AN ABSTRACT OF THE THESIS OF

PAUL B. SAMOLLOW for the degree of Doctor of Philosophy

in ZOOLOGY presented on August 30, 1978

Title: DYNAMICS OF ENZYME POLYMORPHISM IN A NATURAL POPULATION OF THE BOREAL TOAD, *BUFO BOREAS BOREAS* BAIRD AND GIRARD: EVIDENCE OF NATURAL SELECTION VIA DIFFERENTIAL MORTALITY IN EARLY LIFE HISTORY STAGES

Abstract approved: Redacted for privacy

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The genetic effects of heavy mortality during early life history stages in a natural population of *Bufo boreas boreas* were investigated by examining variation at nine polymorphic enzyme coding loci. All reproduction in this population takes place during a single breeding episode each year, hence, all young are of the same age; i.e., they constitute a single cohort. Gene and genotype frequencies were determined in twenty-seven samples of pre-reproductive *Bufo* from three cohorts, over a three year period; 1973, 1974 and 1975. Frequencies in two samples of adult *Bufo*, collected in 1973 and 1975, were also determined.

There was no evidence of change among adult gene frequency distributions during the period examined. Allele and genotype frequencies among young *Bufo* were significantly different than those in the adult population. Within cohorts, distinct shifts in allele and

genotype frequency distributions were evident at six of the nine loci. Five loci showed temporal heterogeneity among genetic proportions in at least two of the three cohorts studied.

After consideration of the possible influences of other evolutionary forces, it was concluded that natural selection was the primary cause of the observed inter-generation and intra-cohort genetic heterogeneities. The inter-generation differences in genetic distributions may have resulted from selection at the level of the adult (late) fitness components and/or early zygotic survival. The data appear to support the former alternative.

Intra-cohort (temporal) heterogeneity among genetic proportions resulted from selection via the differential survival of genotypes. The effects of differences in developmental rates were examined and found insufficient to account for the observed temporal heterogeneity. Dispersal was, in general, random with respect to genotype and not a significant contributor to temporal genetic change in the cohorts examined. Evidence of heterozygote superiority in survivorship was found at only one of the nine loci. Heterozygote proportions observed at this locus showed a convincing tendency to increase over time relative to their expected proportions. A preliminary investigation of genetic differentiation among microgeographically distinct subpopulations of a single cohort revealed significant divergence at half of the eight loci examined in two independent comparisons.

It was concluded that the heavy, early life history stage mortality, characteristic of this (and other) *Bufo boreas* populations, is not

genetically random. In addition, there appear to be differences among genotypes with regard to the late fitness components.

The data indicate that genotypic differences in survivorship (and late fitness) vary in time and in space, presumably in response to changing environmental conditions. These findings are discussed in the context of current theory regarding the maintenance of genetic polymorphism. In particular, the implications of variation in relative fitness differences and rankings among genotypes are examined with regard to multiple-niche polymorphism and the "evolutionary significance" of enzyme variation.

An extensive listing of electrophoretic techniques useful with Bufo boreas and data regarding genetic (Mendelian) analysis of electrophoretic phenotypes in this species are included as appendices.

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ACKNOWLEDGEMENT

I first wish to thank my wife, Catherine M. Samollow, for her dauntless support and cheerful assistance during virtually every aspect of this project. Throughout long days and nights in the field and laboratory, and countless hours at the typewriter, her help and encouragement never ceased.

Special thanks are due my major professor, Peter S. Dawson, for his interest and enthusiastic support of my research. Pete's willingness to discuss my project or any aspect of population biology, has greatly influenced, but never stifled, my academic development in general and the execution of my research interests in particular.

I also thank those persons from whom I and the project have benefitted during many enlightening discussions concerning population phenonena. Especially, I thank Charles L. Davis, C. Eric Orr, Russel A. Riddle and John A. Wiens.

I am indebted to Suh Y. Yang for basic instruction in electrophoretic techniques and to the U.S. Forest Service; Willamette National Forest, for its cooperation during the field phase of this research. Financial support for this work came, in part, from a Sigma Xi grant-in-aid and grants from the Oregon State University and Humboldt State University computer centers.

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DYNAMICS OF ENZYME POLYMORPHISM IN A NATURAL POPULATION OF THE BOREAL TOAD, *BUFO BOREAS BOREAS* BAIRD AND GIRARD: EVIDENCE OF NATURAL SELECTION VIA DIFFERENTIAL MORTALITY IN EARLY LIFE HISTORY STAGES

INTRODUCTION

Examination of the extent and impact of natural selection in nature is often hampered by our inability to clearly delineate the various components of Darwinian fitness. Modern population genetic theory and empirical findings too numerous to cite (but see Lewontin, 1974; Ford, 1975; Dobzhansky et al., 1977 for reviews) emphasize the importance of differences in fertility, fecundity, developmental rate and mating success as major determinants of differential reproductive success. The naive view that fitness is primarily determined through differences in survivorship has long been discarded. Yet, it is obvious that relative rates of genic reproduction can be strongly influenced by differential survival among alternative genotypes.

In many organisms a substantial fraction of deaths probably do occur at random with regard to the genetic makeup of individuals, but the contention that probability of death via predation, disease, climatic conditions, starvation or a host of other factors should be equal for all genetic combinations seems untenable. Particular genotypes may be more or less likely than others to suffer elimination due to mortality. Information regarding such genetic differences in survivorship could be important in furthering our understanding of evolutionary and ecological processes. Consequently, there is compelling reason to ask how the genetic effects of mortality might be monitored in natural populations.

Field observation of differential mortality rates based on morphological or behavioral variation is of little value in understanding the genetic ramifications of mortality since the genetic bases of such characters are usually unknown. Even if the genetic nature of certain complex visible characters were well understood, our ability to quantify the genetic correlates of mortality would be little enhanced unless the characters had very high heritabilities. We are forced into the position of necessarily investigating genetically simple, well defined phenotypic differences. Genetic differences to be considered in this context should fulfill certain criteria. These criteria were nicely outlined by Lewontin (1974, p 96) and include (1) unambiguous detection of single allelic substitutions in individuals, (2) distinction of substitutions at one locus from those at another, and (3) the discrimination of allelic substitutions regardless of the magnitude of physiological effect.

Few, if any, morphologically distinguishable variants satisfy these criteria. Polygenic characters are certainly unsatisfactory. Single and multiple major gene variants in natural populations are legion (Ford, 1975) but certainly not ubiquitous. Furthermore, with a few exceptions the confounding nature of dominance prevents unambiguous classification of genotypes directly from the phenotypic condition. Shell color, banding pattern and body color in a large number of pulmonate snail species including _Cepaea_, (Cain and Sheppard, 1952, 1954; Cain, King and Sheppard, 1960), _Arianta_ (Cook and King, 1966), _Limicolaria_ (Owen, 1965) and others, seem to be controlled by a small

number of segregating loci locked into supergenes, but direct identification of genotypes is hindered by dominance. The famed mimetic polymorphs in lepidoptera, while under the control of simple switch-genes, are often sex-limited and virtually always confounded by dominance (reviewed extensively by Ford, 1975). Even the seemingly clear distinction of melanic and normal morphs of many industrial melanics such as _Biston betularia_ (Kettlewell, 1973, reviews this subject) is based on an allelic series displaying full dominance and the actions of modifier loci.

In some populations of the frog, _Rana pipiens_, the maintenance of an unspotted morph among the normally spotted phenotypes is believed to result from the varying survivorship of phenotypes during different seasons, the unspotted morph having better over-wintering ability but lower survival during active periods. The unspotted phenotype is due to a single dominant allelic substitution (Merrell and Rodell, 1968). Body coloration and spotting in another frog, _Pseudacris triseriata_, is known to be controlled by three genetic loci, each with two alleles and simple dominance at each locus. Evidence presented by Matthews (1971) indicates a shift in allelic frequencies between life history stages and age classes in a year's cohort.

Even these examples fail our requirements; dominance precludes the fulfillment of criterion (1) since heterozygotes are not unambiguously distinguishable from homozygous dominant individuals. Nevertheless, we may infer a great deal about genetic changes in these populations from changes in morphological frequencies if we are willing to make some

simplifying assumptions regarding the relationship of allelic to genotypic frequencies. The real failure of these examples is not the confounding of genotypes as much as the basic lack of generality. The genetic basis of each morphological difference in a given species is very likely to differ from that in another species, and may even differ between conspecific populations (Clarke and Sheppard, 1960a, b, c and d). While we can make progress utilizing these rarely found genetically tractable differences in morphology, the progress is slow, the techniques are tedious and the findings are restricted in application.

Differences between individuals detectable through electrophoretic techniques fulfill (or at least approximate) the criteria outlined above and are applicable (theoretically) to all organisms. Most importantly, almost all of the great number of populations (species) studied to date are polymorphic at many or most of their electrophoretically examined loci (Lewontin, 1974; Selander, 1976). Hence, we may unambiguously identify genotypes of individuals through a method which is virtually universal in application.

It seems reasonable that this technique should have been widely applied to questions regarding differential mortality among genotypes. Yet, such studies are so few as to be effectively hidden in the voluminous literature dealing descriptively with the levels of enzyme polymorphism in natural populations and the recent flood of papers supporting (with or without data) one or the other side of the controversy surrounding the significance of electrophoretic variation. The research described in this paper was designed to investigate genetic involvement

in heavy pre-reproductive mortality in a natural population through the use of electrophoretically detectable variation.

For theoretical (Haldane, 1957, 1960; Lewontin, 1974; Williams, 1975) and practical reasons, populations of organisms with very high fecundities present the best opportunity for the detection of genetically correlated differences in rates of morbidity. Obviously if population size is to remain reasonably stable, pre-reproductive mortality must be tremendous. Textbook discussions intimate that high fecundities and great numbers of offspring evolve as a response to high mortality rates. It is implicit in this tautology that mortality is density-independent, with the absurd result that mortality and natality rates are adjusted to provide stable population sizes. It should be noted that production of large numbers of offspring in no way preclude density-independent or density-dependent mortality as factors acting at the genetic level; in fact it has been suggested by Williams (1975) and Wallace (1975) that the contrary may be the generality. Consequently, populations of organisms with greater fecundities might be expected to successfully tolerate higher levels of "genetic death" than those of organisms with lower potentials of offspring production (Haldane, 1957, 1960). It is not unreasonable to suppose this difference to be reflected as variation in survival probabilities as well. Of equal significance, the large sample sizes necessary for the detection of relatively small selective differences (Lewontin, 1974, pp 239-242) may be obtained with greater facility from larger populations of juveniles than from smaller ones.

Bufonid anurans are, as a group, among the more fecund of vertebrates. _Bufo boreas boreas_ Baird and Girard, a widespread species of western North America, is no exception (Stebbins, 1962). Estimates from my laboratory indicate that females produce an average of 12,000 eggs per spawn. Fertility appears variable but very high. Although spawning but once annually, females (and males) are probably reproductive for a number of years. Obviously a stable population of these organisms must sustain tremendous pre-reproductive mortality and, indeed, it appears that this species does display a classic concave (type 3) survivorship curve (personal observations).

In addition to heavy pre-reproductive mortality, the particular _Bufo boreas_ population examined in this study possesses several attributes which suit it to an investigation of selective mortality. Breeding in this population is explosive. Virtually all breeding activities take place within a short period (less than a week) in spring. Only one episode of breeding takes place in a given year, hence all juveniles are of approximately the same age (ie., a cohort). The lake, which is the focal point of the population (Lost Lake, Linn Co., OR), is isolated by distance from other bodies of water suitable for substantial _B. boreas_ populations. It is fed only by two springs and a single stream (unsuitable for _B. boreas_ breeding), and is without any known outlet (percolation and evaporation are the only "drains" on the water volume). Contamination of the gene pool by immigrants is impossible in the larval stage and must be minimal in juvenile (toadlet) stages. Adult mediated gene flow may occur but considering the

distances involved (several kilometers) it, too, must be of small magnitude.

The life history and particular ecological conditions outlined above provide an opportunity to study the viability component of natural selection in a natural population exclusive of the effects of other components.

Literature bearing directly upon the question of genetic change in natural populations is restricted mainly to chromosomal analysis (Dobzhansky, 1970; Dobzhansky et al., 1977) and the monitoring of simple morphological differences (Ford, 1975). Surprisingly few studies have addressed the problem utilizing the electrophoretic technique although it has been successfully employed to monitor genetic changes in laboratory populations (Kojima and Yarbrough, 1967; Yarbrough and Kojima, 1967; Kojima, 1971; Powell, 1971; MacDonald and Ayala, 1974; Marinković and Ayala, 1975; Gromko and Richmond, 1978). In situations where genetic changes have been successfully monitored in natural populations the contributions of several fitness components have been confounded due to immigration, generation mixing and studying populations over several rounds of reproduction (Fujino and Kang, 1968; Berry and Murphy, 1970; Gains and Krebs, 1971; Tinkle and Selander, 1973; Mitton and Koehn, 1975).

Hebert (1974a, b) and Smith and Fraser (1976) have studied a number of populations of parthenogenetically reproducing cladocerans by means of electrophoresis. These studies have revealed striking intra and inter-seasonal changes in genotypic frequencies. However, since

many generations were produced within the periods embraced by the study, the separate effects of several fitness components could have been involved. Watt (1977) has detected consistent temporal increases in heterozygote excess at an enzyme coding locus (phosphoglucose isomerase) in *Colias* butterflies. These changes were apparently due to mortality and/or dispersal, but immigration effects could not be ruled out. Differential mating success and superior heterozygote survival have been detected by Sassaman (1978) in the terrestrial isopod *Porcellio scaber* by monitoring frequencies of genotypes at the lactate dehydrogenase locus in that species. With the possible exception of this last study, no attempt seems to have been successful at clearly demonstrating the isolated action of a single fitness component. To my knowledge, no one has attempted to serially monitor the genetic effects of mortality *per se* on a clearly defined cohort. With the Lost Lake *Bufo boreas* population this is a relatively simple task. Due to the brief breeding season, and isolated nature of the lake, it may be assumed that all young are from the same cohort and that immigration is inconsequential. In addition, because of the restricted breeding site (the water) all dispersal may be viewed as originating from a single location which greatly simplifies examination of the differential dispersal of genotypes. As a consequence of these conditions, any genetic change which takes place in early pre-reproductive stages must result solely from differential rates of mortality (and/or dispersal in later stages) among genotypes.

If mortality is random, no significant changes in genotypic and

allelic frequencies should occur as a result of that process. Thus, monitoring of genetically variable enzyme systems should reveal no significant changes in these frequencies over time. More rigorously, the null hypothesis states that the distributions of genotypes and alleles are uniform over time. In this case, the series of samples drawn from the population (pre-reproductive) should be homogeneous. Alternatively, detection of change in genotype and allelic frequencies as revealed by heterogeneity among the samples taken over this period would lead to the rejection of the null hypothesis and provide *prima facie* evidence of selection in the pre-reproductive mortality.

Due to complications of epistasis and linkage, one must not conclude that mortality related genetic changes at the locus examined ("marker" locus) give evidence of selection at that locus itself (Clarke, 1975; Thomson, 1977). It does; however, provide evidence of selection on genes somewhere within the linkage group in which the particular "marker" gene is located. Contrarily, a lack of change would not only suggest that the "marker" locus was uninvolved in the observed mortality, but that genes closely linked to it were likewise uninvolved.

The remainder of this thesis is arranged into two major sections. The first section is in the form of a manuscript dealing with the detection and interpretation of temporal genetic change in the Lost Lake *Bufo boreas* population. The second section consists of a series of appendices. These appendices contain information supplementary to that in the manuscript as well as discussion of findings relevant to the

question of genetic change in the Bufo gene pool, but inappropriate for inclusion in the manuscript itself. A list of appendices may be found in the Table of Contents. A list of literature cited in the manuscript section of the thesis is listed at the end of that section. Additional references, cited in the Introduction and Appendices but not in the manuscript, are compiled in a second bibliography entitled "Additional Literature Cited."

EVIDENCE OF DIFFERENTIAL REPRODUCTION AND MORTALITY IN *BUFO BOREAS*.

Until the employment of biochemical techniques had revealed the extent of genetic variation in natural populations, theoretical treatment of genetic polymorphism was largely problematic. Today, controversey surrounding the evolutionary relevance of enzyme variation has added new impetus to theoretical investigations into the forces responsible for all kinds of genetic polymorphism (e.g., Christiansen and Feldman, 1975; King and Ohta, 1975; Ohta and Kimura, 1975; Hedrick, 1976; Gillespie, 1977; Lewontin, Ginzburg and Tuljapurkar, 1978). If variation is a result of mutation and random processes or mutation-selection equilibria, we cannot expect to find evidence of selective differences among the bearers of different genotypes in nature. But, as Lewontin has stated (Lewontin, 1974, p 239), "If alleles are segregating at intermediate frequencies and any selection at all can be demonstrated to operate on them, it would be difficult to avoid the conclusion that they are held by some form of balancing selection." It is, then, important to seek evidence of selection in natural populations if we hope to fully understand the maintenance of genetic polymorphisms.

Survivorship, as a component of fitness, must not be regarded as the sole determinant of reproductive success, yet it is clearly important: The cessation of reproduction as a result of death has an absolute and certainly most obvious effect upon overall reproductive success of the affected organism. A substantial fraction of deaths may be completely random with regard to the genetic makeup of individuals, but the idea that carriers of all genic combinations should be equally

predisposed to the more lethal effects of climatic inclemency, starvation, disease, predation or a host of other factors is scarcely credible. Consequently, there is reason to seek genotype-specific differences in rates of mortality in natural populations.

There are theoretical and practical reasons for seeking a relationship between genes and mortality rates among numerically stable populations of highly fecund organisms. Such populations might be expected to successfully tolerate higher levels of "genetic death" than those of organisms with lower potentials of offspring production (Haldane, 1957, 1960; Lewontin, 1974). It is not unreasonable to suppose this difference to be reflected as variation in survivorship probabilities as well (Wallace, 1975; Williams, 1975). Procedural difficulties including the effects of non-replacement sampling and obtainment of sample sizes adequate for the detection of small mortality differentials decrease dramatically as the sampling base is increased.

Bufonid anurans are, as a group, among the more fecund of vertebrates. *Bufo boreas boreas* Baird and Girard, a widespread species of western North America, is no exception (Stebbins, 1962). Estimates from my laboratory indicate that females produce an average of 12,000 eggs per spawn. Although spawning but once annually, females (and males) are probably reproductive for a numer of years. High fecundity, tremendous larval (tadpole) and juvenile (toadlet) mortality rates and long life expectancy (Stebbins,1962; personal observations) make *Bufo boreas* a likely subject for an investigation of the genetic component of mortality.

In addition to heavy pre-reproductive mortality, the Bufo boreas population considered in this study possesses several attributes which suit it to an investigation of selective mortality. Breeding in this population is explosive. Virtually all breeding takes place within a several day period (less than a week) in the spring. Only one reproductive episode takes place in a given year, hence all juveniles are of approximately the same age (i.e., a cohort). The lake, which is the focal point of the population (Lost Lake, Linn Co., OR), is isolated by distance from other bodies of water suitable for substantial B. boreas populations. It is fed only by springs and one stream (unsuitable for B. boreas breeding), and is without any known outlet (percolation and evaporation are the only "drains" on the water content). Consequently, contamination of the gene pool by immigrants is impossible in the larval stage and must be minimal in juvenile (toadlet) stages. Adult mediated gene flow may occur but considering the distances involved (several kilometers) it, too, must be inconsequential. These particular life history and ecological conditions have provided an opportunity to study the viability component of natural selection in a natural population exclusive of the effects of other components.

Literature bearing directly upon the question of genetic change in natural populations is restricted mainly to chromosomal analysis (Dobzhansky, 1970; Dobzhansky et al., 1976) and the monitoring of simple morphological differences (Ford, 1975). Surprisingly few studies have addressed the problem utilizing the electrophoretic technique although it has been successfully employed to monitor genetic changes in

laboratory populations (Kojima and Yarbrough, 1967; Yarbrough and Kojima, 1967; Kojima, 1971; Powell, 1971; MacDonald and Ayala, 1975; Marinković and Ayala, 1975; Gromko and Richmond, 1978). Where genetic changes have been successfully monitored in natural populations, the contributions of several fitness components have been confounded due to recruitment and generation mixing (Fujino and Kang, 1968; Berry and Murphy, 1970; Gains and Krebs, 1971; Tinkle and Selander, 1973; Mitton and Koehn, 1975). Hebert (1974a, b) and Smith and Fraser (1976) have used electrophoretic techniques to reveal striking intra and inter-seasonal genetic changes within parthenogenetically reproducing populations of cladocerans and Watt (1977) has detected consistent increases in heterozygote excess over time at an enzyme coding locus (phosphoglucose isomerase) in *Colias* butterflies. In neither of these situations could the specific causes of the genetic changes be identified; in the former situation, many generations were produced during the course of the study while in the latter, the effects of immigration could not be ruled out. Differential mating success and superior heterozygote survivorship, detected by Sassaman (1978) through investigation of genotype frequencies at the lactate dehydrogenase locus in *Porcellio scaber*, may be the only clear demonstration of the effects of individual fitness components in nature.

This paper presents the results of an attempt to serially monitor the genetic effects of mortality *per se* in clearly defined cohorts by means of electrophoretically detectable genetic variation. With the Lost Lake *B. boreas* population this is a relatively simple task. Due

to the brief breeding season, and isolated nature of the lake, it may be assumed that all young are from the same cohort and that immigration is insignificant. As a consequence of these conditions, any genetic changes in early pre-reproductive stages must result solely from differential rates of mortality among genotypes. The complication of differential emigration can be easily examined since all dispersal may be viewed as originating from a single central location; the lake.

