGD) and meat, blood, and serum (LDH) were examined.

Received for publication 29 June 1983; revised manuscript received 1 Sept. 1983; accepted for publication 13 Sept. 1983.

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Materials and Methods

Muscle samples were collected from bear skulls submitted by hunters, or by Fish and Wildlife Protection officers examining bear kills in the field. Blood and serum samples were collected by Alaska Department of Fish and Game biologists from chemically immobilized bears, using both ethylenediaminetetraacetate (EDTA) treated and untreated vacutainers. These samples were collected from bears throughout Alaska. All samples were stored at −20°C for up to two years.

Meat extracts were made by homogenizing the sample with an equal volume of water, then centrifuging for 3 min at 12 000 × *g*. The supernatant was stored at −20°C. For LDH electrophoresis, meat extracts were diluted 1:10 with water and the serum and freeze/thaw hemolyzed blood samples were diluted 1:1. All samples were run undiluted for 6PGD electrophoresis.

Electrophoresis was performed on cellulose acetate membranes (Sartorius 11200B, 12200BB or Beckman "Microzone Plus"), using Grunbaum's [*11*] basic techniques on Sartorius Sartophor or Beckman Microzone equipment (use of a manufacturer's name does not imply endorsement). The microzone tanks were adapted to accomodate an eight-sample applicator [*12*].

For LDH analysis a 0.06*M* barbital, 0.005*M* calcium lactate, pH 8.8 stock buffer was used diluted 1:4 for the tank reservoirs and membrane soak. The reaction mixture consisted of 5-mg nicotinamide adenine dinucleotide (NAD), 0.6 mL of phenazine methosulfate (PMS) (5 mg/mL), 0.6 mL of 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT) (5 mg/mL), 75-μL DL-sodium lactate (60% syrup), in 15 mL of a 0.1 *M* Tris, pH 8.0 buffer, which was added to 0.25-g Noble agar liquified with 10 mL of the same buffer. Samples were applied at Position 4 and run at 350-V for 21 min.

For 6PGD analysis, Grunbaum's [*11*] procedure was adapted by using a 1:7 dilution of a stock 0.9*M* Tris, 0.5*M* boric acid, 0.07*M* Na_2EDTA pH 8.7 buffer for the tank reservoir and a 1:20 dilution for the membrane soak. The reaction mixture consisted of 2.46-mL 0.02*M* magnesium chloride ($MgCl_2$), 0.4-mL MTT (5 mg/mL), 1.0-mL nicotinamide adenine dinucleotide phosphate (NADP) (5 mg/mL), 0.4 mL of PMS (5 mg/mL), and 7 mg of 6-phosphogluconic acid dissolved in 15 mL of a 0.1*M* Tris, pH 8.0 buffer, which was added to 0.25-g Noble agar liquified with 10 mL of the same buffer. The samples were applied at Position 2 and electrophoresed at 350 V for 35 min.

Results and Summary

LDH isozyme variation was found in both brown and black bears (Fig. 1). This enzyme exhibited three phenotypes consistent with a two-allele polymorphism at the LDH-2 locus. LDH is a tetrameric enzyme composed of randomly associating peptides produced from two loci, LDH-1 (H) and LDH-2 (M). In both brown and black bears, LDH-1 is expressed more in the blood and LDH-2 is expressed more in the meat. A third locus, LDH-3 is expressed only in post-pubertal testis and sperm [*13*]. This tetrameric structure results in a 5-banded homozygous phenotype, and a 15-banded heterozygous phenotype. The H_4 isozyme migrates the most cathodal and the M_4 isozyme migrates the most anodal. Brown and black bear isoenzymes appeared to have identical mobilities for all three phenotypes. The variant allele occurs at a much higher frequency in black bear than in brown bear (Table 1). Phenotyping blood, serum, and dried blood depends on observing a two-banded pattern of the H_3M isozymes because these samples contain very low levels of activity of the H_2M_2, HM_3, and M_4 isozymes. LDH activity was detected in meat and blood samples stored at −20°C for over two years.

6PGD isozyme variation was found only in brown bears. This enzyme exhibited six phenotypes (Fig. 2) that are consistent with a three-allele polymorphism at the single 6PGD locus

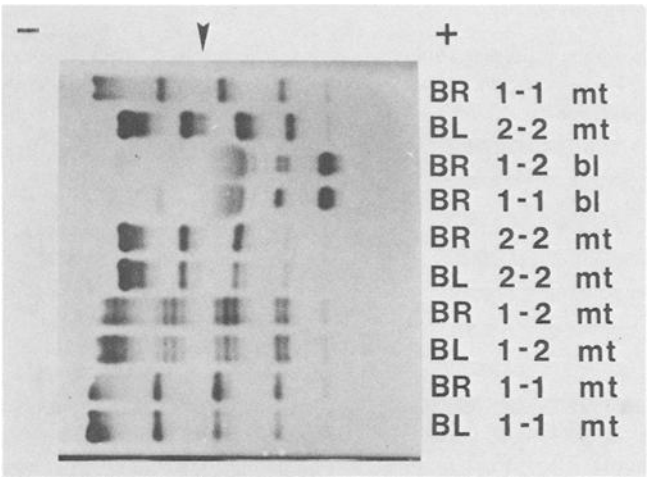

FIG. 1—*Cellulose acetate electrophoretogram of six brown bear* (BR) *and four black bear* (BL) *samples stained for LDH. Both meat* (mt) *and blood* (bl) *samples were analyzed. LDH phenotypes are listed on the right and the application point is indicated by the arrow.*

TABLE 1—*Phenotype frequencies (percent), number of individuals analyzed* (N), *and gene frequencies for LDH and 6PGD in Alaskan brown and black bears.*

		LDH							
	N	1-1	1-2	2-2				LDH^1	LDH^2
Brown bear	285	92.6	7.0	0.4				0.96	0.04
Brown bear	176	42.1	46.0	11.9				0.65	0.35
		6PGD							
	N	1-1	1-2	1-3	2-2	2-3	3-3	$6PGD^1$	$6PGD^2$	$6PGD^3$
Brown bear	144	4.1	24.9	4.1	53.8	11.7	1.4	0.19	0.72	0.09

[*13*]. The observed phenotype and gene frequencies are shown in Table 1. The monomorphic black bear phenotype coincides with the 2-2 brown bear phenotype. 6PGD activity appeared greatest in meat, and tended to disappear within two months of storage at −20°C. 6PGD activity in blood was relatively weak. When stored for more than a month at −20°C, the blood formed degradation products which interfered with interpretation, a problem that was not improved by the addition of Cleland's reagent. Because of this, results from only the meat samples are listed.

Two factors support the assumption that this observed variation in bear LDH and 6PGD is genetic and not a result of post-translational changes. The variant isozyme patterns are those expected for both the tetramer LDH and the dimer 6PGD, and the observed phenotype frequencies agree with the expected phenotype frequencies when calculated assuming the alleles are at Hardy-Weinberg equilibrium (for brown bear LDH $x^2 = 0.20$, $P = 0.67$, degrees of freedom (df) = 1; black bear LDH $x^2 = 0.03$, $P = 0.89$, df = 1; and for brown bear 6PGD, $x^2 = 1.31$, $P = 0.73$, df = 3, phenotypes with frequencies < 10 were combined).

To date, LDH and 6PGD phenotyping has been used in both brown and black bear poaching cases where bear hides in the hunter's possession were compared to bear carcasses found

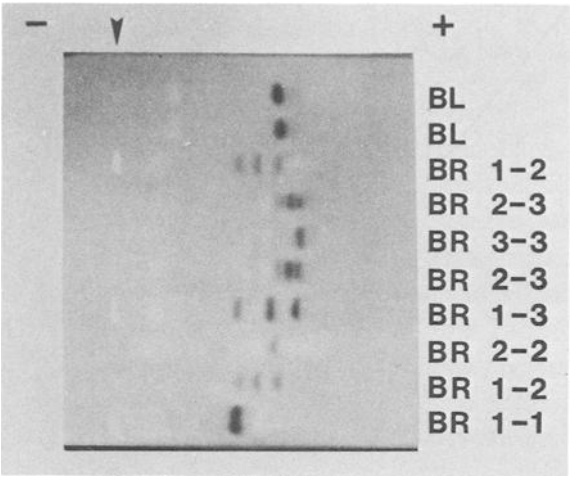

FIG. 2—*Cellulose acetate electrophoretogram showing eight brown bear* (BR) *and two black bear* (BL) *meat extracts stained for 6PGD. Phenotypes are listed at the right, with the arrow marking the application point.*

in the field. One measure of the usefulness of a set of polymorphic enzymes in individualization is the discrimination probability (probability of two randomly chosen individuals having different phenotypes) [*11*]. For Alaskan brown bears the discrimination probability is 0.68, and for Alaskan black bears the discrimination probability is 0.60. Although these probabilities are relatively low, important circumstantial evidence can still be obtained especially if the bears in question exhibit rare enzyme phenotypes.

For this technique to be applied to bear populations other than those examined here, additional studies to investigate the types of enzyme variation present in local populations are needed. The need for this is evident as LDH-2 polymorphism has been reported in Tennessee black bear [*5*] with a variant gene frequency of 0.18, which is much less than the Alaska variant gene frequency of 0.35 found in this study. Continued screening of bears in Alaska is also needed to determine how gene frequencies vary within the state.

Acknowledgments

The author thanks the many Alaska Department of Fish and Game biologists and Alaska Division of Fish and Wildlife Protection Officers who assisted in gathering meat and blood samples.

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