If mortality is random, monitoring of genetically variable enzyme systems should reveal no significant changes in genotypic or allelic frequencies over time and a series of samples drawn from the population (pre-reproductive) should be homogeneous. Alternatively, detection of change in genotype and allele frequencies as revealed by heterogeneity among the samples taken over this period would lead to the rejection of the null hypothesis and be *prima facie* evidence of selection in pre-reproductive mortality.

MATERIALS AND METHODS

Population sampling for this particular investigation was concentrated within one arbitrarily designated sampling area along the north-eastern shore of the lake, with the exception of the adult samples (collected during and shortly after breeding; May 1973 and June 1975) taken from an area encompassing about one-third of the northern and eastern shoreline (500-600 m). In each of the years 1973, 1974, and 1975, tadpoles were collected starting at the free-swimming stage and each two to three weeks subsequently. Annual explosive breeding habits of *Bufo boreas* together with the mixing and swarming behavior characteristic of many bufonid larvae (Wassersug, 1973; Beiswenger, 1975, 1977) ensure that tadpoles from the entire lake mix randomly such that representative samples of a single cohort may be obtained by simple methods. Collection of tadpoles was by dip net. Avoidance behavior of solitary tadpoles was minimal, while swarming individuals seemed completely oblivious to the presence of investigator or net. Larval collections were taken by quickly collecting several large samples of tadpoles within the collecting site and pooling them. The pooled collection was then subsampled to provide a single sample for electrophoresis. The remainder of the tadpoles were returned to the lake unharmed. The procedure continued each two to three weeks until metamorphosis. Newly metamorphosed toadlets (juveniles) were collected by hand and, when abundant, subsampled as described for tadpoles. Toadlet collections were gathered each two to three weeks until late fall when sampling became unproductive as toadlets began hibernation.

Sampling resumed in the spring as toadlets reemerged from hibernation and continued until sufficient sample sizes became unobtainable (as a result of attrition). Information concerning dates and sites of capture, cohort affiliation, life history stage and sample sizes is given in Table 1.

Tadpoles and toadlets were individually frozen in water and stored at -76°C until used for electrophoresis (some were stored at -15°C with no noticeable effect on enzyme characteristics). Adults were either immediately killed by a sharp blow to the head and then frozen at -76°C or first used for breeding purposes to establish the Mendelian basis of the electrophoretic variation and subsequently killed and individually frozen until used for electrophoresis.

Whole tadpoles and toadlets as well as individual livers, hearts, kidneys, eyes and samples of skeletal muscle dissected from adults, were prepared for electrophoresis by first homogenizing in a volume of "grinding buffer" (0.1 M Tris, 1 mM Na_2EDTA, $5x10^{-5}$ M NADP, pH 7.0; Selander et al., 1971). The homogenates were centrifuged (15,000 g; 20 minutes; 0-2°C) to remove debris and used immediately for electrophoresis or stored at -76°C until use. "Tissue" weight to buffer volume varied (tadpoles, 1:0.67; toadlets, 1:1.5; liver, kidneys, eyes, 1:1; heart, skeletal muscle, 1:2).

Horizontal starch gel electrophoresis was carried out using standard methods (Selander et al., 1971; S. Y. Yang, personal communication). Gels (170x170x5 mm) were prepared from Electrostarch (Otto Hiller Co., Madison, Wisconsin) lots 371 and 302 at starch weight to buffer volumes

of 12% and 11.38% respectively. Two types of filter paper "wicks" were used to apply samples to gels: #1 and #3 refer to wicks made from Whatman #1 and Whatman #3 filter paper respectively.

Four gel and electrode buffer systems were utilized. (A), electrode: 0.135 M Tris, 43 mM citric acid, pH 7.0; gel: 9 mM Tris, 2.9 mM citric acid, pH 7.0 (Shaw and Prasad, 1970). (B), electrode: 0.223 M Tris, 86 mM citric acid, pH 5.8; gel: 7.8 mM Tris, 3 mM citric acid, pH 5.8 (Schaal and Anderson, 1974). (C), electrode: 0.3 M boric acid, 60 mM sodium hydroxide, pH 8.2; gel: 76 mM Tris, 5 mM citric acid, pH 8.7 (Poulik, 1957). (D), electrode: 0.138 M potassium phosphate (dibasic), 62 mM sodium hydroxide, pH 6.7; gel: 69 mM potassium phosphate (dibasic), 3.1 mM sodium hydroxide, pH 6.7 (Selander et al., 1971).

Thirty-one enzyme assay techniques were applied. Only seven of the procedures revealed enzyme patterns with the proper combination of variation, reproducibility and activity in all life stages required for this investigation. <u>Alkaline phosphatase</u> (APH): Tadpoles, toadlets, liver; #3 wicks; buffer system A, 1 hour at 175 volts (15-18 v/cm); stain: 50 mg β-napthyl acid phosphate, 50 mg Fast Blue BB, 0.3 ml 10% (w/v) $MgCl_2 \cdot 6\ H_2O$, 0.3 ml 10% (w/v) $MnCl_2 \cdot 4\ H_2O$, 50 ml 0.05 M Tris-HCl buffer, pH 9.0; incubate 37°C (modified from Ayala et al., 1972). <u>Esterase</u> (EST): i) EST-1; toadlets only; #1 wicks, buffer system A, 4 hours at 175 volts (15-18 v/cm) or buffer system B, 4 hours at 150 volts (13-15 v/cm); stain: 1 ml of α-napthyl propionate solution (1 gm α-napthyl propionate dissolved in 50 ml acetone, diluted to 100 ml with

H_2O), 25 mg Fast Blue RR salt, 10 ml isopropanol, 40 ml stain buffer (1880 ml H_2O, 40 ml each of 0.2 M $NaH_2PO_4 \cdot H_2O$ and 0.2 M $Na_2HPO_4 \cdot 7\ H_2O$); incubate at room temperature (modified from Selander et al., 1971).
ii) EST-3; tadpoles, toadlets, heart; #1 wicks (remove after 10 minutes of electrophoresis); buffer system B, 4.5 hours at 150 volts (13-15 v/cm); stain: As per EST-1. Glutamate oxaloacetate transaminase (GOT): Tadpoles, toadlets, eye, kidney; #3 wicks; buffer system C, 3 hours at 250 volts (23-25 v/cm); stain: 25 ml H_2O, 25 ml substrate solution (146 mg α-Keto glutaric acid, 532 mg L-aspartic acid, 2.0 g polyvinyl pyrrolidone, 200 mg Na_2EDTA, 5.68 g $Na_2HPO_4 \cdot 7\ H_2O$, H_2O to 200 ml), 125 mg Fast Blue BB, incubate in the dark at room temperature (Schaal and Anderson, 1974). Lactate dehydrogenase (LDH): Tadpoles, toadlets, heart, eye; #3 wicks for tadpoles and toadlets, #1 for heart and eye (remove wicks after 5-7 minutes of electrophoresis); buffer system A, 5 hours at 175 volts (15-18 v/cm); stain: 5 ml 0.5 M sodium D, L-lactate, 1 ml NAD (10 mg/ml), 1 ml NBT (Nitro-Blue Tetrazolium, 10 mg/ml), 0.5 ml PMS (Phenazine Methosulfate, 10 mg/ml), incubate in the dark at 37°C (modified from Selander et al., 1971). Peptidase (PEP): Tadpoles, toadlets, eye, skeletal muscle; #1 or #3 wicks; buffer system A, 3.5 hours at 175 volts (15-18 v/cm); stain: 10 mg snake venom (*Crotalus atrox* or *C. ademanteus*), 10 mg O-dianisidine, 20 mg peroxidase 35 mg glycyl-l-leucine, 0.5 ml 0.25 M $MnCl_2 \cdot 4\ H_2O$, 50 ml 0.05 M Tris-HCl buffer, pH 8.0, incubate in the dark at 37°C (recipe by S. Y. Yang). Phosphoglucomutase (PGM): Tadpoles, toadlets, eye; #3 wicks; buffer system B, 3.75 hours at 150 volts (13-15 v/cm); stain: 250 mg

Na_2 α-D-glucose-1-phosphate, 25 mg Na_2EDTA, 40 units G-6-PDH (glucose-6-phosphate dehydrogenase), 50 ml 0.1 M Tris-HCl buffer, pH 8.0, incubate in the dark at 37°C (modified from Schaal and Anderson, 1974). Phosphoglucose isomerase (PGI): Tadpoles, toadlets; #1 wicks for tadpoles and toadlets, #3 for kidney (remove wicks after 5-7 minutes of electrophoresis); buffer system D, 5 hours at 250 volts (23-25 v/cm); stain: 4 ml 18 mM Na_2 D-fructose-6-phosphate, 1 ml NADP, 30 units G-6-PDH, 1.5 ml MTT, 10 ml 0.1M $MgCl_2 \cdot 6\ H_2O$, 30 ml 0.2 M Tris-HCl buffer, pH 8.0, incubate in the dark at 37°C (modified from Selander et al., 1971).

The seven enzyme assay techniques have revealed variation encoded by nine genetic loci, APH-2, EST-1, EST-3, GOT-2, LDH-1, LDH-2, PEP-2, PGI-1 and PGM-2. Data from controlled crosses in the laboratory are completely consistent with the hypothesis that the observed enzymatic variation at eight of the loci is inherited in a Mendelian fashion. EST-1 activity, not yet unambiguously identified in adults, is not amenable to Mendelian analysis. However, no evidence contrary to the idea of simple Mendelian inheritance has been discovered at this locus.

The number of allelic differences detected varied from two at the LDH-1, PEP-2 and PGM-2 loci, to as many as seven for EST-3. APH-2, GOT-2 and PGI-1 each had three alleles. Four were detected at the EST-1 and LDH-2 loci. Migration rates of allelic products (enzymes) during electrophoresis serve to distinguish and identify the alleles at a locus. For simplicity, the symbols S, M and F (slow, medium and fast) are substituted for the conventional numerical designation of relative

mobilities (electromorph classes) except where more than three alleles are discussed. Rarity of particular alleles as well as possible confoundment of certain genotypes sometimes necessitated the combining of two or more of them into a single class. At the EST-1 locus, four alleles with mobilities 0.50, 1.00, 1.20 and 1.70 were detected. Heterozygotes EST-$1^{1.00/1.20}$ were not consistently and unambiguously distinguishable from EST-$1^{1.00/1.00}$ homozygotes so the 1.00 and 1.20 classes were combined to create a three allele system: EST-1^{S} (EST-$1^{0.50}$), EST-1^{M} (EST-$1^{1.00}$ and EST-$1^{1.20}$) and EST-1^{F} (EST-$1^{1.70}$). Allelic mobility classes at the EST-3 locus included 1.00, 1.05, 1.09, 1.15, 1.17, 1.19 and 1.21 but low frequency and possible misscoring among genotypes of the last three forced the combining of these with the 1.15 class. At the LDH-2 locus, the classes 1.00, 1.16, 1.19 and 2.30 were grouped to form a three allele system by the pooling of LDH-$2^{1.19}$ and LDH-$2^{2.30}$ alleles. No other genic "lumping" was required at any locus. All reference to these alleles in text, figures and tables applies to the combined allelic classes.

Internal consistency of genotype and allele frequencies of individual collections was examined by chi-square testing of each locus in each sample for goodness-of-fit to Hardy-Weinberg expectations based on Levene's (1949) unbiased formula for small samples. Genotypic and allelic frequencies among sequential samples of a cohort (at a single collection site) were tested for both homogeneity among themselves and agreement with expected values generated from adult (parental) allele frequencies through interaction chi-square analysis (Sokal and Rohlf,

1969, pp 575-585). In order to examine inter-year differences in allele frequencies among the young <u>Bufo</u>, data from all samples of each cohort were combined to form a single pooled sample for each year (data from C20, C23, C48 and C52 were not included). The three pooled samples were inspected for homogeneity through contingency chi-square test. Linear regression of heterozygote deviation ($D = (H_o - H_e)/ H_e$ where H_o and H_e are the observed and expected number of heterozygotes, Rasmussen, 1964; Selander, 1970) against time was performed on the PDP-1145 computer (Digital Equipment Corp.) at Humboldt State University using the LNREG1 program supplied by Digital Equipment Corporation User's Group (DECUS).

RESULTS

Twenty-five collections of pre-reproductive (larval and juvenile) and two collections of adult *Bufo boreas* from Lost Lake were examined with respect to electrophoretically detectable variation encoded by nine loci: APH-2, EST-1, EST-3, GOT-2, LDH-1, LDH-2, PEP-2, PGI-1 and PGM-2. The nature of genetic variation at these loci cannot be accurately represented through nine sets of genotype or allele frequencies: these proportions varied temporally within cohorts as well as between generations. Perhaps the best estimator of this genetic variation is that obtained from adult samples. The adult segment contains the genetic base from which subsequent generations will issue. That is, the adult component includes the breeding population gene pool. As such, it may adequately serve as a reference point for the investigation of variation and change in pre-reproductive cohorts.

Genetic Variation in Adult *Bufo boreas*.

Adult toads were collected during and shortly after the breeding period in 1973 and 1975 (C10 and C36 respectively). Sex-ratio in adults was male-skewed in all years: Combined 1973 and 1975 samples; 161:38. No allelic frequency heterogeneity was discovered at any locus when males and females were compared, hence sex has been ignored in this investigation. Observed allele frequencies (Table 2) were used to compute the genotypic proportions expected in the absence of evolutionary forces, using Levene's (1949) unbiased formula for small samples. All loci showed good agreement between actual and expected genotypic

proportions in both collections except EST-3. In both years, a substantial (though significant at the $\alpha = 0.05$ level only in 1973) departure from expectation was seen to result from the excess of $EST\text{-}3^{1.06/1.06}$ and $EST\text{-}3^{1.09/1.09}$ homozygotes and deficiency of $EST\text{-}3^{1.06/1.09}$ and $EST\text{-}3^{1.09/1.15}$ heterozygotes (data available from author upon request).

As there were no differences between the two adult samples with respect to gene frequencies (homogeneity at each locus examined by contingency chi-square), the two collections were combined to form a single "adult" collection. Allele frequencies from this pooled adult collection (hereafter called "parental" allele frequencies) are given in the last column of Table 2. These estimates of true adult population allele frequencies yield the best estimate of allele frequencies of the breeding population gene pool. Therefore, the "parental" frequencies were used to generate a set of expected "zygotic" frequencies based on the assumptions of random mating and no selection (Table 3). The "parental" and "zygotic" frequencies are herein considered the best estimates of allele and genotype frequencies expected among the young in the population in the absence of perturbing (evolutionary) influences.

Dynamics of Genetic Variation in Pre-reproductive *Bufo boreas*.

Temporal patterns of allele and heterozygote frequency variation for the 1973, 1974 and 1975 cohorts of young *Bufo* are presented in Figures 1 through 9. Genotype frequencies, D (standardized heterozygote deviation) values and chi-square for goodness-of-fit of observed genotypic proportions to those expected under Hardy-Weinberg conditions

("intrinsic"; i.e., computed from sample allele frequencies, not from "parental" frequencies) may be found in Table 3. In general, there is good agreement of observed genotype frequencies to expected distributions. In only a few instances (e.g., APH-2 in 1973 and EST-1 and PGI-1 in the 1975 cohort) do large proportions of cohort samples show poor "intrinsic" Hardy-Weinberg fit. This impression of general stability vanishes, however, when the data are viewed as a series of independent estimates of the true zygotic distribution of a single population.

Estimates of genotype and allele frequencies from multiple samples drawn at different times from a single cohort will differ only as a result of sampling error unless actual genetic alteration occurs during the sampling period. Significant change in the genetic composition of cohorts was sought via interaction (heterogeneity) chi-square analysis of gene and genotype frequency distributions among the samples, a procedure which partitions the total chi-square in a manner analogous to the partitioning in the analysis of variance. At each locus in each sample, a goodness-of-fit chi-square was computed for the observed frequency distribution (allele or genotype) versus the expected one ("parental" or "zygotic"). While they are, themselves, informative, individual chi-squares may be summed over the cohort to yield a total chi-square of much greater power in testing overall goodness-of-fit. This total chi-square (T) can be partitioned into the pooled and heterogeneity components. A significant pooled chi-square (PO) suggests a consistent directional deviation from expectation in most or all

samples (although a single huge deviation may cause significance), while significance in the heterogeneity chi-square (H), indicative of inter-sample heterogeneity, is reason to reject the hypothesis that the genetic frequencies had been stable during the sampling period.

Table 4 lists interaction chi-square results for gene and genotype frequencies observed in cohort samples versus "parental" and "zygotic" expectations (except for EST-1, as explained below). Evaluations were conducted only on the series of samples collected at the shoreline and do not include those samples contemporaneously collected in other than this standard collection area (i.e., "dispersing" collections C20, C23, C48 and C51). These latter are examined individually in the Discussion section. Because chi-square values are not strictly additive, the H value, obtained by H = T - PO, is only an approximation (albeit a close one) to the true heterogeneity chi-square obtainable by analysis of the contingency table. In situations where borderline H values were encountered, actual contingency chi-square values were computed. The probabilities indicated in the tables reflect the true contingency table chi-square probabilities in these cases.

The most striking feature revealed by the interaction chi-square analysis is the general lack of agreement between adult generated expected frequencies ("parental" and "zygotic") and those observed at almost every locus in each cohort. The only exceptions are in the 1973 cohort which shows good fit for PEP-2 and PGM-2 in 1975 where PEP-2 also shows nice agreement. Even when EST-1 genotypes are considered, poor agreement in two of the three years is strongly indicative of

overall genetic instability. Parsimony precludes detailed examination of these rather astounding results but a locus by locus overview is informative.

APH-2. Poor agreement between adult and observed offspring frequencies is obvious at the APH-2 locus (Table 4). In all cohorts, $APH\text{-}2^{M}$ frequency exceeds the "parental" level. This excess is compensated by a lower than expected frequency of $APH\text{-}2^{F}$. The allelic differences are reflected by the elevated frequency of the $APH\text{-}2^{M/M}$ genotype and depressed heterozygote proportions, especially $APH\text{-}2^{M/F}$. These consistent deviations contribute heavily to T through the significant PO values clearly indicating that the gene and genotype proportions differ between young *Bufo* and the adults from which they arose. The direction and magnitude of differences between parents and offspring are about equal each year (= cohort) as no evidence of differences in allele frequencies are evident among cohorts ($X^2 = 4.17$, $P > 0.30$).

Evidence of temporal genetic change within cohorts of young *Bufo* comes from the H component. Allele frequencies at the APH-2 locus were not homogeneous in 1973. It is highly unlikely that the 1973 sample series was drawn from a distribution that was stable through time. Variation in genotype frequencies in 1973, though not statistically significant ($P < 0.1$) contributes substantially to the poor agreement between adult and offspring genotype proportions.[1] The nature of

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[1] It should be remembered that while allele and genotype frequencies are highly correlated, the correlation need not be perfect except under Hardy-Weinberg conditions. Accordingly, significance of

genetic change at the APH-2 locus is best seen by examining Figure 1. Most notable was the initial rise of APH-2^M frequency from near parity with the "parental" proportion followed by a gradual decline through the metamorphic period and on into hibernation with little change occurring over winter. Although changes in heterozygosity closely mirrored those in APH-2^M frequency, all genotype frequency changes cannot be attributed to changes in allelic proportions since several of the samples show poor fit to "intrinsic" expected genotypic proportions (computed from sample gene frequencies; see chi-square results, Table 3). These poor fits result mainly from higher APH-$2^{S/S}$, APH-$2^{F/F}$ and APH-$2^{S/F}$ genotype frequencies than would be expected under Hardy-Weinberg conditions. No temporal genetic change was evident in 1974. Among the 1975 samples, significant heterogeneity among genotypes indicates genetic instability over time and the metamorphosis-to-hibernation trends of APH-2^M and heterozygote frequency are similar to those in 1973.

EST-1. Because "parental" and "zygotic" frequencies were lacking for this locus, contingency chi-squares for homogeneity were computed for observed allele frequencies within cohorts. Mean allele frequencies of all iso-cohort samples were used to generate expected genotypic proportions for each year for interaction chi-square analyses of genotypes. The observed genotype proportions are in close agreement

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pooled or heterogeneity chi-squares for either of the allele or genotype frequency distributions is sufficient evidence of genetic instability and differentiation.

with these expected frequencies in 1973 but not in 1974 or 1975 (Table 4). Departures from expectation are difficult to interpret since no consistent inter-year pattern is obvious and average allele frequencies are not equal among cohorts ($X^2_4 = 19.13$, $P < 0.001$).

Genetic change is evident in the genotypic heterogeneity among young toads collected in 1974 and 1975. The 1973 cohort appears genetically stable. Caution should be exercised in the interpretation of the 1974 data since a single sample, C30, is responsible for the bulk of the heterogeneity as a result of a very low $EST\text{-}1^{M/F}$ frequency and elevated $EST\text{-}1^{M/M}$ and $EST\text{-}1^{F/F}$ proportions. In 1975, $EST\text{-}1^{M}$ showed a considerable drop in frequency accompanied by a rise in the frequency of $EST\text{-}1^{F}$ over a two month period. Genotype frequencies for this cohort are consistently out of "intrinsic" Hardy-Weinberg fit because of an excess of $EST\text{-}1^{M/M}$ and strong deficiency of $EST\text{-}1^{M/F}$. It was a drop in the former with compensatory increase in the latter which were responsible for most of the change in allele frequencies. This post-metamorphic pattern is very similar to that seen in 1973 (compare with C17, C18, C21 and C22) as are the over-winter patterns of the first two years (compare C26 and C32 with C30, C37 and C39).

<u>EST-3</u>. Genetic differentiation of adults and young each year results from generally lower than "parental" frequencies of $EST\text{-}3^{1.00}$ and $EST\text{-}3^{1.06}$ with high relative proportions of the alternative alleles. Genotype frequencies observed generally agree with "intrinsic" Hardy-Weinberg expectations hence it is not surprising to find significant disparity between "zygotic" and observed offspring

proportions. Although the directions of offspring differentiation relative to "parental" and "zygotic" frequencies are similar each year, the magnitudes of the allelic shifts clearly differ between cohorts as seen in the marked heterogeneity among cohort average allele frequencies ($X^2_6 = 19.97$, $P < 0.005$).

Little evidence of intra-cohort change was seen in 1974 or 1975 but a radical increase in EST-3$^{1.09/1.15}$ frequency in C12 was sufficient to produce significant H values for both allele and genotype proportions in 1973. The huge disparity of C12 compared to the other collections may have resulted, in part, from sampling error, but removal of C12 from the analysis still yields a significant H chi-square for allele frequencies. Furthermore, patterns of gene frequency changes in 1975 are virtually identical with those in 1973, albeit of reduced magnitude (Figure 3). Changes in the proportions of EST-3$^{1.06}$ and EST-3$^{1.15}$ occurred at almost identical periods in the life history during both years. Patterns for the other two alleles are remarkably similar as well.

<u>GOT-2</u>. The GOT-2 locus is characterized by a rather low level of genetic variation. Even so, distinct genetic change is evident between generations and within cohorts. For both 1974 and 1975, lack of fit may be explained entirely by single disparate samples (C27 and C47, respectively). If these samples are ignored, overall agreement between adult and young frequencies appears excellent indeed. In 1973, however, GOT-2^{M} and GOT-2^{F} proportions are too high and too low, respectively, throughout the year as a result of the deficiency of

GOT-$2^{M/M}$ and excesses of GOT-$2^{M/F}$ genotypes. Actually this pattern is repeated in 1974, significantly so if C27 is considered, but the allelic frequencies among all years are quite variable (X^2_4 = 15.74, $P < 0.005$) indicating a lack of consistency among the determinants of gene frequencies in each year's cohort. The temporal changes in the genetic composition of the 1973 cohort were clear cut. They resulted from changes in genotype proportions relative to the allele frequencies due primarily to an overall rise in GOT-$2^{M/M}$ levels and corresponding drop in heterozygote frequencies prior to metamorphosis. Subsequently there was a gradual return to earlier levels. The same pre-metamorphosis rise in GOT-$2^{M/M}$ (and heterozygote drop) is seen in 1974 but cannot be discerned in 1975 without considerable exercise of the imagination.

LDH-1. The temporally stable and annually similar (X^2_2 = 0.99, $P > 0.50$) allelic patterns at the LDH-1 locus are illustrated in Figure 5. Genotype distributions in good agreement with allelic proportions reflect the stability within cohorts. Although stable and similar, the average allele and genotype frequencies among cohorts differ significantly from those of adults. LDH-1^F, the major allele, is significantly less frequent in the former than latter life stage resulting in higher than "zygotic" heterozygote frequencies. All genotype frequencies, however, are in excellent agreement with sample gene frequencies (i.e., show good fit to "intrinsic" Hardy-Weinberg expectations).

LDH-2. Disparity between adults and young at this locus results primarily from higher than "parental" LDH-2^S and LDH-2^F (and corresponding genotypic) proportions among most of the samples each year. Major shifts in genotype proportions (especially LDH-$2^{S/S}$, LDH-$2^{S/M}$, LDH-$2^{M/M}$ and LDH-$2^{M/F}$) account for heterogeneity among alleles in the 1974 and 1975 cohort samples. Mean allele frequencies are very similar among the three cohorts ($X^2_4 = 2.87$, $P > 0.20$) and certain dynamic patterns of allele and genotype frequencies appear upon close inspection. In all years, LDH-$2^{M/M}$ started at low frequency and rose to double or triple its original level by the metamorphic period. Concurrently, falling frequencies of LDH-2^M-carrying heterozygotes dampened the rise in LDH-2^M allele frequency. In 1973, shifts in overall heterozygosity were greater than would be expected solely due to changes in allelic proportions. No additional similarities are obvious. One must conclude that patterns of heterogeneity and genetic change are distinct among years.

PEP-2. Genetic frequencies at this locus were very stable during the periods examined, but significant differentiation was obvious between generations in 1974 and 1975. The same pattern developed non-significantly in 1973. Lower than "parental" PEP-2^F frequencies characterize most samples, though the deviation is significant only in the 1974 cohort. The temporal stability of these patterns is further emphasized by the good agreement between sample allele and genotype proportions and the overall similarity of the cohort gene frequency deviations each year ($X^2_2 = 2.58$, $P > 0.20$). Even the suspiciously

large decline in the frequency of the PEP-2$^{S/F}$ genotype at the beginning of 1973 was insufficient to produce heterogeneity among genotypes.

Despite the statistically homogeneous nature of these patterns, certain trends were seen to occur each year. The most notable was a sharp decline in PEP-2$^{F/F}$ frequency during the period including metamorphosis. This pattern occurred in all years accompanied by a rise in PEP-2$^{S/S}$. Heterozygote proportions were stable. Translating this to alleles revealed a drop in PEP-2^{F} and a rise in its allele at this period (Figure 7). Interestingly, while there was no similarity in direction, the winter period appeared to cause a rather drastic change in allele and especially genotype frequencies in both 1974 and 1975 purely due to shifts in homozygote frequencies.

<u>PGI-1</u>. Each year, PGI-1^{S} frequency in young toads was consistently in excess of that expected from consideration of adult genic proportions. This excess was compensated by lower frequencies of both alternate genes and the overall effect is highly significant. Even so, the effects were not equal each year as differences in the magnitude of allelic frequency departures are obvious among the three cohorts (X^2_4 = 19.39, $P < 0.001$). No significant trends or patterns of temporal genetic change within cohorts emerge from visual inspection of these data (Table 3 and Figure 8), an impression reinforced by the statistical analysis. Nevertheless, the pattern of genotype and allele frequency change among samples of the 1974 cohort are biologically interesting. The abrupt drop in PGI-1$^{S/S}$ frequency and rise in

heterozygote proportions among early samples of the cohort result in corresponding shifts among PGI-1^S and alternative allelic distributions. The low (but non-significant) probabilities of both allele frequency diversity (X^2_{10} = 16.46, P = 0.087) and variation in heterozygote proportions (X^2_5 = 9.41, P = 0.087) are suggestive of less than perfect temporal stability at this locus.

PGM-2. In 1973, cohort data agree very nicely with those from adults and there is no evidence of change over time within the young toad segment of the population. In the 1974 and 1975 cohorts, there is striking heterogeneity among samples contributing to significant differences between adults and offspring. In both years, remarkable shifts in allelic frequencies resulted from changes in the relative proportions of PGM-2$^{S/S}$ and PGM-2$^{S/F}$ genotypes just prior to and during the period of transformation from the aquatic to terrestrial stages. Although this pattern is totally lacking in 1973, the average allele frequency of each cohort is similar in each year (X^2_2 = 3.18, $P > 0.20$).

Conclusions regarding genetic stability. The data undeniably demonstrate the dynamic nature of the gene pool under consideration. Genetic change occurring concomitantly with heavy mortality is manifest at six of the nine loci at least once during the three year period examined. At five loci, there are at least two years in which such genetic activity is detectable. Additional confirmation of genetic change may be seen in the general lack of agreement of genetic constitution among young and adult toad populations. At five of eight

loci, there are distinct differences between generations in each year examined, and all loci show significant inter-generation differences in two of the three years considered. Even where no parental data are available (EST-1), there is poor agreement of genotype frequency data with those expected under Hardy-Weinberg conditions. Finally, in four of nine comparisions, significant inter-year differentiation in allele frequencies is evident among the young animals. The conclusion of genetic change, not stability, is inescapable.

Overdominance in Survivorship. The data provide little evidence of higher survivorship among heterozygotes in the early life history stages of *Bufo boreas*. Heterozygotes at several loci are distinctly less frequently encountered than would be expected if alleles were distributed according to Hardy-Weinberg relationships (Table 3). At the APH-2 and EST-1 loci, samples with negative D values (heterozygote deficiencies) significantly outnumber those with excesses (sign test based on equal binomial probabilities of positive and negative D; $P < 0.025$ and $P < 0.005$, respectively) while not a single locus has a significant excess of positive ones. Considering all pre-reproductive *Bufo* samples, only 92 of the 226 D values are positive ($P < 0.005$).

Heterozygote deficiencies (or excesses) *per se* have no bearing on the question of overdominance in survivorship. A deficiency of heterozygotes among newly conceived zygotes may result from a variety of phenomena and be maintained into later stages even if heterozygotes are somewhat more viable than homozygotes. Superior heterozygote

survivorship will be seen as a temporal increase in D, regardless of sign. This was investigated by calculating the least-squares regression of $\frac{D\ (\arcsin\sqrt{|D|})}{|D|}$ on time (this transformation corrects for non-normality of variance encountered in frequency data, but maintains the original sign of D). The regression was computed for tadpole stages alone, toadlet stages alone, and the entire year for each locus in each cohort (excluding C20 and C23 in 1973 and C48 and C51 in 1975). Only eight of the 72 regressions were significant at the $\alpha = 0.05$ level:

a) EST-1, toadlets, 1973[2]: $y = -20.02 + 0.119x$; $F_{1,4} = 15.68$, $P < 0.05$.

b) EST-1, toadlets, 1975: $y = -35.86 + 0.121X$; $F_{1,1} = 41542.00$, $P < 0.005$.

c) EST-3, all, 1974: $y = -21.18 + 0.101x$; $F_{1,3} = 98.89$, $P < 0.005$.

d) GOT-2, tadpoles, 1973: $y = -22.25 + 0.644x$; $F_{1,3} = 47.42$, $P < 0.01$.

e) LDH-2, toadlets, 1975: $y = -14.98 + 0.229x$; $F_{1,2} = 112.48$ $P < 0.01$.

f) PGM-2, all, 1975: $y = -26.65 + 0.445x$; $F_{1,4} = 9.85$, $P < 0.05$.

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[2]Let $y = \frac{D\ (\arcsin\sqrt{|D|})}{|D|}$; x = time.

g) PEP-2, tadpoles, 1973: $y = 27.17 - 0.642x$; $F_{1,3} = 16.37$, $P < 0.05$.

h) PEP-2, toadlets, 1975: $y = 22.72 - 0.122x$; $F_{1,1} = 189.62$, $P < 0.05$.

Since so few significant relationships may be expected due to chance alone, there seems no support for the concept of overdominance in survivorship among young *Bufo boreas*. However, the first two relationships, EST-1, 1973 and 1975 are worthy of additional discussion. Data on EST-1 are available only for toadlets, hence fortuitous regressions are much less likely for this locus than for any of the others. Yet, two of the three regressions were significant. Furthermore, the regression coefficients for these two relationships are identical. Finally, the observed (absolute) frequencies of heterozygotes did increase with time both years. It seems difficult to attribute these particular relationships entirely to chance.

DISCUSSION

Change and differentiation are characteristic of the genetic constitution of the Lost Lake Bufo boreas population. The results of this investigation leave little doubt of the dynamic nature of that particular gene pool. This in itself is interesting, but the central queries of this study were formulated in terms of natural selection. In particular, the study was concerned with selective differences in survivorship. There are clearly several alternative causes of evolutionary change. Examination of the involvement of selective differences in the observed genetic changes must bear upon two distinct aspects of the data. First, how can the nearly ubiquitous differences between adult and offspring gene pools be interpreted? Second, what evolutionary force(s) may best explain the changes in the genetic composition of individual cohorts? In either case the effects of mutation must be miniscule. In view of population size (2000 - 5000 or more adults), fecundity of adult toads and the vast numbers of young in each cohort, it is difficult to seriously entertain the notion that random processes (genetic drift) could be effective agents of change in this regard. The remaining alternatives must be more closely examined.

Perhaps the simplest explanation for the observed inequalities of adult and young genetic distributions is that the adults sampled were not representative of the parental population at all. This would be so if the adult collections were, as a result of sampling error, biased samples of the adult population. Considering the almost perfect

agreement of allele frequencies among the two samples and between sexes, sampling error of this kind seems an unlikely explanation for the observed inter-generation differences. Alternatively, if only a genetically non-random segment of the adult population generally reproduces, then a perfectly "representative" sample of all adults would yield a poor estimate of the breeding gene pool. In this case the samples would be biased, not as a result of sampling error, but as a result of natural selection.

Genetic differences between breeding adults and their offspring may arise through non-random gametic union *per se* or via unequal contribution in the formation of the gamete pool itself. I shall first examine non-random mating. Local inbreeding in this population is an intuitively unappealing concept since the swarming and mixing behavior of *Bufo* larvae during the several weeks prior to metamorphosis should effectively randomize sibships. At the genetic level, it proves entirely inadequate to explain lower than "zygotic" heterozygote proportions at some loci while other loci have heterozygotes in excess of these levels (this should not be confused with the overall deficiency of heterozygotes based on "intrinsic" expectations). Nor is inbreeding expected to affect change in allele frequencies, but it is precisely allelic disparity between parents and offspring which requires explanation. These differences in allelic proportions also rule out assortative mating as a cause for genetic disagreement.

At loci with complete dominance, disassortative mating may be associated with rather strong selection (Li, 1976; Spiess, 1977) that

can be expected to rapidly alter gene frequencies toward equilibrium values. Yet, even assuming enzymatic differences to be somehow manifested in a completely dominant manner at the level of inter-individual recognition, this mode of non-random mating is irreconcilable with the data. The affected loci should show overall excesses of heterozygotes relative to "intrinsically" derived Hardy-Weinberg expectations (show positive D values in excess of random binomial expectations). None do. Clearly, non-random mating is unable to explain the observed genetic differences between parents and offspring. I next consider selection.

Inequality of adult and offspring gene and genotype frequencies may result from any combination of genotypic variance in fertility, fecundity or mating success (late components of fitness) such that zygotes result via union of gametes from a selectively non-random subset of the adult (potential) gene pool. Genotype proportions among such zygotes would be in perfect agreement with Hardy-Weinberg expectations based on sample allele frequencies (i.e., "intrinsic" frequencies). But these frequencies would not agree with those expected assuming parity of adult and offspring allelic proportions. This is precisely the situation at hand. Excluding all cohort series showing significant temporal heterogeneity, the overall agreement of sample allele and genotype proportions under the assumption of random genetic union is remarkably good (Table 3). The only exception is PGI-1 (This may indicate very early zygotic selection in addition to the possibility of selection among adults. It may also be indicative

of the added contribution of assortative mating. At any rate, strong selection among PGI-1 genotypes must have occurred at some point each year.). Early zygotic selection cannot be entirely ruled out, nor do the data permit discrimination between the forementioned sources of differential gametic contribution, but it seems clear that bearers of different, electrophoretically distinguishable, genotypes vary with regard to some late component of fitness. For whatever reason, the adults present at Lost Lake during the breeding periods examined differentially contributed gametes to the subsequent generations.

The genetic changes detected among young *Bufo boreas* will now be discussed. The examination of temporal genetic change was conducted during the period beginning shortly after conception, through the year and into the subsequent spring (except in 1975 when the study ceased with the onset of winter). It has been my experience that at an absolute minimum, 95 to 99% of the young *Bufo* die during these life stages. It is more accurate to say that they disappear, but since dispersal is rather limited and easily witnessed, I find it inconceivable that the bulk of this disappearance is the result of emigration. If this view is correct, most of the genotypic differences in survival are probably expressed during this period. This is no proof that the genetic changes observed in these cohorts were due primarily to differences in survival probabilities. However, as I will attempt to show, this seems to be the only logical explanation for these observations. Mutation and random processes have already been discounted in this regard but there remain additional alternatives which must be carefully assessed.

It is extremely improbable that differential recruitment of genotypes via immigration had any effect on the genetic constitution of pre-reproductive _Bufo_ cohorts examined. Certainly immigration was impossible for tadpoles. In the terrestrial stages, young _Bufo_ are somewhat vagile, but high-speed, long-distance travel of significant numbers of toadlets from distant localities during the first few weeks post-metamorphosis seems incredible. Differential recruitment through asynchronous reproduction was not likely to have had much effect either. No "late" reproductive activities were observed in any year although such activities certainly could have escaped notice. But if significant numbers of "late" recruits had entered the population surely some rather conspicuous size disparity should have been noted in the tadpoles. More importantly, unless "late" recruits possessed unusually high developmental rates, compensating for their late start, some bimodality of metamorphic activities should have been obvious. There simply was no evidence of substantial "late" recruitment via late reproduction.

Two additional agents of non-selective (although potentially important in a selective sense) genetic changes among the cohorts of young _Bufo_, differential dispersal and divergence in developmental rates, will be considered in order. Dispersal of toadlets from the lakeshore was evident in all years. The basic flow of animals was strikingly perpendicular to the shoreline. Dispersal was not a universal tendency among all individuals nor was it synchronous among those toadlets that did disperse. Since the evidence of temporal

genetic change in cohorts comes entirely from those animals collected at the lake shore, it is important to see if an extraordinary tendency for particular genotypes to disperse may explain some major portion of the observed temporal heterogeneity. Differential dispersal per se is not selectively relevant, but survival rates or probabilities of successful maturation and return to the lake for reproduction might differ between those animals which disperse quickly and those dispersing more slowly or not at all. If so, and if there are genetic differences between groups, the differences are selective. Naturally, the data do not provide sufficient information to examine all aspects of this question. What may be examined is whether or not the two groups ("disperser" and "main" sub-populations) did, indeed, differ with regard to the loci under consideration and if so, whether the temporal changes in the "main" population might be attributable to differential dispersal.

As may be seen in Table 1, there are four pairs of collections (two pairs in each of 1973 and 1975) which bear upon the question of differential dispersal. Each pair of collections consists of a sample collected at the lake shore ("main") and one collected among individuals having dispersed 80-100 m (perpendicularly) from the lake shore ("dispersers"). These pairs, called "1973 early" (C21/C20), "1973 late" (C22/C23), "1975 early" (C47/C48) and "1975 late" (C50/C51) were compared with regard to allele and genotype frequencies. There was evidence of differentiation between "dispersers" and "main" only at the EST-3, LDH-2 and PEP-2 loci. PEP-2 showed significant

heterogeneity among genotype proportions in the "1975 late" pair ($X^2_2 = 7.86$, $P < 0.025$) but since there was no temporal heterogeneity at this locus, no further discussion is warranted. "Dispersers" in the "1975 early" comparison carried LDH-2^S less often than "main" population individuals, a difference which contributed heavily to the overall heterogeneity between these two groups ($X^2_2 = 9.11$, $P < 0.025$). Part of the observed temporal heterogeneity at this locus was in fact due to the sharp rise in LDH-2^S and drop in LDH-2^M frequencies between metamorphosis (C42 and C43) and the period in question (C47). This might have been the result of differential dispersal such that bearers of LDH-2^M tended, on the average, to disperse more frequently and LDH-2^S less frequently than expected (under the assumption that dispersal was LDH-2-genotype independent). If so, C48 LDH-2^M frequency should have been relatively higher, and LDH-2^S frequency relatively lower than the "metamorphic" frequencies. This was not the case. The disparity between C47 and C48 was not due to symmetrical differentiation of allele frequencies but to a unilateral change (relative to the previous and subsequent samples) in allelic proportions. Apparently "dispersers" were different from "main" individuals, but as a result of different selective pressures, not differential dispersal. The "1975 late" pair did not differ significantly for LDH-2. This should not be surprising since the "dispersers" of this pair were partially drawn from the distribution present in the "main" population prior to the

sampling period. In fact, both the "main" and "disperser" populations in the "1975 late" pair had converged somewhat upon the original "metamorphic" frequencies.

Allele frequencies of "dispersers" and "main" differed significantly at the EST-3 locus for both the 1973 and 1975 "early" pairs ($X^2_3 = 9.77$, $P < 0.025$; $X^2_3 = 10.21$, $P < 0.025$, respectively). In both cases "dispersers" carried EST-$3^{1.09}$ in higher and EST-$3^{1.06}$ in lower proportion than did the "main" individuals. The difference disappeared after a greater proportion of individuals had dispersed ("late" pairs). In 1973, the trend for higher rates of dispersal among EST-$3^{1.09}$ bearing genotypes was consistent with the drop in this allele's frequency in the "main" population between metamorphosis and the sampling period in question (C22). In 1975, the same trend in "dispersers" was seen but there was no change in the "main" population. At best, there is some evidence that differential dispersal of genotypes may have been a reality and somewhat contributory to temporal genetic heterogeneity within cohort sample series. There is no support, however, for the idea that differential dispersal was a major cause of temporal genetic change in the "main" population.

It has become obvious from field and laboratory observation (Samollow, unpublished) that developmental synchrony is less than perfect among young Bufo boreas. This has the effect of subdividing the population into two distinct but dynamic segments during the emergence period: An "aquatic" phase (semi-aquatic, early stages of transformation) and a "terrestrial" phase (able to hop about near water's

edge but not fully transformed). As a result, representative sampling became a problem during these intervals. Samples collected during metamorphosis should have included representative numbers of both phases, but accurate assessment of the relative proportions of individuals in each phase would have been very difficult and subject to enormous error. If developmental rates in Bufo boreas are correlated with genotype and the sampling problems are ignored, one might detect a huge but ephemeral shift in the genetic composition of a particular population and erroneously attribute it to a cyclic shift in relative survivorships. However, since such shifts in genetic frequencies would be symmetrical among the two phases, they should be easily detected if both phases are sampled separately but simultaneously. This is exactly what was done in 1973 and 1975 and there was little evidence of association between developmental rate and the genetic variation at the particular loci in question. I examined allele and genotype frequency homogeneity among fast and slow developers in 1973 and 1975: C17 and C18, C43 and C42, respectively, in these years. There were no instances of heterogeneity in 1973. In 1975, there was some differentiation between fast and slow developers with respect to the EST-3 and LDH-2 loci. EST-3 genotype frequencies were quite distinct among fast and slow developing individuals ($X^2_7 = 27.71$, $P < 0.001$). The differences appeared symmetrical such that the simple averages of the genotype frequencies for both samples fell approximately into line with the values for the previous and subsequent samples. This is precisely what should be expected if

differences between fast and slow developing individuals are in fact due to developmental rates and not differential selective pressures. Hence, developmental rate differences might explain the heterogeneity of EST-3 frequencies in 1975. Unfortunately, there is no heterogeneity to explain. Also in 1975, LDH-2 allele frequencies appeared significantly different among fast and slow developers ($X^2_2 = 6.92$, $P < 0.05$). This was entirely due to the relatively low LDH-2^S and high LDH-2^M frequencies in slow developers. There was no evidence of a symmetrical shift in fast developers when compared to previous and subsequent sample frequencies. Therefore it is difficult to conclude that the differences between these samples are due to developmental rates. Obviously then, differences in developmental rates are insufficient to account for the observed heterogeneity within cohorts. Having finally exhausted the alternatives, differential survival rates among genotypes seems the only logical explanation for the observed genetic changes within cohorts.

Evidence of differential mortality among genotypes in young *Bufo boreas* is by no means tantamount to evidence of selection on the particular marker locus (loci) examined (Lewontin, 1974; Clarke, 1975; Thomson, 1977). However, since the relative probabilities of death are constant neither between nor within years, the results address a more general question than that regarding the physiological differences between the bearers of different genotypes.

It is well known that relative fitnesses may vary dramatically with changes in environmental conditions. The relative intra-populational competitive performances of particular genotypes have been shown to be profoundly affected by the frequencies of competing genotypes in several laboratory populations (reviewed by Lewontin, 1974; Ayala and Cambell, 1974; Spiess, 1977). Abiotic factors must be of equal or greater importance. Changes in the proportions of chromosomal arrangements are known to occur in response to changes in abiotic conditions (Dobzhansky, 1970; Lewontin, 1974). Shifts in fitnesses among morphologically distinguishable genotypes in lepidoptera and several molluscs have been well documented (Ford, 1975). It is hardly precedental to discover that the survivorship potentials of particular genotypes are inconstant among young Bufo boreas. What seem surprising are the magnitude and rapidity of these changes and of the environmental heterogeneity which must underlie them. However, there is really no basis for astonishment: The Lost Lake environment is very dynamic.

Considering a single year, environmental conditions change rapidly. Young tadpoles encounter seasonally increasing average water temperatures and declining water levels. Aquatic floral diversity and abundance change rapidly during the early summer. Conspecific interactions which are very obvious in larvae of this species, become more and more pronounced as the tadpoles grow. These interactions are apparently quite important in their effects on developmental rates and competitive abilities of larval bufonids (Brockelman, 1969; Wassersug,

1973; Wilbur, 1977). The environmental constraints faced by individuals passing from the aquatic to the terrestrial phase must differ radically from those encountered previously. Fully transformed individuals must deal with new predators, temperature fluctuations previously unknown, and an entirely new trophic mode. In general, not only does the environment change radically throughout this period, so too, does the organism. Metabolic and developmental changes may occur in direct response to the changing external evnironment, or in spite of it. In either case, they create a series of novel internal environments. Some of these internal changes undoubtedly include the expression of previously unexpressed genes or the inactivation of some of those previously active, i.e., they cause alteration of the "effective" genetic background upon which other genes must face the test of selection. Yet, considering the population on a long term basis, the situation is even more complex. The conditions encountered by individuals of a particular stage vary from year to year. This was clearly the case for the years during which this investigation took place. One need only consider the dates at which the first tadpoles were collected and when metamorphic activities occurred in each cohort to be convinced that the environmental factors affecting breeding and developmental rates varied between years. 1973 appeared to me to be the most productive year in terms of the number of young toads as well as the total biotic productivity of the lake. The following year was the poorest of the three and 1975 was approximately intermediate. Doubtless, _Bufo_ that start life early in May face considerably

different biotic and abiotic environmental conditions than those spawned in late June or July. It appears that survivorship in young Bufo is responsive to these internal and external environmental changes.

Much of the current thinking about genetic variation in natural populations favors the view that stochastic changes in fitness rankings among genotypes are crucial in the maintenance of genetic polymorphism (Gillespie and Langley, 1974; Gillespie, 1976, 1977). It is an inescapable truism that if various genotypically distinguishable individuals differ in survival probabilities, they must differ in some physiologically (biochemically) important way. Then it must also be the case that if relative differences in these survival probabilities are inconstant, the biochemical differences must vary in their physiological impact. Indeed then, the evolutionary relevance of the biochemical differences which underlie the observed differences in survivorship depends upon the environmental conditions: Genotypes most favored under one set of conditions may be the least favored under another. In addition, some conditions may effectively favor no genotypes at all. In other words, genetic differences at some loci may be selectively important or neutral, depending on the environmental conditions. A concrete, albeit overly simplistic, example may be helpful. Genotypic differences in developmental rates or dispersal proved unimportant in producing temporal genetic change in the cohorts studied. They could have been extremely important had some environmental factor caused heavy mortality unilaterally upon the

"aquatic" and "terrestrial" subdivisions. Unseasonably cold weather (not unknown in the Lost Lake area) could easily have resulted in the deaths of a great number of "terrestrial" phase individuals, while having little effect on the "aquatic" ones. In this case differences in developmental rates would have been indirectly responsible for selection which would not have occurred in the absence of the cold weather. One might construct other equally plausible stories concerning this developmental dichotomy or that of "disperser" and "main" population phases, but this would be superfluous. It is quite evident that survivorship probabilities among genotypes need not differ, nor need they be constant when they do.

There is an additional consideration with regard to the question of variable fitness: Selection via differential gametic contribution. The directions of deviation between adults and young were strikingly uniform each year. However, differences in allele frequencies at some loci among cohorts of different years indicate annual variation in relative gametic contributions by different genotypes. The magnitudes, but not directions, of these late fitness differences apparently varied widely at several loci.

Taken together, the results of this investigation agree nicely with the theoretical concept of stochastic variation in relative fitnesses. It is plain that differences in the survival component of fitness among young *Bufo* carrying certain marker genotypes are not constant and that the factors affecting changes among them are less than deterministic. This may be true for adult late fitness components

as well. In addition to this evidence of a temporally stochastic element, there are indications of microgeographic variance in survivorship probabilities as well (Samollow, unpublished).

Bryant (1976) has criticized some of the forementioned theoretical concepts by pointing to what he considered some rather restrictive assumptions about the nature of fitness relationships. In particular, Bryant believes that true homeostatic biochemical superiority of heterozygotes may better explain genetic polymorphism than theories based upon genotype-environment interactions. The present data do not support the idea that heterozygotes are generally superior as would be expected under Bryant's contention of homeostatic "marginal" overdominance. If this were the case, heterozygotes would be expected to have increased in corrected relative frequency (i.e., D should have increased over time). Except for the rather convincing increase in EST-1 heterozygotes, this was not the case. Perhaps it is unreasonable to assume all polymorphisms to be maintained via the same processes.

It was stressed earlier that it is not possible to conclude that selection has occurred upon the particular enzyme-coding marker loci studied during this investigation. This is true. But it is also true that selection has occurred at some locus (loci) and while the product(s) of this locus may not be an enzyme, it is some biochemical product nonetheless. The implication of this is clear. The biochemically different products of competitive alleles may or may not be functionally different at the physiological level, depending upon

the exact environmental conditions. Experimental procedures designed to detect functional differences in the products of allelic variants must be considered in view of the relevant environmental heterogeneity and possible genotype-environment interaction. This underscores the pleas of Clarke (1975) and Johnson (1976) to redirect the approach to the question of evolutionary signficance of allozyme variation. Expanding knowledge of the biochemical roles of genic products is likely to lead to increased awareness of the environmental constraints germane to particular biochemical systems. Controlled manipulation of relevant environmental variation should permit direct testing of hypotheses concerning the physiological impact of biochemical differences based on allelic substitutions. This will surely be a more effective strategy in ending the controversy over the adaptive significance of enzyme variation than the search for correlation among environmental and genotypic variation or the examination of evolutionary distances between species.

SUMMARY

The genetic effects of heavy mortality during early life history stages in a natural population of Bufo boreas boreas were investigated by examining variation at nine polymorphic enzyme coding loci. All reproduction in this population takes place during a single breeding episode each year, hence, all young are of the same age; i.e., they constitute a single cohort. Gene and genotype frequencies were determined in twenty-five collections of pre-reproductive Bufo from three cohorts, over a three year period; 1973, 1974 and 1975. Frequencies in two samples of adult Bufo, collected in 1973 and 1975, were also determined.

There was no evidence of change among the adult gene frequencies during the period examined. However, allele and genotype frequencies in young Bufo were significantly different than those in the adults (= average of 1973 and 1975 adult frequencies) at every locus during at least two of the three years. At five loci, adults and young differed each year. Within cohorts, distinct shifts in allele and/or genotype frequency distributions were evident at six of the nine loci examined for temporal genetic change. Five of these six loci showed change in two of the three cohorts but only one was significantly heterogeneous in all cohorts.

After consideration of the possible influences of various evolutionary forces, it was concluded that natural selection was the primary cause of the observed inter-generation and intra-cohort

genetic heterogeneities. The effects of mutation and random processes were dismissed as negligible in this regard. Physical isolation (distance) of the study site precluded significant emigration. Non-random mating systems could not have brought about the allele frequency differences detected between adults and offspring. This left only selection at the level of adult (late) fitness components (fertility, fecundity or mating success) and/or early zygotic survival as the possible causes of inter-generation differences. The data appear to support the former alternative.

Intra-cohort (temporal) heterogeneity among genetic distributions appears to have resulted from selection via differential survival among genotypes. The effects of genetic differences in developmental rates were discussed and found insufficient to account for the genetic heterogeneity within cohorts. Dispersal was, in general, random with respect to genotype. Weak evidence of differences in rates of dispersal was found at three loci but was not significantly contributory to temporal genetic change in the cohorts examined. It was concluded that the heavy, early life history stage mortality, characteristic of this (and other) _Bufo boreas_ populations, is not genetically random.

Evidence of heterozygote superiority in survivorship was sought among all loci. Only one showed any convincing tendency for the proportion of heterozygotes observed to increase over time relative to the proportion expected.

The data indicate that genotypic differences in the survivorship component of fitness and among the late fitness elements vary in time, presumably in response to changes in environmental conditions. Fitness rankings shift relative to one another. Under some conditions, there appear to be no fitness differences among the bearers of different genotypes. These observations agree with theoretical considerations regarding the maintenance of genetic polymorphism in natural populations. In particular, the data support the concept of multiple-niche polymorphism.

ACKNOWLEDGEMENTS

I am grateful to Peter S. Dawson and Russel A. Riddle for helpful criticism of the manuscript and lively discussion of several aspects of this project. Thanks are due Suh Y. Yang for his cheerful instruction in basic electrophoretic technique and to Joseph J. Beatty, R. A. R. and John T. Rotenberry for their help with the collection of adult toads. I especially thank Catherine M. Samollow for her assistance in the field and laboratory as well as her editorial comments during preparation and typing of the manuscript. The cooperation of the U.S. Forest Service; Willamette National Forest, was invaluable. Financial support came in part from a Sigma Xi grant-in-aid and grants from the Oregon State University and Humboldt State University computer centers.

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Table 1. Samples from the Lost Lake *Bufo boreas* population utilized in this study.

Collection ID Number	Cohort Year	Date of Capture Day/Mo/Yr	Area of Collection[a]	Life Stage[b]	Sample Size
10	?	6-13/5/73	W, S	AD	94
36	?	1-30/6/75	W, S	AD	105
13	1973	28/5/73	W	TP	144
14	1973	13/6/73	W	TP	146
12	1973	1/7/73	W	TP	144
17	1973	20/7/73	W	TP	144
18	1973	20/7/73	S	META, TL	144
21	1973	6/8/73	S	TL	144
20	1973	6-11/8/73	A	TL	144
22	1973	3/9/73	S	TL	140
23	1973	3/9/73	A	TL	143
26	1973	4/10/73	S	TL	114

Table 1 continued.

Collection ID Number	Cohort Year	Date of Capture Day/Mo/Yr	Area of Collection	Life Stage	Sample Size
32	1973	11-17/7/74	S	TL	64
27	1974	3/8/74	W	TP	144
28	1974	21/8/74	W	TP	200
46	1974	13/9/74	S	TL	144
30	1974	29/9/74	S	TL	144
37	1974	13/6/75	S	TL	144
39	1974	2/7/75	S	TL	142
40	1975	20/7/75	W	TP	144
41	1975	13/8/75	W	TP	144
42	1975	24/8/75	S	META, TL	144
43	1975	24/8/75	W	TP, META	144

Table 1 continued.

Collection ID Number	Cohort Year	Date of Capture Day/Mo/Yr	Area of Collection	Life Stage	Sample Size
47	1975	15/9/75	S	TL	150
48	1975	15/9/75	A	TL	149
50	1975	18/10/75	S	TL	150
51	1975	18/10/75	A	TL	95

[a]W = captured in water (or on objects floating in water).

S = captured on land, close to shoreline (non-dispersing).

A = captured on land, away from shoreline (dispersing).

[b]TP = Tadpole, META = Transforming, TL = Toadlet, AD = Adult.

Table 2. Allele frequencies in adult Bufo boreas from Lost Lake; 1973, 1975 and both years combined.

Locus	Allele	1973	1975	Pooled[a]
APH-1	Sample size	94	105	199
	S	.091	.081	.085
	M	.771	.776	.774
	F	.138	.143	.141
EST-3	Sample size	94	104	198
	1.00	.117	.106	.111
	1.06	.298	.322	.311
	1.09	.457	.442	.449
	1.15	.128	.130	.129
GOT-2	Sample size	94	105	199
	S	.053	.033	.043
	M	.931	.933	.932
	F	.016	.033	.025
LDH-1	Sample size	94	105	199
	S	.080	.048	.063
	F	.920	.952	.937

Table 2 continued.

Locus	Allele	1973	1975	Pooled
LDH-2	Sample size	94	104	198
	S	.511	.418	.462
	M	.308	.337	.323
	F	.181	.245	.215
PEP-2	Sample size	94	105	199
	S	.527	.571	.550
	F	.473	.429	.450
PGI-1	Sample size	94	104	198
	S	.665	.712	.698
	M	.197	.192	.195
	F	.138	.096	.116
PGM-2	Sample size	94	105	199
	S	.904	.843	.872
	F	.096	.157	.128

[a]Discrepencies between pooled frequencies and weighted averages of 1973 + 1975 frequencies are due to rounding error.

Table 3. Genotypic variation in larval and juvenile *Bufo boreas* from Lost Lake: 1973, 1974 and 1975 cohorts. Chi-squares are for goodness-of-fit of observed genotypic distributions to those expected based on individual sample allele frequencies (using Levene's unbiased formula for small samples). "Zygotic" frequencies are genotype frequencies expected based on n-nomial expansion of pooled adult "parental" allele frequencies (see text) and are not used for these chi-square tests.

Collection		Sample Size	Genotype						df[a]	χ^{2}[b]	D[c]
APH-2			S/S	M/M	F/F	S/M	S/F	M/F			
1973	14	121	.008	.587	.033	.190	.025	.157	2	1.45	-.058
	12	142	.007	.739	.000	.049	.042	.162	2	16.08***	-.073
	17	109	.018	.716	.018	.101	.000	.147	2	6.78*	-.126
	18	143	.007	.636	.014	.140	.014	.189	2	0.27	+.009
	21	145	.014	.676	.021	.090	.028	.172	2	4.43	-.123
	22	138	.022	.601	.044	.094	.007	.232	2	10.24**	-.134

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
APH-2 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1973	26	114	.053	.561	.018	.193	.009	.167	2	7.22*	-.118
	32	63	.016	.635	.032	.127	.016	.175	2	0.22	-.122
	20	141	.021	.773	.007	.064	.021	.114	2	10.97**	-.202
	23	120	.000	.725	.017	.083	.033	.142	2	5.63	-.095
1974	27	124	.000	.686	.008	.129	.008	.169	2	0.78	+.055
	46	133	.008	.699	.015	.120	.023	.135	2	1.66	-.079
	30	142	.000	.704	.028	.148	.014	.106	2	4.01	-.097
	37	144	.000	.660	.028	.154	.000	.160	2	4.53	-.017
	39	140	.000	.607	.029	.193	.043	.129	2	3.31	-.053

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
APH-2 continued			S/S	M/M	F/F	S/M	S/F	M/F			
1975	40	144	.007	.667	.014	.215	.000	.097	2	2.82	+.019
	41	67	.015	.552	.015	.209	.000	.209	2	0.59	+.057
	43	140	.021	.650	.021	.107	.014	.186	2	4.21	-.111
	42	129	.000	.698	.016	.093	.023	.171	2	1.25	-.035
	47	144	.035	.688	.007	.118	.035	.118	2	9.75**	-.270
	50	146	.007	.630	.048	.130	.034	.151	3	10.06*	-.171
	48	166	.007	.621	.012	.133	.018	.211	2	0.03	+.030
	51	91	.022	.648	.022	.143	.044	.121	2	5.19	-.166
"Zygotic"			.007	.599	.020	.132	.024	.218			

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
EST-1			S/S	M/M	F/F	S/M	S/F	M/F			
1973	17	47	.000	.499	.149	.000	.021	.340	1	2.21	-.216
	18	141	.007	.504	.099	.050	.014	.326	2	3.02	-.132
	21	138	.007	.435	.087	.029	.043	.399	2	4.36	-.028
	22	133	.015	.368	.150	.060	.023	.383	2	1.49	-.113
	26	112	.000	.482	.071	.054	.027	.366	2	0.09	-.007
	32	63	.000	.365	.111	.063	.016	.444	2	0.70	+.042
	20	141	.007	.511	.050	.071	.028	.333	2	0.39	-.010
	23	142	.000	.465	.134	.085	.021	.296	2	8.51*	-.172
1974	46	131	.014	.518	.050	.078	.014	.326	2	0.18	-.030
	30	136	.000	.640	.118	.037	.015	.191	2	23.50***	-.387
	37	143	.007	.583	.063	.021	.035	.336	2	7.06*	-.078

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
EST-1 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1974	39	135	.015	.511	.081	.044	.022	.326	2	2.75	-.126
1975	43	17[d]	.000	.882	.000	.059	.000	.059	-	-	-
	42	125	.000	.696	.088	.032	.008	.176	2	18.55***	-.343
	47	140	.000	.550	.136	.093	.000	.221	2	23.65***	-.300
	50	144	.028	.493	.118	.028	.014	.319	2	8.49*	-.238
	48	160	.013	.550	.100	.056	.050	.231	2	17.17***	-.263
	51	91	.011	.516	.132	.066	.011	.264	2	7.88*	-.265
"Zygotic"					NONE						

Table 3 continued.

Collection		Sample Size	Genotype								df	χ^2	D
EST-3			1.06/1.06	1.09/1.09	1.00/1.06	1.00/1.09	1.06/1.09	1.06/1.15	1.09/1.15	Others[e]			
1973	14	130	.100	.231	.054	.054	.262	.093	.108	.100	4	7.54	-.065
	12	143	.070	.140	.049	.063	.196	.098	.280	.105	4	5.09	+.044
	17	143	.084	.287	.028	.077	.217	.126	.133	.049	4	6.32	-.052
	18	135	.067	.244	.059	.089	.385	.052	.052	.052	4	7.04	+.070
	21	145	.076	.200	.028	.055	.310	.117	.124	.090	4	1.32	-.002
	22	136	.052	.309	.015	.074	.265	.096	.147	.044	4	3.24	+.002
	26	114	.105	.237	.009	.053	.342	.096	.113	.035	4	2.21	-.010
	32	62	.016	.290	.016	.032	.355	.097	.145	.048	4	4.22	+.092
	20	143	.049	.294	.007	.056	.231	.084	.224	.056	4	2.20	+.028
	23	141	.064	.340	.036	.071	.241	.071	.149	.028	4	1.39	-.029

Table 3 continued.

Collection		Sample Size	Genotype								df	χ^2	D
EST-3 continued.			1.06/1.06	1.09/1.09	1.00/1.06	1.00/1.09	1.06/1.09	1.06/1.15	1.09/1.15	Others			
1974	27	130	.138	.245	.046	.077	.231	.046	.131	.077	4	9.55*	-.150
	46	143	.119	.259	.042	.070	.245	.049	.182	.035	4	7.47	-.093
	30	143	.098	.252	.021	.084	.259	.133	.105	.049	4	7.45	-.050
	37	142	.070	.239	.028	.070	.303	.092	.176	.021	4	3.21	+.058
	39	142	.049	.317	.042	.077	.303	.056	.113	.042	4	1.23	+.025
1975	40	139	.085	.302	.043	.065	.317	.058	.094	.036	4	1.57	-.043
	41	143	.035	.308	.049	.077	.273	.056	.161	.042	4	1.58	+.054
	43	143	.049	.252	.007	.056	.336	.112	.154	.035	4	5.91	+.098
	42	144	.139	.361	.042	.069	.229	.028	.097	.035	4	13.07*	-.206
	47	143	.084	.294	.021	.084	.315	.084	.098	.021	4	3.46	+.012

Table 3 continued.

Collection		Sample Size	Genotype								df	χ^2	D
EST-3 continued.			1.06/1.06	1.09/1.09	1.00/1.06	1.00/1.09	1.06/1.09	1.06/1.15	1.09/1.15	Others			
1975	50	144	.049	.285	.021	.035	.347	.056	.181	.028	4	3.98	+.111
	48	165	.048	.352	.024	.024	.273	.030	.182	.067	4	9.54*	-.021
	51	82	.037	.268	.024	.049	.244	.012	.293	.073	4	7.23	+.076
"Zygotic"			.097	.202	.069	.100	.279	.080	.117	.057			
GOT-2			S/S	M/M	F/F	S/M	S/F	M/F					
1973	13	145	.014	.766	.014	.097	.000	.110			2	8.21*	-.127
	14	142	.007	.796	.007	.099	.007	.085			1	1.13	-.082
	12	144	.000	.861	.000	.063	.000	.076			1	1.12	+.052
	17	143	.000	.783	.000	.161	.000	.056			1	2.04	+.092

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
GOT-2 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1973	18	143	.000	.825	.000	.112	.000	.063	1	1.25	+.069
	21	145	.000	.841	.000	.062	.000	.097	1	0.97	+.061
	22	138	.007	.826	.000	.094	.000	.073	1	0.12	-.015
	26	114	.000	.790	.000	.097	.000	.114	1	1.50	+.081
	32	63	.000	.730	.000	.206	.000	.064	1	1.43	+.116
	20	143	.014	.811	.000	.112	.000	.063	1	0.55	-.067
	23	142	.017	.831	.000	.106	.007	.049	1	0.76	-.050
1974	27	140	.007	.764	.007	.086	.007	.129	1	0.36	-.049
	28	140	.007	.821	.000	.100	.000	.071	1	0.13	+.014
	46	143	.000	.832	.000	.126	.000	.042	1	1.16	+.070
	30	143	.007	.853	.000	.070	.000	.070	1	0.22	-.040

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
GOT-2 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1974	37	144	.000	.833	.000	.104	.000	.063	1	1.14	+.064
	39	142	.000	.873	.000	.092	.007	.028	1	0.35	-.002
1975	40	144	.000	.896	.000	.090	.000	.014	1	0.39	+.045
	41	143	.000	.804	.007	.070	.000	.119	1	0.24	+.007
	43	143	.000	.874	.007	.063	.000	.056	1	0.49	-.062
	42	144	.000	.903	.007	.042	.014	.035	1	15.04***	-.203
	47	144	.000	.903	.000	.049	.000	.049	1	0.34	+.035
	50	146	.000	.863	.000	.096	.000	.041	1	0.74	+.053
	48	166	.006	.904	.000	.060	.000	.030	1	0.60	-.083
	51	91	.000	.868	.000	.099	.000	.033	1	0.41	+.051
"Zygotic"			.002	.869	.001	.079	.002	.047			

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
LDH-1[f]			S/S	F/F	S/F			
1973	13	145	.014	.800	.186	1	0.12	-.028
	14	142	.010	.802	.190	1	0.16	+.033
	12	143	.000	.853	.147	1	0.11	+.075
	17	142	.021	.789	.190	1	0.87	-.078
	18	143	.000	.832	.168	1	1.14	+.088
	21	145	.007	.828	.165	1	0.02	+.011
	22	138	.007	.848	.145	1	0.04	-.016
	26	114	.000	.851	.149	1	0.69	+.076
	32	63	.000	.810	.190	1	0.10	+.097
	20	143	.007	.783	.210	1	0.40	+.052
	23	142	.007	.824	.169	1	0.02	+.013

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
LDH-1 continued.			S/S	F/F	S/F			
1974	27	141	.014	.816	.170	1	0.38	-.052
	28	150	.020	.867	.113	1	6.35*	-.202
	46	143	.014	.846	.140	1	1.29	-.093
	30	143	.007	.881	.112	1	0.44	+.025
	37	144	.028	.799	.173	1	3.21	-.147
	39	142	.000	.831	.169	1	1.16	+.083
1975	40	144	.014	.812	.174	1	0.30	-.045
	41	143	.000	.790	.210	1	1.90	+.113
	43	143	.007	.860	.133	1	0.11	-.021
	42	144	.000	.813	.187	1	1.48	+.100
	47	144	.021	.785	.194	1	0.72	-.070

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
LDH-1 continued.			S/S	F/F	S/F						
1975	50	146	.000	.822	.178				1	1.34	+.094
	48	166	.024	.735	.241				1	0.14	-.029
	51	91	.033	.868	.099				1	11.86***	-.350
"Zygotic"			.004	.878	.118						
LDH-2			S/S	M/M	F/F	S/M	S/F	M/F			
1973	13	135	.244	.030	.051	.304	.244	.126	3	3.66	+.089
	14	137	.212	.037	.044	.336	.270	.102	3	7.31	+.152
	12	128	.273	.016	.023	.320	.250	.117	3	7.46	+.160
	17	142	.254	.042	.070	.239	.253	.141	3	0.78	+.012
	18	143	.217	.105	.049	.266	.259	.105	3	2.51	-.012
	21	145	.241	.069	.062	.269	.249	.110	3	0.46	-.007

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
LDH-2 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1973	22	137	.241	.066	.066	.270	.263	.095	3	1.35	+.008
	26	114	.281	.088	.061	.246	.211	.114	3	1.68	-.089
	32	63	.238	.079	.048	.286	.254	.095	3	0.42	+.006
	20	143	.266	.070	.063	.252	.259	.091	3	1.31	-.024
	23	142	.282	.085	.056	.254	.247	.078	3	2.98	-.052
1974	27	128	.281	.016	.079	.250	.219	.156	3	6.34*	+.011
	46	143	.350	.098	.021	.189	.217	.126	3	10.02*	-.105
	30	143	.175	.105	.049	.308	.259	.105	3	4.17	+.045
	37	144	.236	.090	.049	.264	.216	.146	3	0.40	-.018
	39	140	.243	.100	.036	.164	.250	.207	3	11.76**	-.042

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
LDH-2 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1975	40	144	.278	.056	.042	.264	.236	.125	3	0.48	+.022
	41	126	.206	.040	.087	.262	.230	.175	3	2.77	+.030
	43	143	.210	.126	.042	.322	.161	.140	3	0.31	-.019
	42	144	.201	.083	.076	.257	.264	.118	3	1.28	-.008
	47	144	.292	.063	.049	.257	.259	.083	3	0.91	-.003
	50	146	.226	.062	.069	.322	.219	.103	3	3.28	+.026
	48	164	.207	.085	.079	.232	.207	.189	3	2.15	-.042
	51	91	.242	.121	.055	.264	.165	.154	3	1.89	-.091
"Zygotic"			.213	.105	.046	.299	.198	.139			

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
PEP-2			S/S	F/F	S/F			
1973	13	142	.268	.141	.591	1	5.61*	+.198
	14	142	.282	.197	.521	1	0.30	+.046
	12	141	.255	.220	.525	1	0.32	+.047
	17	111	.351	.198	.540	1	0.75	-.082
	18	124	.379	.186	.435	1	1.22	-.099
	21	144	.305	.160	.535	1	1.14	-.089
	22	133	.346	.188	.466	1	0.30	-.048
	26	114	.342	.184	.474	1	0.12	-.033
	32	63	.413	.095	.492	1	0.47	+.086
	20	81	.383	.160	.457	1	0.17	-.045
	23	98	.388	.153	.459	1	0.11	-.033

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
PEP-2 continued.			S/S	F/F	S/F			
1974	27	112	.366	.188	.446	1	0.76	-.087
	28	118	.297	.229	.474	1	0.30	-.051
	46	143	.398	.154	.448	1	0.38	-.051
	30	143	.378	.119	.503	1	0.82	+.075
	37	144	.306	.187	.507	1	0.09	-.087
	39	142	.394	.169	.437	1	1.00	-.083
1975	40	144	.306	.222	.472	1	0.39	-.052
	41	81	.346	.234	.420	1	1.97	-.155
	43	118	.348	.186	.466	1	0.26	-.047
	42	142	.359	.148	.493	1	0.11	+.028
	47	111	.396	.162	.442	1	0.55	-.070

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
PEP-2 continued.			S/S	F/F	S/F						
1975	50	146	.308	.274	.418				1	4.06*	-.166
	48	122	.369	.107	.424				1	1.83	+.122
	51	59	.322	.102	.576				1	2.43	+.201
"Zygotic"			.303	.202	.495						
<u>PGI-1</u>			S/S	M/M	F/F	S/M	S/F	M/F			
1973	13	130	.531	.054	.000	.208	.200	.008	3	10.99*	-.023
	12	143	.490	.021	.021	.245	.175	.049	3	0.57	+.004
	17	143	.511	.035	.000	.224	.210	.021	3	5.02	+.046
	21	143	.566	.049	.007	.182	.182	.014	3	8.82*	+.077
	22	138	.536	.020	.000	.268	.138	.029	2	0.53	+.043
	26	114	.597	.026	.035	.167	.123	.053	3	8.43*	-.183

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
PGI-1 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1973	32	63	.492	.064	.000	.302	.127	.016	2	1.10	-.013
	20	143	.580	.063	.000	.168	.161	.028	3	12.91**	-.134
	23	142	.528	.035	.000	.197	.225	.014	3	7.49	+.038
1974	27	136	.515	.044	.000	.250	.169	.022	3	3.43	+.013
	28	148	.635	.034	.007	.162	.149	.013	3	5.09	-.083
	46	143	.601	.084	.000	.161	.154	.000	2	21.92***	-.120
	30	143	.483	.070	.014	.217	.189	.028	3	7.17	-.097
	37	143	.518	.077	.007	.238	.140	.021	3	6.95	-.119
	39	142	.493	.084	.000	.261	.162	.000	2	11.56**	-.067

Table 3 continued.

Collection		Sample Size	Genotype						df	χ^2	D
PGI-1 continued.			S/S	M/M	F/F	S/M	S/F	M/F			
1975	40	143	.615	.042	.000	.210	.133	.000	2	5.73	-.039
	41	143	.650	.077	.000	.189	.070	.014	2	13.11**	-.242
	43	142	.648	.049	.000	.127	.162	.014	2	9.01*	-.138
	42	144	.583	.063	.014	.160	.046	.035	3	12.29**	-.196
	47	144	.604	.063	.000	.194	.139	.000	2	11.42**	-.115
	50	146	.658	.048	.007	.164	.110	.014	2	8.82*	-.170
	48	166	.578	.030	.006	.211	.157	.018	3	1.65	-.014
	51	91	.604	.022	.000	.264	.110	.000	2	2.31	+.070
"Zygotic"			.475	.038	.014	.268	.160	.045			

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
PGM-2 [f]			S/S	F/F	S/F			
1973	13	135	.793	.007	.200	1	0.22	+.039
	14	140	.743	.029	.228	1	0.70	+.070
	12	143	.748	.028	.224	1	0.78	+.073
	17	143	.727	.028	.245	1	0.30	-.045
	18	143	.769	.000	.231	1	2.29	+.127
	21	145	.724	.021	.255	1	0.01	+.007
	22	138	.783	.043	.174	1	7.89**	-.236
	26	114	.746	.026	.228	1	0.41	-.059
	32	63	.730	.000	.270	1	1.44	+.147
	20	143	.755	.007	.238	1	0.86	+.077
	23	142	.711	.014	.275	1	0.61	+.065

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
PGM-2 continued.			S/S	F/F	S/F			
1974	27	117	.692	.026	.282	1	0.01	+.011
	28	85	.812	.012	.176	1	0.06	+.003
	46	143	.839	.007	.154	1	0.00	-.003
	30	143	.748	.021	.231	1	0.08	-.024
	37	144	.646	.056	.297	1	1.16	-.087
	39	142	.662	.014	.324	1	1.84	+.113
1975	40	144	.646	.048	.306	1	0.42	-.053
	41	48	.833	.021	.146	1	1.21	-.154
	43	143	.706	.042	.252	1	1.51	-.102
	42	143	.776	.014	.210	1	0.00	-.002
	47	144	.708	.028	.264	1	0.06	-.020

Table 3 continued.

Collection		Sample Size	Genotype			df	χ^2	D
PGM-2 continued.			S/S	F/F	S/F			
1975	50	146	.712	.000	.288	1	4.01*	+.164
	48	166	.705	.030	.265	1	0.15	-.030
	51	91	.802	.000	.198	1	0.21	+.104
"Zygotic"			.760	.016	.224			

[a] Degrees of freedom. Genotypic classes with expected numbers less than 1.0 were pooled. Where necessary, such classes were pooled with classes which had expected numbers greater than 1.0.

Table 3 continued.

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[b]Asterisks indicate chi-square values, the probabilities of which are less than; 0.05 (*), 0.01 (**) and 0.001 (***) when the true genotypic distribution and expected distribution are identical.

[c]$D = \frac{H_o - H_e}{H_e}$, where H_o and H_e are the observed and expected numbers of heterozygotes, respectively.

[d]Enzyme becomes detectable only in later stages of metamorphosis. This group had few individuals in these stages.

[e]Pooled: 1.00/1.00, 1.15/1.15 and 1.00/1.15.

[f]In order to preserve one degree of freedom, classes were not pooled.

Table 4. Results of interaction chi-square analysis performed on pre-reproductive Bufo boreas from Lost Lake: T = total chi-square, PO = pooled chi-square, H = heterogeneity chi-square (see text for further explanation). Asterisks indicate probability levels as in previous tables.

Locus	Year	Type of Chi-square	Alleles Value of Chi-square	Alleles Degrees of Freedom	Alleles Probability	Genotypes Value of Chi-square	Genotypes Degrees of Freedom	Genotypes Probability
APH-2	1973	T	37.03	16	**	43.15	24	**
		PO	7.63	2	*	13.17	3	**
		H	29.40	14	**[a]	29.98	21	-
	1974	T	25.53	10	**	39.09	15	***
		PO	18.81	2	***	28.14	3	***
		H	6.72	8	-	10.95	12	-
	1975	T	33.60	12	***	49.85	18	***
		PO	16.60	2	***	21.29	3	***
		H	17.0	10	-	28.50	15	*

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
EST-1[b]	1973	T				17.73	18	-
		PO				2.91	3	-
		H	9.26	8	-	14.82	15	-
	1974	T				27.14	12	*
		PO				9.19	3	*
		H	6.34	6	-	17.95	9	*
	1975	T				54.72	9	***
		PO				39.97	3	***
		H	13.84	4	**	14.75	6	*

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
EST-3	1973	T	116.96	24	***	146.57	56	***
		PO	47.13	3	***	53.06	7	***
		H	69.83	21	***	93.51	49	***
	1974	T	46.68	15	***	68.58	35	***
		PO	36.70	3	***	35.36	7	***
		H	9.98	12	-	33.22	28	-
	1975	T	103.44	18	***	132.42	42	***
		PO	90.33	3	***	89.71	7	***
		H	13.11	15	-	42.71	35	-

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
GOT-2	1973	T	61.38	18	***	85.73	18	***
		PO	45.97	2	***	46.88	3	***
		H	15.41	16	-	38.85	15	***
	1974	T	38.63	12	***	40.66	12	***
		PO	12.65	2	**	13.60	2	**
		H	25.98	10	**	27.06	10	**
	1975	T	30.84	12	**	27.01	12	**
		PO	4.36	2	-	2.19	2	-
		H	26.48	10	**	24.82	10	**

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
LDH-1	1973	T	40.31	9	***	36.93	9	***
		PO	32.04	1	***	31.01	1	***
		H	8.27	8	-	5.02	8	-
	1974	T	24.39	6	***	17.99	6	**
		PO	16.55	1	***	11.44	1	***
		H	7.84	5	-	6.55	5	-
	1975	T	40.26	6	***	37.96	6	***
		PO	32.88	1	***	33.14	1	***
		H	7.38	5	-	4.83	5	-

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
LDH-2	1973	T	56.08	18	***	75.40	45	**
		PO	48.95	2	***	52.83	5	***
		H	7.13	16	-	22.57	40	-
	1974	T	29.06	10	**	59.85	25	***
		PO	15.51	2	**	22.97	5	***
		H	13.55	8	-	36.88	20	*
	1975	T	40.66	12	***	49.41	30	*
		PO	19.13	2	***	20.40	5	**
		H	21.53	10	*	29.01	25	-

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
PEP-2	1973	T	16.07	9	-	22.80	18	-
		PO	3.50	1	-	4.12	2	-
		H	12.57	8	-	18.68	16	-
	1974	T	19.37	6	**	22.76	12	*
		PO	11.76	1	***	20.69	2	***
		H	7.61	5	-	2.07	10	-
	1975	T	9.79	6	-	17.18	12	-
		PO	1.89	1	-	6.09	2	*
		H	7.90	5	-	11.09	10	-

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
PGI-1	1973	T	24.78	14	*	33.56	21	*
		PO	14.02	2	***	17.62	3	***
		H	10.76	12	-	15.94	18	-
	1974	T	29.59	12	**	34.51	18	**
		PO	14.52	2	***	18.52	3	***
		H	15.07	10	-	15.99	15	-
	1975	T	73.42	12	***	93.76	18	***
		PO	63.93	2	***	80.87	3	***
		H	9.49	10	-	12.89	15	-

Table 4 continued.

Locus	Year	Type of Chi-square	Alleles: Value of Chi-square	Alleles: Degrees of Freedom	Alleles: Probability	Genotypes: Value of Chi-square	Genotypes: Degrees of Freedom	Genotypes: Probability
PGM-2	1973	T	5.05	9	-	3.38	9	-
		PO	0.84	1	-	0.37	1	-
		H	4.21	8	-	3.01	8	-
	1974	T	30.53	6	***	27.05	6	***
		PO	5.10	1	*	4.22	1	*
		H	25.43	5	***	22.83	5	***
	1975	T	22.36	6	**	18.19	6	**
		PO	9.44	1	**	7.70	1	**
		H	12.92	5	*	10.49	5	-

Table 4 continued.

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[a]Heterogeneity chi-square values are approximate only, but in cases where values border on the $\alpha = 0.05$ level of significance, exact tests were performed. Significance levels shown indicate the results of the exact tests in these cases.

[b]Expected genotype frequencies derived by trinomial squared expansion of average cohort allele frequencies.

Figure 1. APH-2 allele and heterozygote frequencies in samples of pre-reproductive Bufo boreas from Lost Lake. Circles = S allele, triangles = M allele, inverted triangles = F allele, squares = heterozygotes. Tadpoles are represented by open symbols. Toadlets are represented by solid symbols. Symbols (solid) on the ordinate represent "parental" allele frequencies and "zygotic" heterozygote proportions. Half-solid symbols represent the simple average of frequencies among "aquatic" and "terrestrial" sub-populations during the metamorphic period.

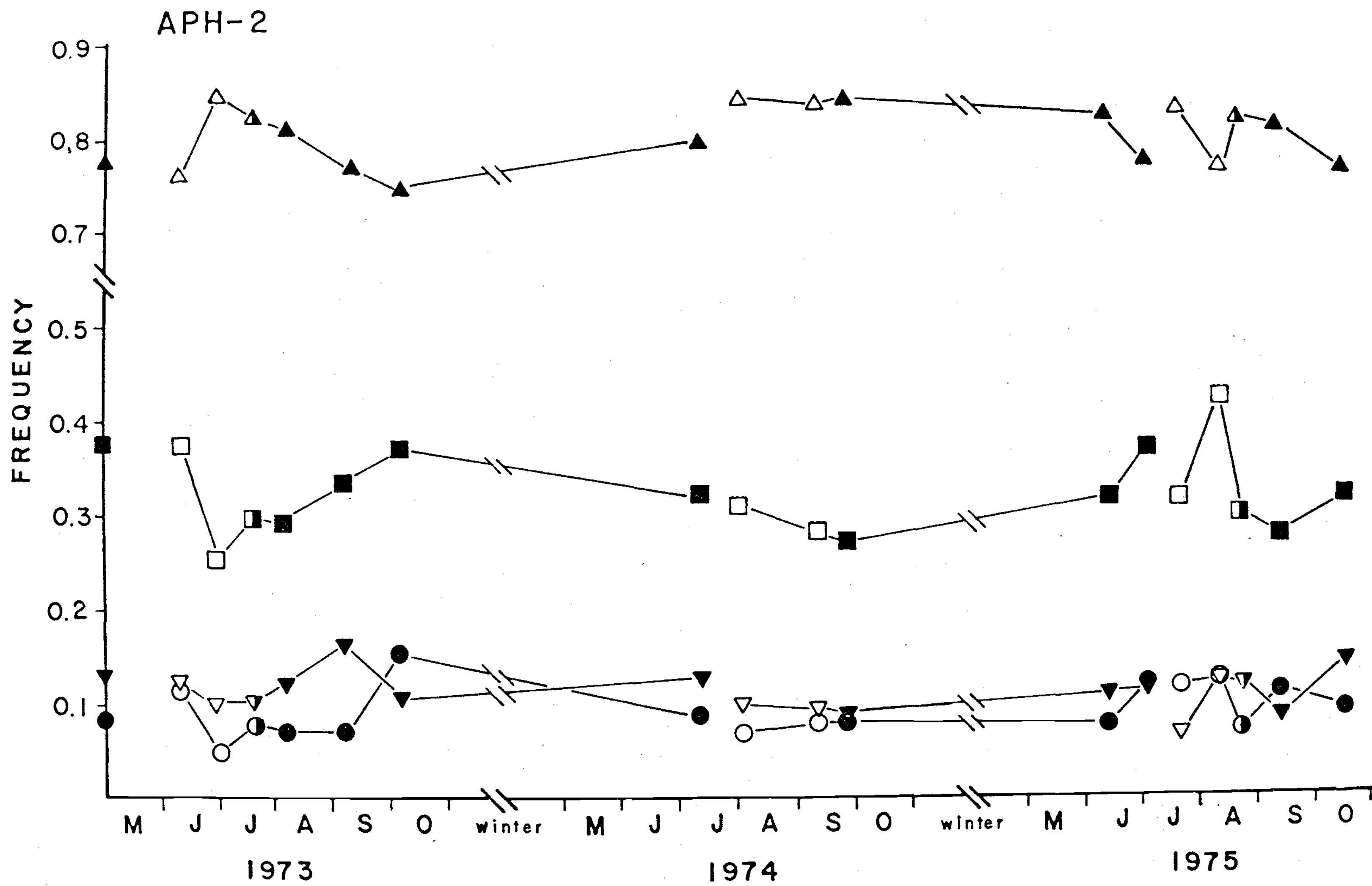

APH-2
FREQUENCY
0.9
0.8
0.7
0.5
0.4
0.3
0.2
0.1
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 2. EST-1 allele and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.

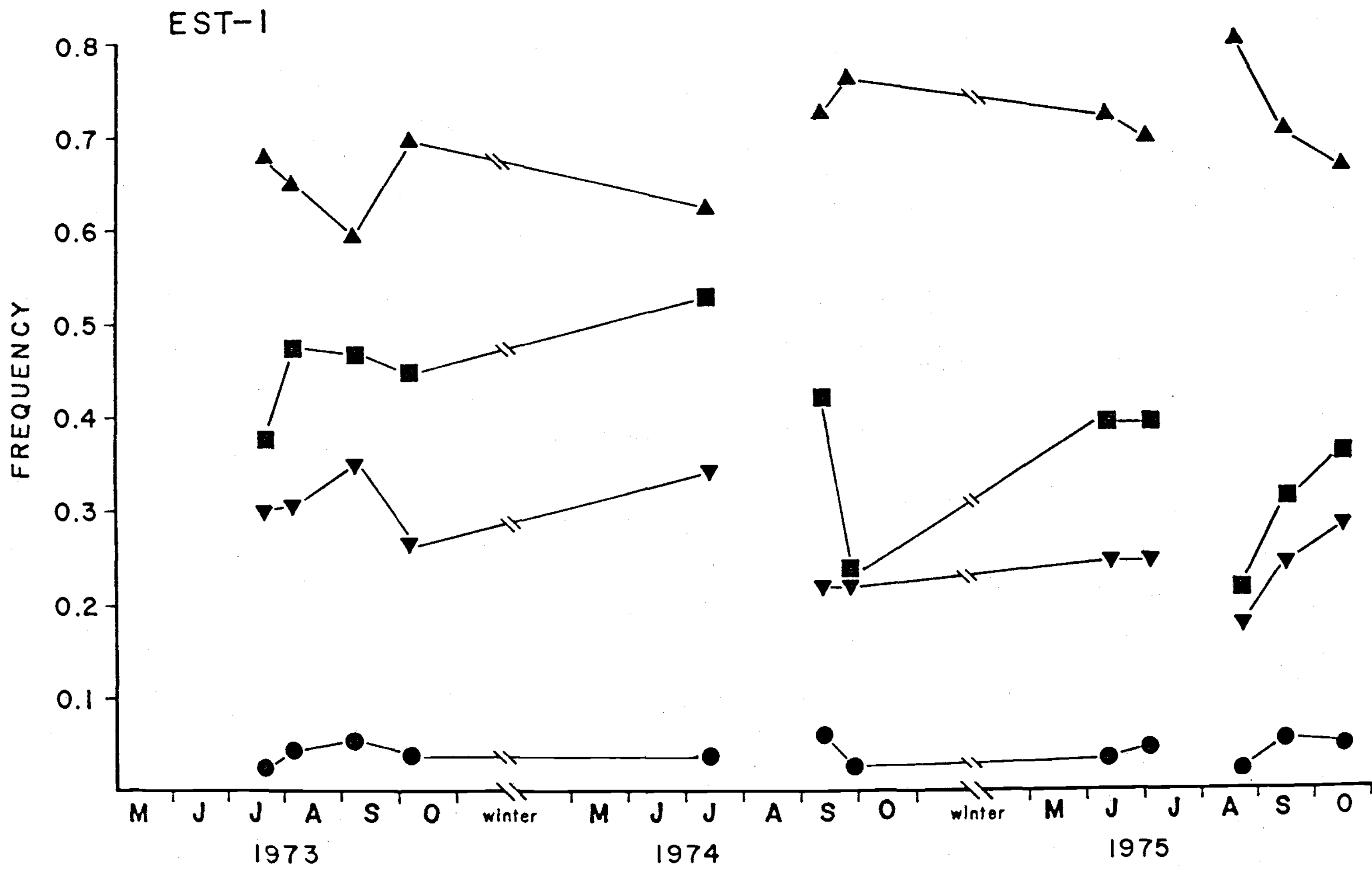

EST-1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FREQUENCY
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 3. EST-3 allele and heterozygote frequencies in samples of pre-reproductive Bufo boreas from Lost Lake. Circles = 1.00, triangles = 1.06, inverted triangles = 1.09, diamonds = 1.15. Tadpoles are represented by open symbols. Toadlets are represented by solid symbols. Symbols (solid) on the ordinate represent "parental" allele frequencies and "zygotic" heterozygote proportions. Half-solid symbols represent the simple average of frequencies among "aquatic" and "terrestrial" sub-populations during the metamorphic period.

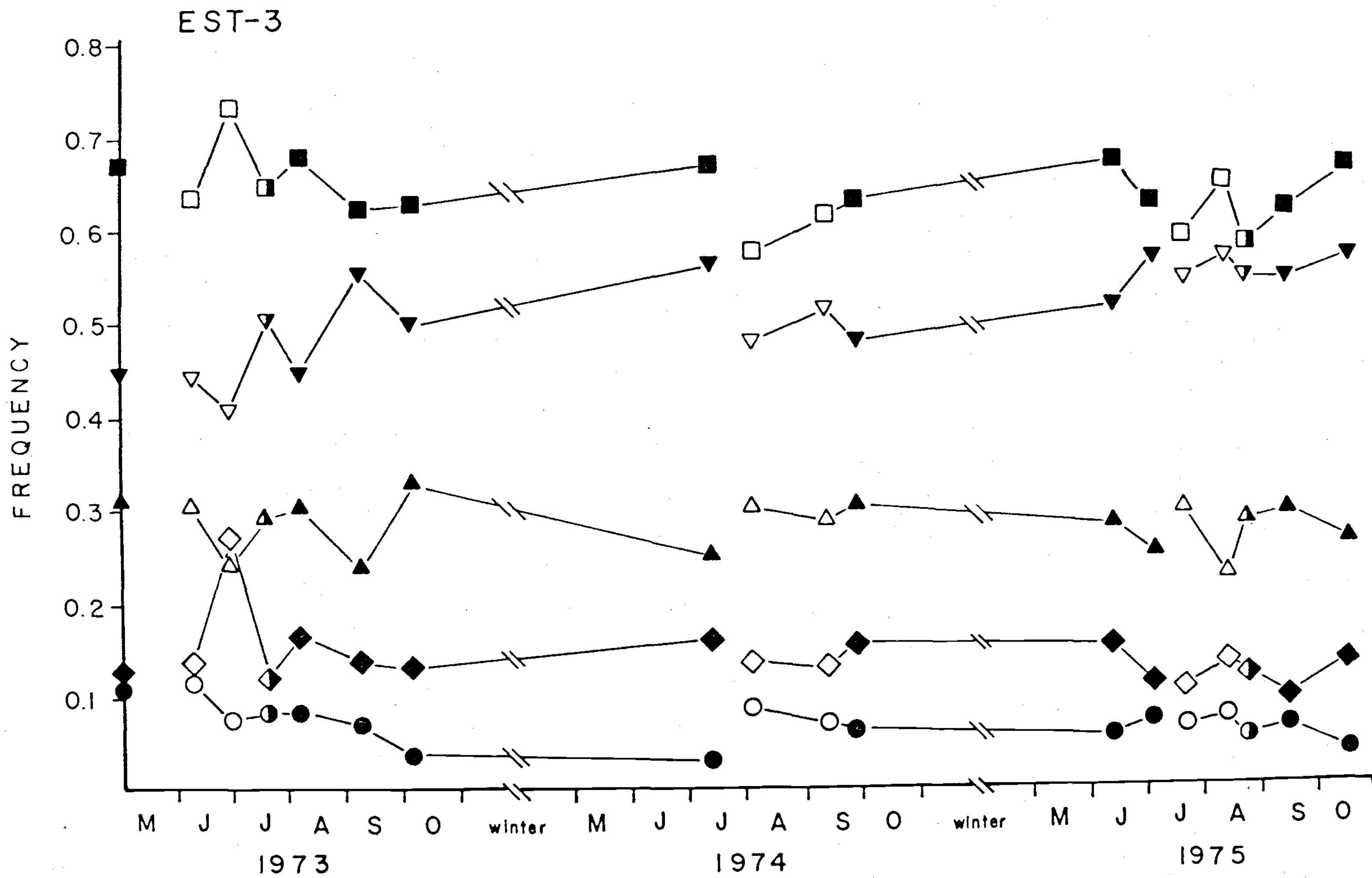

EST-3
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FREQUENCY
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 4. GOT-2 allele and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.

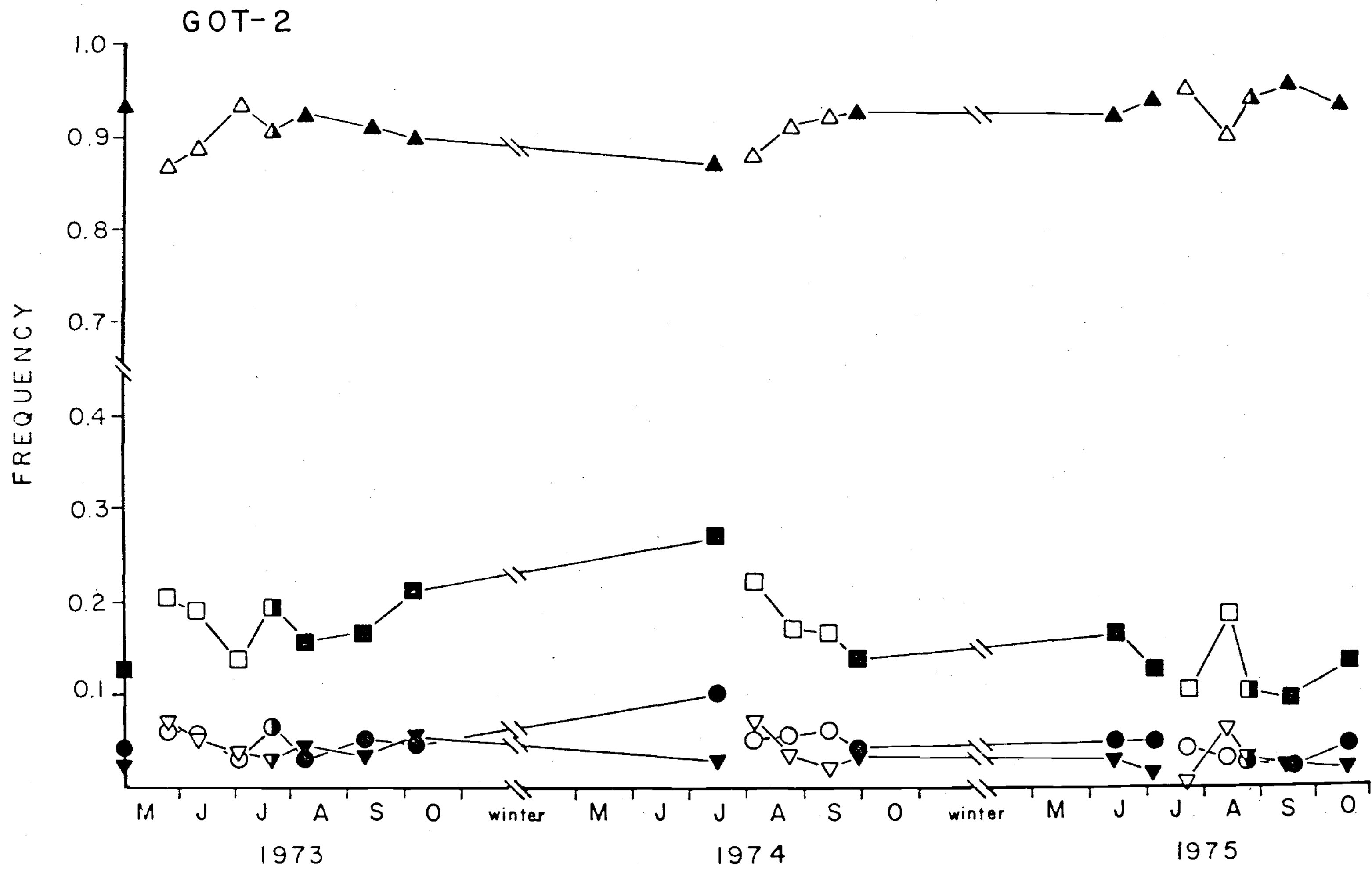
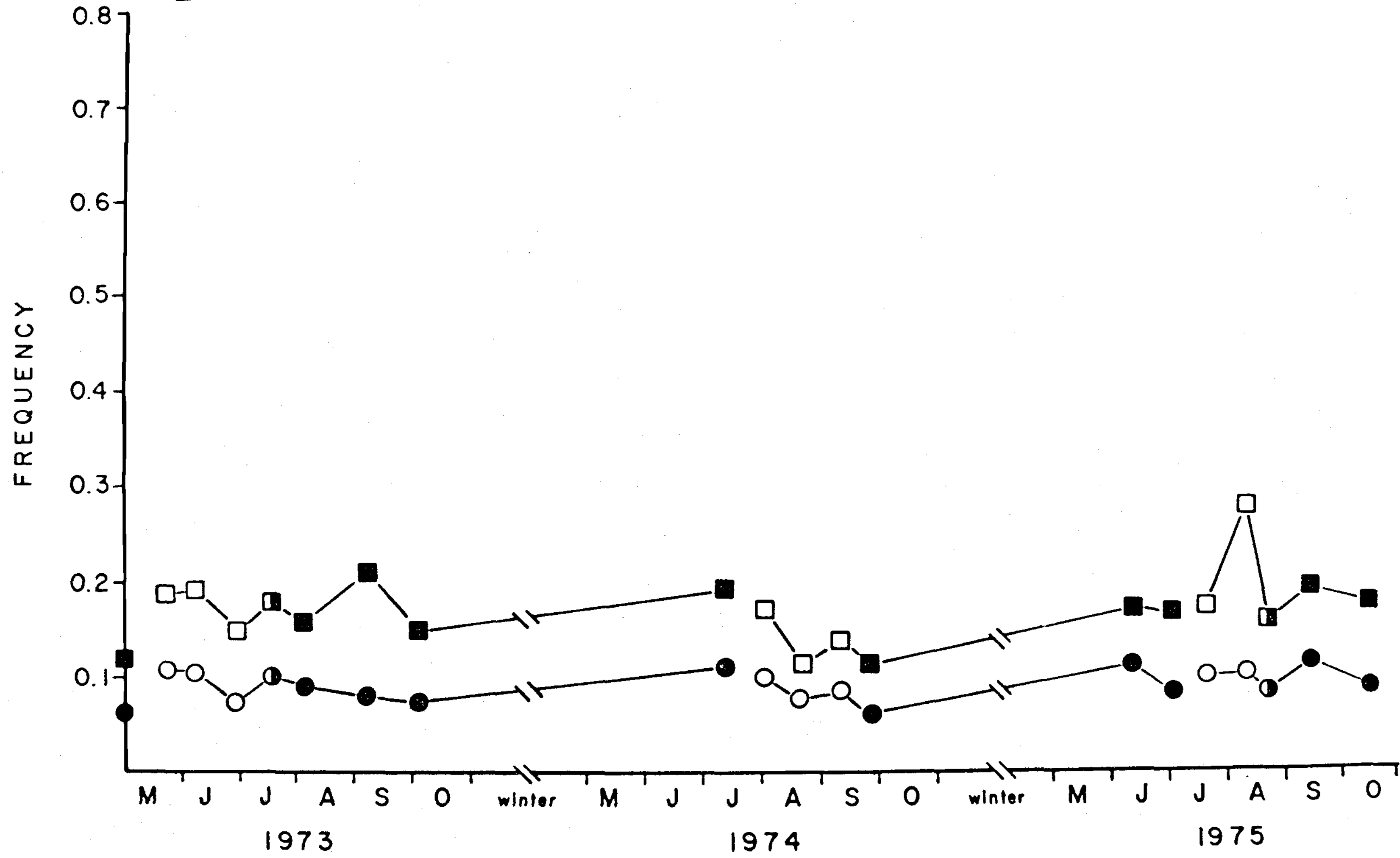

GOT-2
FREQUENCY
1.0
0.9
0.8
0.7
0.4
0.3
0.2
0.1
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 5. LDH-1 allele and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.



LDH-1
FREQUENCY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
M
J
J
A
S
O
winter
M
J
J
A
S
O
winter
M
J
J
A
S
O
1973
1974
1975

Figure 6. LDH-2 allele and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.

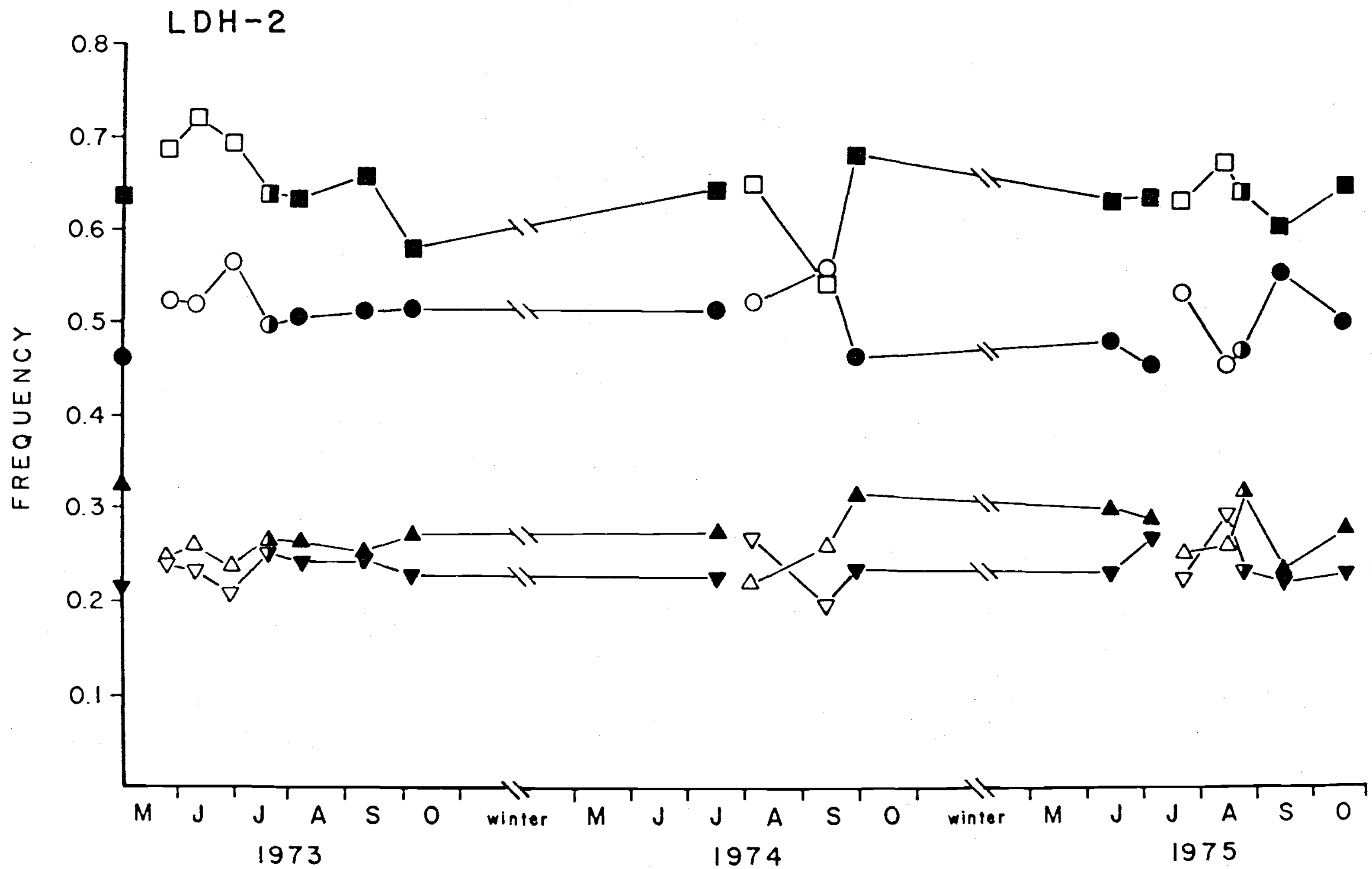

LDH-2
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FREQUENCY
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 7. PEP-2 allele and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.

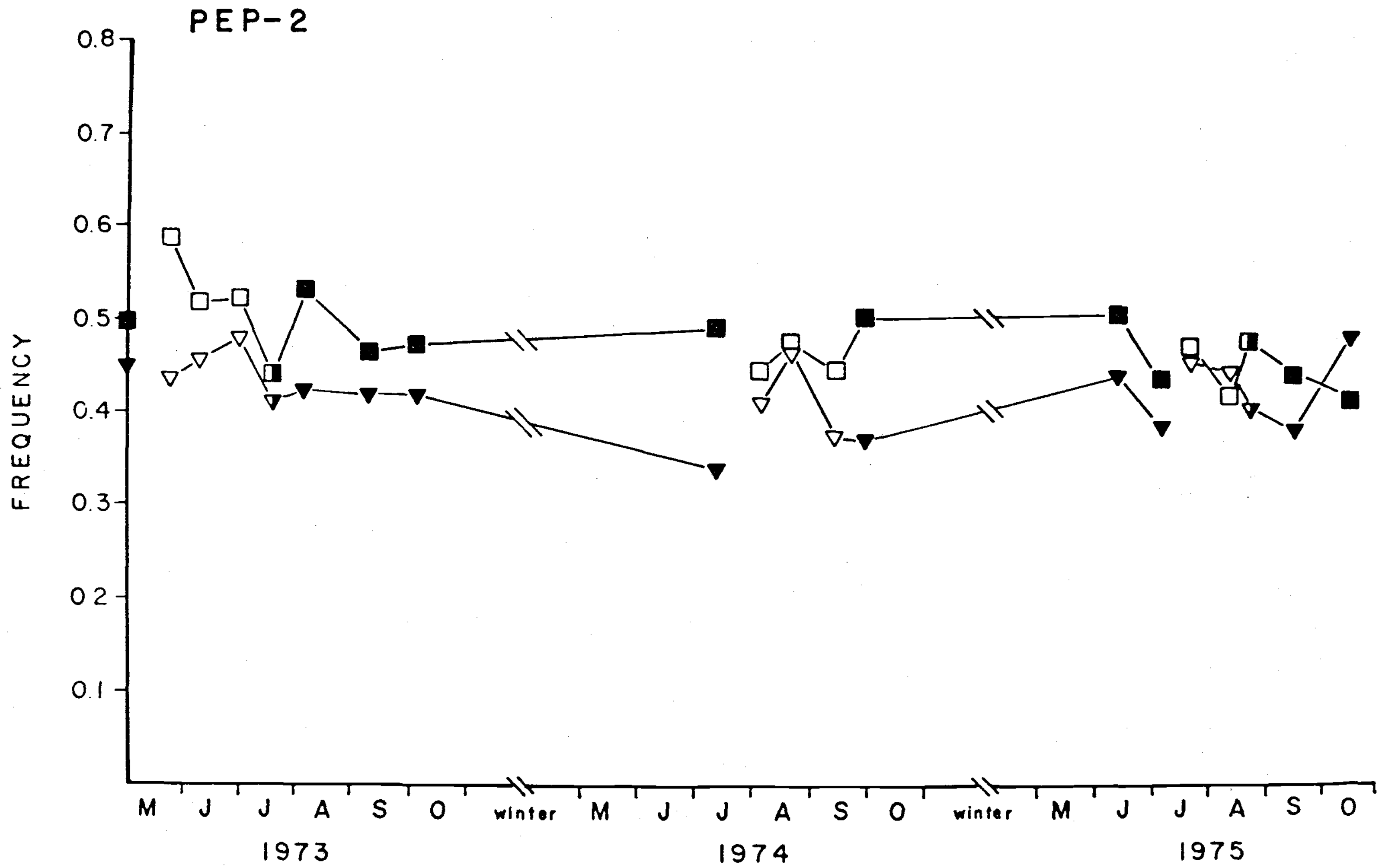

PEP-2
FREQUENCY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 8. PGI-1 allele and heterozygote frequencies in sample of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.

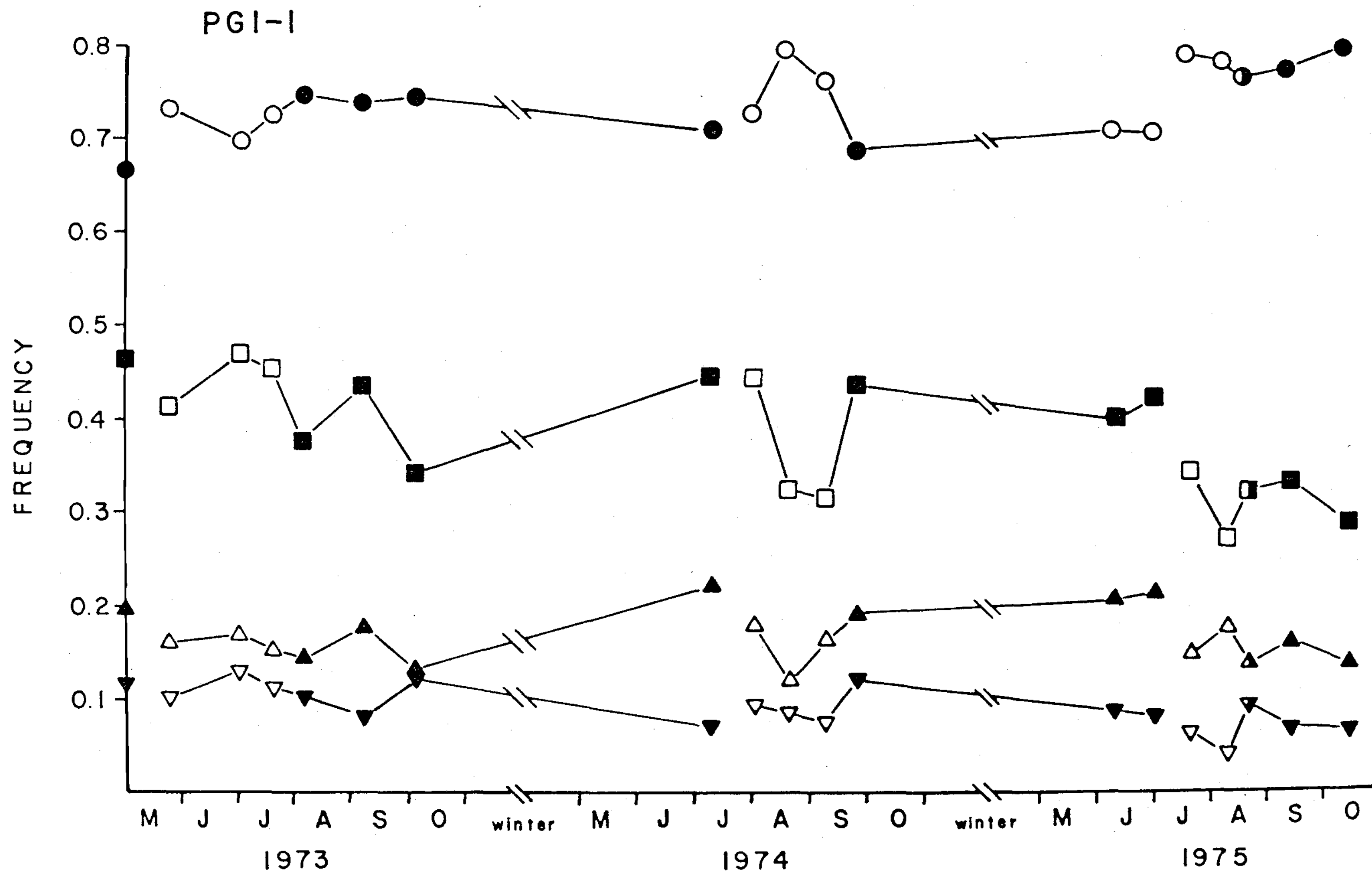
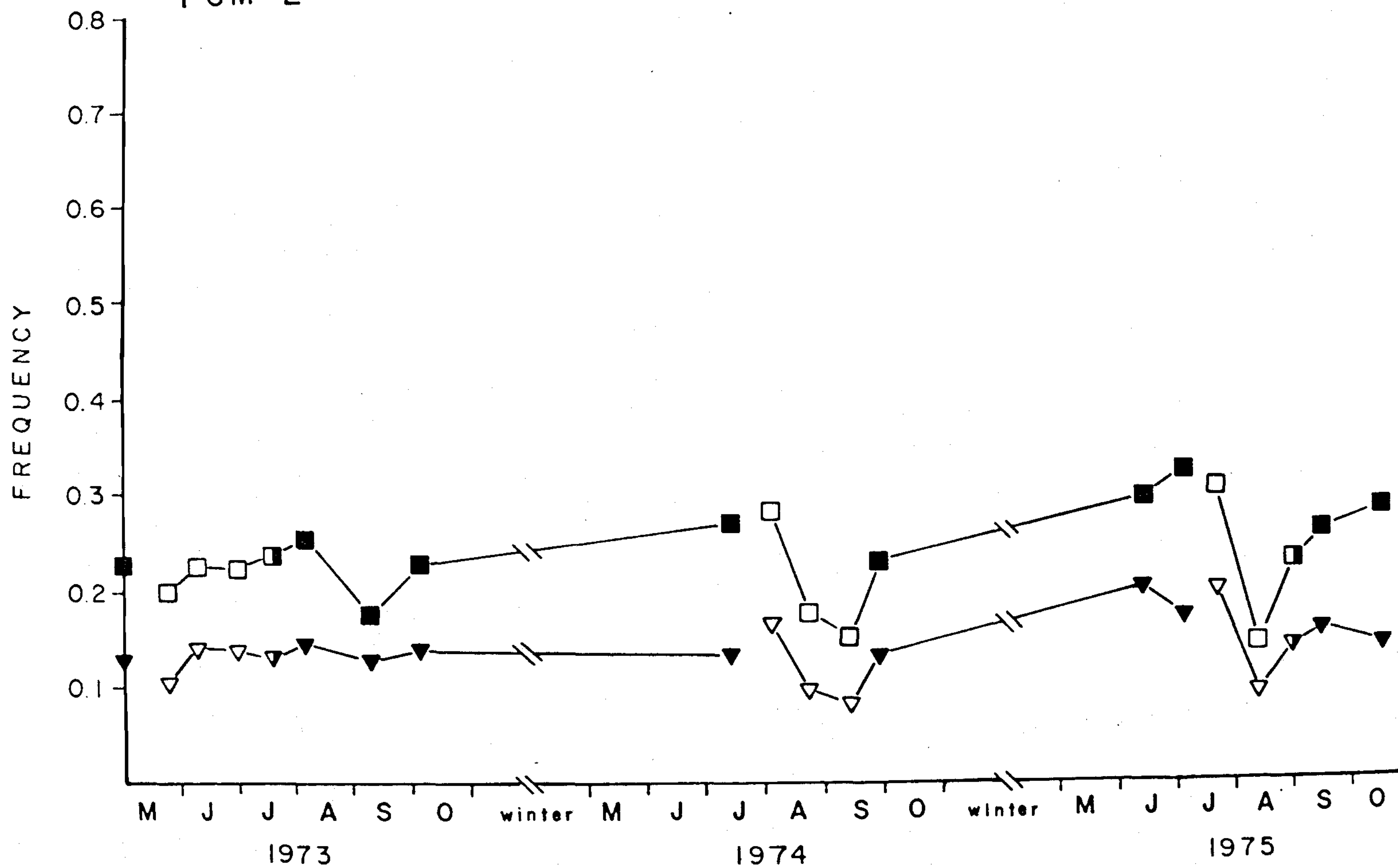

PGI-1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FREQUENCY
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

Figure 9. PGM-2 alleles and heterozygote frequencies in samples of pre-reproductive *Bufo boreas* from Lost Lake. Symbols are explained in Figure 1.


PGM-2
FREQUENCY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
M J J A S O winter M J J A S O winter M J J A S O
1973
1974
1975

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APPENDICES

APPENDIX I

THE BOREAL TOAD AND THE LOST LAKE STUDY SITE

The Boreal Toad: Bufo boreas boreas, the boreal subspecies of the western toad, is an excellent organism for genetic studies involving electrophoretic variation. The animals are geographically widespread, being found from Alaska to southern Nevada (the subspecies B. b. halophilus extends from the southern range of B. b. boreas well into Baja California), and from the Pacific Ocean into Montana, Colorado and Wyoming. Altitudinal distribution is remarkable. Populations may be found from sea level to over 10,000 feet (Stebbins, 1962, 1966). The animals are easily captured and transported at any life stage. Adults may be induced (without harm) to breed under laboratory conditions, facilitating genetic analysis of enzyme variation.

B. b. boreas is highly fecund. I have been unsuccessful in finding fecundity estimates for this species in the literature, but have personally investigated egg (zygote) number in masses from single pair matings in the laboratory. A conservative average estimate is over 12,000 eggs per mass (average from four masses). Livezey and Wright (1947) attribute an egg mass in nature containing approximately 16,500 ova to a single female of the subspecies B. b. exsul. The boreal toad reaches maturity at two to three years of age (Stebbins, 1951), and reproduces for several years although exact lifespan is unknown.

Lifetime reproductive output is, consequently, impressive; clearly a desirable feature with regard to this investigation.

The Population: Although I have found it impossible to ascertain the actual size of the Lost Lake adult *Bufo* population, I estimate it at no less than 2,000 and probably in excess of 5,000 individuals. Clearly this population can (and does) produce several million offspring each year. Furthermore, all of the breeding takes place in an explosive episode lasting only four to seven days during spring. Therefore, all young found at a given time may be considered to be of the same cohort. Obviously the mortality of larval and juvenile toads is very high. Few of the young appear to survive their first winter. Mortality prior to metamorphosis is also substantial, as it is during early post-metamorphosis, but the bulk of the disappearances (due to death or dispersal, which is genetically equivalent to death within the population) of young animals are during the late fall and early winter.

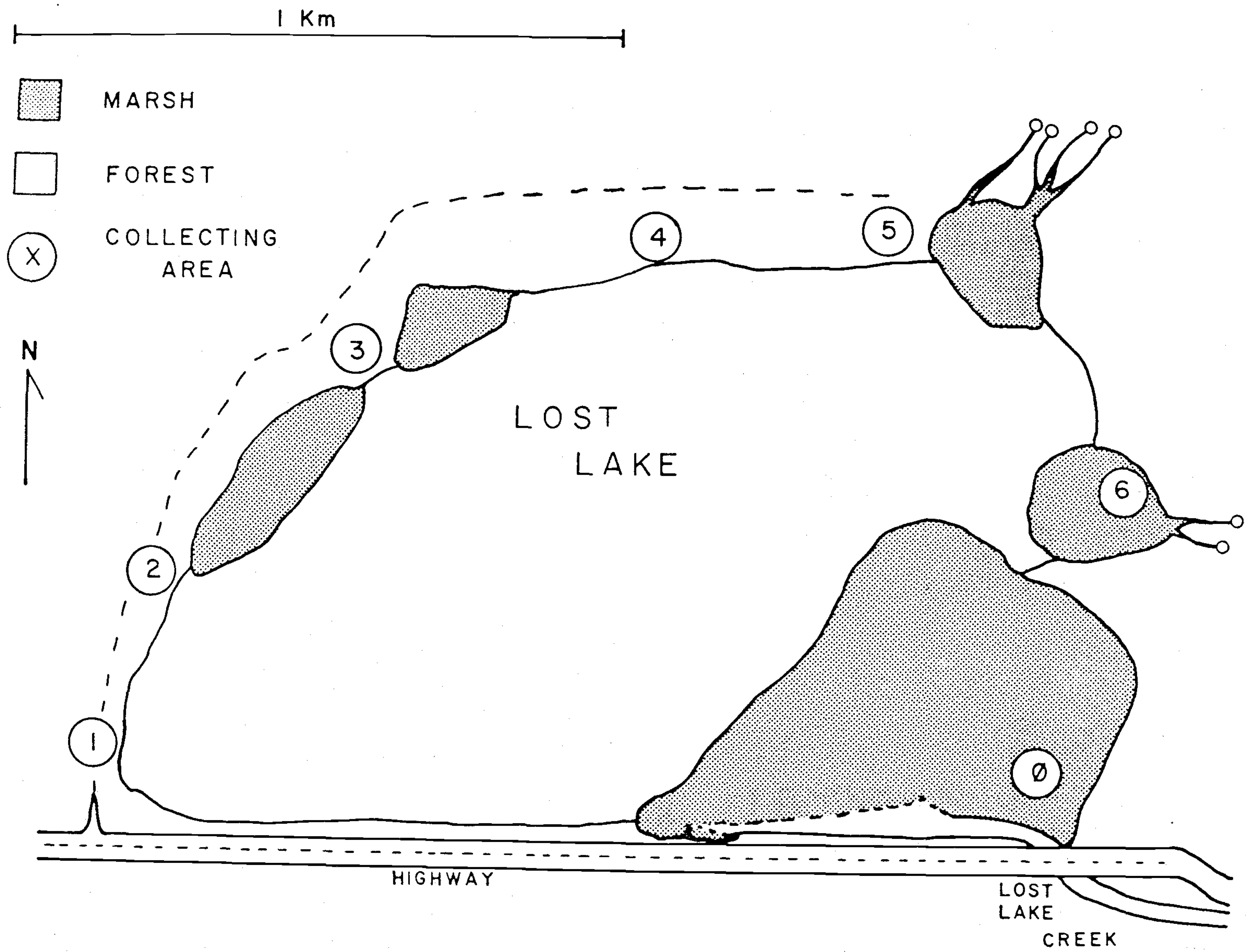
The Study Site: Certain ecological attributes of the study site suit it particularly well to this investigation. Lost Lake is located in the central Cascade Range at approximately 1200 m altitude. It lies in a small valley 130 km east of Corvallis, Oregon, near the Santiam pass. The volumes of flow from the lake's three water sources, two springs and a small creek, vary greatly due to seasonal variation in ground water and snowpack. Lost Lake has no surface outlet nor any known subsurface one. As a result of input variations, the depth and surface area of the lake vary tremendously, ranging from 5 m in depth

and 90-130 ha in area in the spring, to under 2 m depth and as little as 40-80 ha area in the late fall. The lake is isolated from other sizable bodies of water by approximately 10 km, a considerable distance when the mountainous terrain is considered. Although adult Bufo are somewhat vagile, distances of this magnitude assure that gene flow through immigration of small juveniles will be relatively unimportant. Immigration by larvae is impossible (without human intervention). Another notable feature of this site is the existence of two qualitatively different habitat types (Figure A1.1). The vegetation of the forest habitat consists of an unusually large number of coniferous species, the most obvious being Douglas fir (Pseudotsuga menziesii) and Grand fir (Abies grandis), with scattered angiosperm tree and shrub species in evidence. The marsh habitat consists of wet areas with patches of grass mixed with patches of willow (Salix sp.), cottonwood (Populus sp.), alder (Alnus sp.), and a variety of thorny shrubs. The marsh habitat is patchily distributed around the lake periphery, where it separates the lake from the forest. Between these patches, the forest reaches to the lake shore. However, the south-eastern lake edge is covered by a continuous marshy habitat of approximately 20-25 ha in area. The existence of qualitatively distinct "patch" types at the lake suggests investigation of population differentiation within and between habitats. Several investigations at the theoretical level (Levene, 1953; Haldane and Jayakar, 1963; Levins and MacArthur, 1966; Levins, 1968; Prout, 1968; Maynard Smith, 1970; Christiansen, 1974; Gillespie, 1974a, 1974b, 1977) indicate the

importance of population subdivision and the maintenance of polymorphism as a result of environmental patchiness. (My investigation was not designed to examine population subdividion, nevertheless, it has laid groundwork for more specifically designed work on this aspect of the genetic structure of the population.)

Juvenile toads (toadlets) seem restricted to moist areas during the first season. This limits them to the immediate vicinity of the lakeshore, the areas near the springs and the streams flowing from them, the marshy areas and inlet stream bank. The moist areas are rather discrete and, except in the case of the marsh, distinctly linear with orientation perpendicular to the shoreline. This feature facilitates the capture of dispersing individuals as they travel away from the lake along these moisture trails.

Figure AI.1. The Lost Lake study site. The area surrounding the Lake consists of "forest" and "marsh" habitat types (described in text) as indicated. Collecting areas refer to those described in Appendix II. The broken line indicates the position of an access road (dirt).



1 Km
MARSH
FOREST
X
COLLECTING AREA
N
LOST LAKE
1
2
3
4
5
6
Ø
HIGHWAY
LOST LAKE CREEK

APPENDIX II

POPULATION SAMPLING

Adult toads were captured in 1972 (June), 1973 (May) and 1975 (June). Excessive mortality among the 1972 specimens rendered the samples useless. Fifty collections of tadpoles and toadlets were made from 1972 to 1975. Of these, 27 collections were used in this investigation. Table 1 (main text) gives the identification code, date of collection, sample size, life stage and capture site for each of the adult collections and 25 of the 27 pre-reproductive samples. Data regarding the two remaining samples (C44 and C52) may be found in Appendix VIII. All samples listed in Table 1 were from collecting area 5 (collecting areas are described below). C44 was from area 3, while C52 came from area Ø.

The lake was arbitrarily divided into seven sampling areas: Ø, 1, 2, 3, 4, 5 and 6 as shown in Figure AI.1. Tadpole and toadlet occurrence at 1, 3, 4, and 5 was high while area 2 was seldom utilized by the young animals. Tadpoles were not seen at area 6, although toadlets were common. Area Ø is entirely terrestrial and had only toadlets. Sampling was restricted mainly to area 5. Sampling in other areas was carried out on a less rigorous schedule with the intention of making occasional genetic comparisons between young *Bufo* from these areas and those from the more extensively studied site (area 5). Sampling area 1 was not used.

Tadpole swarms frequented area 5 several times each day during the first several weeks post-hatching. At the beginning of metamorphosis, the tadpoles became more sedentary and spent longer periods at a given site. As individuals progressed to the semi-terrestrial stage; migratory movement ceased. Such transformation and emergence activities were restricted to a few specific areas each year: 1, 3 and 5. Presumably these activities went on in the large marsh as well.

Metamorphosis was not synchronous within the tadpole population. Individuals emerged from the water over a period of approximately two weeks. Undoubtedly there were a few stragglers that took even longer. During emergence, aquatic and terrestrial stages were individually sampled.

Dispersal of toadlets from the emergence areas was mainly perpendicular to the shore at area 5 due to the moist ground and lush vegetation provided by the springs located some distance from the lake itself (Figure A1.1). Some immigration from area 3 and area 1 to area 5 probably occurred but my impression is that this was a minor influx. The toadlet population at area 5 was divided into two distinct groups. Those individuals found close to shore were designated as non-dispersing individuals. Individuals found at a great distance from the shoreline (nearer the springs) were designated as dispersing individuals. An arbitrary dividing line between dispersing and non-dispersing sub-populations was set at approximately 65 m, but in

practice, non-dispersing individuals were captured within 10 m of shore while only animals collected at 80 m or more from shore were considered dispersing.

Toadlets utilized in this study were collected exclusively by hand capture. An autoentrapment method utilizing drift fences and pit-fall traps was unsuccessfully employed during the 1974 and 1975 seasons in an attempt to quantify dispersal and increase capture rates of dispersing individuals. Drift fences of 0.152 mm (6 mil) black plastic approximately 25 m long and 0.5 m high were constructed parellel to the lake shore at five locations; two at area 3, one at area 4 and two at area 5. Lake-to-fence distances were initially: Area 3, 45 m and 90 m; Area 4, 90 m; Area 5, 45 m and 90 m, but due to drastic seasonal changes in waterline position, these are only approximate relative distances and mean little on an absolute scale. Five pit-fall traps (three pound coffee cans) were set into the ground at regular intervals under each fence (lids were closed when traps were not in use to prevent inadvertent capture of animals). The concept of the method was that a dispersing individual would encounter the fence during movement away from the lake and attempt to move laterally along the fence in order to find a way around the barrier. During this lateral movement, the animal would encounter and fall into a pit-fall trap. The method was very successful in capturing numerous arthropods and small mammals but proved poor in toadlet entrapment.

Hand capture rates of toadlets far exceeded drift fence capture rates. The absolute numbers of toadlets captured by the latter method were so low that the method was abandoned.

APPENDIX III

BIOCHEMICAL TECHNIQUES

Electrophoretic Procedures: Starch-gel electrophoresis was conducted on apparatus constructed in our laboratory, modified from a design kindly supplied by Dr. S. Y. Yang. It consisted of the gel, in a lucite mold, supported over two electrode chambers, filled with buffer. Continuity between electrode chambers and gel was supplied by cellulose sponge, "sponge cloth" (Dupont), bridges. Gels were cooled during electrophoresis by a pan of ice supported above the gel mold by a glass plate. Additionally, the entire apparatus was placed into a refrigerator or cold room during operation. Several different constant-voltage power supplies were utilized but the most extensively employed were the Heathkit IP-17 and Buchler 3-1014A. All types performed satisfactorily and gave indistinguishable results.

Gels were prepared by mixing a slurry of Electrostarch (Otto Hiller, Madison, Wisconsin), lot 371 (which became unavailable) or lot 302, in an aqueous buffer at the ratio of 12 g/100 ml (lot 371) or 11.38 g/100 ml (lot 302). The slurry was swirled constantly while being heated to a rapid boil in a 1 L erlynmeyer flask. After about 10 seconds of vigorous boiling, the solution was removed from heat and degassed by application of vacuum to remove bubbles, then poured into the lucite mold and allowed to cool to room temperature. Molds employed in this study were generally 170 x 170 x 5 mm inside dimensions requiring a buffer volume of 225 ml. Particular

applications requiring deeper gels utilized a 170 x 150 x 10 mm mold requiring 325 ml of buffer for the gel solution. The thinner gels produced in the shallow molds dissipated heat far better than thicker gels and were also more economical of chemical supplies. It was possible to assay a maximum of three different enzyme systems on thin gels but up to five on thicker gels due to the different number of horizontal slices obtainable from each gel type (as explained below). After cooling, gels were covered with plastic film and kept at room temperature up to 24 hours until ready for use. Just prior to use, gels were refrigerated to 2-4°C.

Frozen adult toads were thawed and dissected to remove liver, kidneys, heart, eyes, skeletal muscles and gonads. All tissues (organs) were rinsed in distilled water, blotted and weighed. Using a teflon and glass tissue grinder, tissues were homogenized in a volume of grinding buffer (0.1 M tris, 1 mM Na_2EDTA, $5x10^{-5}$ M NADP, pH adjusted to 7.0 with hydrochloric acid). Tissue weight to buffer volumes used were; liver, kidney, eye and gonad, 1:1; heart and skeletal muscle, 1:2. Homogenates were centrifuged (at 0-2°C) for 20 minutes at 15,000 g to remove cellular debris. Supernatant extracts were placed in vials, stoppered and labelled. Tadpole and toadlet extracts were prepared in similar fashion except that whole animals were homogenized. The tissue to buffer ratio for tadpoles was 1:0.67 and for toadlets, 1:1.5. Each extract was placed in a separate vial, stoppered and labelled. Extracts were stored until needed at -76°C.

For electrophoresis, extract samples were absorbed onto filter paper "wicks" (Whatman #1 or #3 depending on enzyme system) of appropriate size (generally 4.5 x 4.5 mm or 2.5 x 4.5 mm for shallow molds, 9 x 4.5 mm for deep molds). Wicks were blotted to remove excess liquid, then inserted into the gel. The insertion of the wicks was at a site known as the origin, a slit cut across the gel approximately 25 to 35 mm from what was to be the anodal or cathodal end of the gel. The slit ran the full width and depth of the gel. The origin was positioned cathodally for anodally migrating proteins and anodally for cathodally migrating proteins. Twenty-five to 35 wicks (one extract sample per wick) could be inserted across the length of the origin, depending on width of wick.

After electrophoresis, the parts of the gel which were in contact with the bridge sponges were removed and discarded. The remainder of the gel was sliced horizontally into one to five 1.5 mm slices by means of tightly stretched 0.152 mm steel wire (g-banjo string; 0.006 inch diameter) held over 1.5 mm guides. It was then possible to stain each slice for a different enzyme system.

<u>Gel and Electrode Buffers:</u> The following is a partial list of buffer systems investigated during the course of this study. In all, 16 basic systems and numerous modifications were utilized. Those listed below give the best resolution, to date, of one or more of the enzyme systems studied.

A. Electrode: 0.135 M Tris, 43 mM citric acid, pH 7.0.

Gel: 9 mM Tris, 2.9 mM citric acid, pH 7.0.

Source: Shaw and Prasad, 1970.

Current: 175 volts, (15-18 v/cm).

B. Electrode: 0.223 M Tris, 86 mM citric acid, pH 5.8.

Gel: 7.8 mM Tris, 3 mM citric acid, pH 5.8.

Source: Schaal and Anderson, 1974.

Current: 150 volts, (13-15 v/cm).

C. Electrode: 0.3 M boric acid, 60 mM sodium hydroxide, pH 8.2.

Gel: 76 mM Tris, 5 mM citric acid, pH 8.7.

Source: Poulik, 1957.

Current: 250 volts, (22-25 v/cm).

D. Electrode: 0.138 M potassium phosphate (dibasic), 62 mM sodium hydroxide, pH 6.7.

Gel: 69 mM potassium phosphate (dibasic), 3.1 mM sodium hydroxide, pH 6.7.

Source: Selander et al., 1971.

Current: 110 volts, (9-11 v/cm).

E. Electrode: 0.687 M Tris, 0.157 M citric acid, pH 8.0.

Gel: 22.89 mM Tris, 5.22 mM citric acid, pH 8.0.

Source: Selander et al., 1971.

Current: 100 volts, (8-10 v/cm).

F. Electrode: 30 mM lithium hydroxide, 0.19 M boric acid, pH 8.1.

Gel: 3 mM lithium hydroxide, 19 mM boric acid, 45 mM Tris, 7.2 mM citric acid, pH 8.2.

Source: Selander et al., 1971.

Current: 250 volts, (22-25 v/cm).

G. Electrode: 0.169 M potassium phosphate (dibasic), 27 mM citric acid, pH 6.3.

Gel: 6.2 potassium phosphate (monobasic), 1.21 mM citric acid, pH 3.1.

Source: P. B. Samollow, unpublished.

Current: 50 volts (4-5 v/cm).

H. Electrode: 0.10 M Tris, 0.10 M maleic acid, 10 mM Na_2EDTA, 10 mM magnesium chloride, pH 7.4.

Gel: 10 mM Tris, 10mM maleic acid, 1 mM Na_2EDTA, 1 mM magnesium chloride, pH 7.4.

Source: Selander et al., 1971.

Current: 80 volts, (6-8 v/cm).

I. Electrode: 0.5 M Tris, 20 mM Na_2EDTA, 0.65 M boric acid, pH 8.0.

Gel: 50 mM Tris, 2 mM Na_2EDTA, 65 mM boric acid, pH 8.0.

Source: Selander et al., 1971.

Current: 200 volts, (18-20 v/cm).

J. Electrode: 87 mM Tris, 8.7 mM boric acid, 1.22 mM Na_2EDTA, pH 9.0.

Gel: Same as Electrode.

Source: S. Y. Yang, personal communication.

Current: 400 volts, (37-40 v/cm).

K. Electrode: 0.3 M boric acid, 60 mM sodium hydroxide, pH.8.2.

Gel: 10 mM Tris, pH 8.5 adjusted with hydrochloric acid.

Source: Selander et al., 1971.

Current: 250 volts, (22-25 v/cm).

Enzyme Assays: Assay techniques for 31 different catalytic activities were utilized during some part of this investigation. Nine of these techniques (acid phosphatase, adenylate kinase, aldehyde oxidase, alchol dehydrogenase activities utilizing ethanol and isopropanol substrates, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase and fumarase) revealed no useful electrophoretic patterns. Reasons for failure included; lack of enzyme activity, lack of substrate-specific activity, poor resolution. The remaining 22 techniques revealed one or more substrate specific areas of activity per gel which were nicely resolved or believed to be resolvable with minimal additional effort.

Receipes of these 22 "stains" and the description of the zones of enzymatic activity are given below. A clearly resolved zone is called a "locus". Each locus may be best resolved by a unique combination of

technical procedures. In the following table, information on technique and descriptions of the particular loci is provided in coded form.

FORM: a/b/c/d/e/f/g

a/ Tissue type used: TP = tadpole
TL = toadlet
L = liver
K = kidney
H = heart
S = skeletal muscle
G = gonad
E = eye

b/ Buffer system, run time in hours.

c/ Wick type: 1 = Whatman #1
3 = Whatman #3

In some cases, the symbol RW accompanied by two numerals may follow wick code. These denote that the wicks should be removed after the amount of run time indicated by the numerals. For example; 1, RW05 indicates that the #1 Whatman wick should be removed from the gel after about 5 minutes of electrophoresis.

d/ Migration of system: A = anodal migration
C = cathodal migration

This applies only for the indicated buffer system.

e/ Variation level: M = no variation detected
L = few variants detected
P = polymorphic
? = unknown

f/ Number of alleles: May be a single integer or a range if number of alleles is uncertain.

g/ Probable number of subunits of enzyme molecule:

M = monomer
D = dimer
T = tetramer
? = unknown

As an example: TP, TL, L/A, 3.5/2/3, RW10/A/P/4-6/D. This indicates that tadpoles, toadlets, and livers are suitable tissues. The system is best resolved on buffer system A when run for 3.5 hours. Whatman #3 wicks should be used and removed from gel after 10 minutes of electrophoresis. Migration is anodal. The system is polymorphic with four to six alleles and the molecule is apparently a dimer.

In the following receipes, NAD, NADP, NBT (Nitro-Blue Tetrozolium) and PMS (phenazine methosulfate) are in solution at a concentration of 1 mg per ml. MTT tetrazolium may be substituted for NBT in many applications.

Aldolase: 100 mg $Na_2AsO_4 \cdot 7\ H_2O$, 100 mg Na_4 fructose-1, 6-diphosphate, 50 units glyceraldehyde-3-phosphate dehydrogenase, 1 ml NAD, 2 ml NBT (MTT), 0.3 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (S. Y. Yang, personal communication).

ALD-1: TP, H, E/E, 3.5-5/3, RW10/A/M/1/?. Possible null allele.

ALD-2: Unresolved, anodal to ALD-1. Activity in TP, H.

Alakaline phosphatase: 50 mg β-napthyl acid phosphate, 50 mg Fast Blue BB, 0.3 ml 10% (w/v) $MgCl \cdot 6\ H_2O$, 0.3 ml 10% (w/v) $MnCl_2 \cdot 4\ H_2O$, 50 ml 0.05 M Tris-HCl buffer, pH 9.0. Incubate at 37°C until pink bands appear (modified from Ayala et al., 1972).

APH-1: Unresolved, anodal activity.

APH-2: TP, TL, L/A, 1/3/A/P/3/M.

Catalase: Soak gel in 0.5% H_2O_2 for 1 minute. Pour off H_2O_2 and rinse in tap water. Pour on 0.5% KI solution acidified with 0.5 ml of glacial acetic acid. Activity quickly appears as white bands on dark blue-black background. Watch carefully as bands fade rapidly. (Schaal and Anderson, 1974).

CAT-1: E/G, 3/3/C/M/1/?.

Esterase: 1 ml α-napthyl propionate solution (1 gm α-napthyl propionate dissolved in 50 ml acetone, diluted to 100 ml with H_2O), 25 mg Fast Blue RR salt, 10 ml isopropanol, 40 ml stain buffer (40 ml 0.2 M $NaH_2PO_4 \cdot 7\ H_2O$, H_2O to 1960 ml). Incubate at room temperature until dark bands appear (modified from Selander et al., 1971).

EST-1: TL/B or A, 3-5/1/A/P/4/M.

EST-2: Weakly staining locus.

TP/A or B, 3-5/3/A/L/2/M.

H/D, 5/3/A/L/2/M.

EST-3: TP, TL, H/B, 4.5/1, RW10/A/P/5-7/M.

EST-4: TL, L, K, H/B, 3.5-4/1/A/L/2/M. Little studied.

EST-5: Cathodal area, unstudied.

EST-6: G/B or A, 3.5/1/A/?/?/?. Little studied.

EST-7: SK/B, 3-4/A/?/?/?. Overlaps EST-3, use care in interpretation; little studied.

Glutamate dehydrogenase: 10 ml 0.1 M KH_2PO_4 buffer pH 7.0, 15 ml 1.0 M monosodium glutamate, 10 ml H_2O, 3 ml NAD, 2 ml NBT (MTT), 0.2 ml PMS. Incubate in dark at room temperature until blue bands appear (S. Y. Yang, personal communication).

GDH-1: K/1, 5/3, RW05/A/?/?/?. There is but one band per individual but bands vary in width and placement among individuals. This indicates hidden variation. Probably resolvable with more work. Very good bands.

Glutamate oxaloacetate transaminase (Aspartate Aminotransferase): 25 ml substrate solution (146 mg α-keto-glutaric acid, 532 mg L-aspartic acid, 2.0 g polyvinyl pyrrolidone, 200 mg Na_2EDTA, 5.68 g Na_2HPO_4, H_2O to 200 ml), 25 ml H_2O, 125 mg Fast Blue BB. Incubate in dark at room temperature until dark bands appear

(Schaal and Anderson, 1974).

GOT-1: TP, TL, E, K/C, 2-3/3, RW05/C/M/1/?.

GOT-2: TP, TL, E, K/C, 2-3/3or 1/A/P/3/D.

α-Glycerophosphate dehydrogenase: 1 ml 0.1 M $MgCl_2 \cdot 6\ H_2O$, 30 mg disodium α-D, L-glycerophosphate, 1 ml NAD, 1.5 ml NBT (MTT), 0.5 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Incubate in dark until blue bands appear (modified from Selander et al., 1971).

α-GPDH-1: K/1, 4-6/3, RW05/A/M/1/?.

Hydroxybutyrate dehydrogenase: 5 mg $MgCl_2 \cdot 6\ H_2O$, 287 mg NaCl, 630 mg sodium D, L-β-hydroxybutyric acid, 2.5 ml NAD, 1 ml NBT (MTT), 0.5 ml PMS, 50 ml 0.15 M Tris-HCl buffer, pH 7.0. Incubate in dark at 37°C until blue bands appear (F. J. Ayala, personal communication).

HBDH-1: SK/J, 4/?/A/?/?/?.

HBDH-2: K/J, 4/?/A/P/2-3/D?.

These loci should resolve with a bit of work.

Isocitrate dehydrogenase: 3 ml 0.1 M $MgCl_2 \cdot 6\ H_2O$, 3 ml 0.1 M trisodium D, L-isocitric acid, 1 ml NADP, 1 ml NBT (MTT), 0.5 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (modified from S. Y. Yang, personal communication). Add 1 ml of 2-mercaptoethanol to

gel when preparing gel for IDH.

IDH-1: TL, L, K, SK, H, E/E, 4-6/3, RW10/A/L/2/D.

IDH-2: TL, L, K/E, 4-6/3, RW10/A/L/2/D.

Lactate dehydrogenase: 5 ml 0.5 M sodium D, L-lactate, 1 ml NAD, 1 ml NBT (MTT), 0.05 ml PMS, 50 ml 0.2 M Tris-HCl buffer, pH 8.0. Inbubate in dark at 37°C until dark blue bands appear (modified from Selander et al., 1971).

LDH-1: TP, TL/A, 4-5/3, RW10/A/P/2/T.
H, E, G/A, 4-5/1, RW10/A/P/2/T.

LDH-2: TP, TL/A, 4-5/3, RW10/A/P/4/T.
H, E, G/A, 4-5/1, RW10/A/P/4/T.

LDH pattern are very complex due to the formation of heterotetramers among the subunits produced by both loci. A double homozygote has 5 bands.

Leucine amino peptidase: Soak gel 20 minutes in 0.5 M boric acid at room temperature. Pour off boric acid, rinse gel in water. Pour on a solution of 25 ml LAP buffer A (8 g NaOH, 19.6 g maleic anhydride, H_2O to 1 liter), 5 ml of LAP buffer B (0.32 M NaOH), 20 ml H_2O, 35 mg L-leucyl-β-napthylamide HCl, 15 mg Black K salt. Incubate in dark at 37°C until dark bands appear (Schaal and Anderson, 1974).

LAP-1: G/A or C, 3-4/3/A/M/1/?.

LAP-2: All Adult/F, 3-4/3/A/M/1/?.

Malate dehydrogenase (cytosol): 7 ml 2.0 M D,L-malic acid (pH adjusted to 7.0 with sodium hydroxide), 1 ml NAD, 1 ml NBT (MTT), 0.3 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (modified from Selander et al., 1971).

MDH-1: TP, TL, E/D, 6(C, 4)/1/A/L/4/D.

Malic enzyme (NADP dependent Malate dehydrogenase): Stain same as MDH except substitute NADP for NAD. Also tried at other pH's. Activity exactly same as MDH on all buffer systems. Appears that one enzyme catalyzes the reaction with NAD or NADP as the oxidative coenzyme.

"Nothing" dehydrogenase: A dehydrogenase activity which appears on many gels stained for other, specific, dehydrogenase activities. It requires no added substrate. Best activity revealed by the stain: 7 ml 0.1 M $MgCl_2 \cdot 6\ H_2O$, 0.1 ml NADP, 0.5 ml MTT (not NBT), 0.1 ml PMS, 10 ml 0.2 M Tris-HCl buffer, pH 8.0. Add this solution to 15 ml of a solution of 2% agar in water, cooled to 50°C, and mix thouroughly. Pour this mixture over gel. Incubate in dark at room temperature until blue bands appear.

NDH-1: L/B, 3.75/3, RW10/A/M/1/?.

NDH-2: L/B,3.75/3, RW10/A/P/3-4/M.

NDH activities appear sporadically in TP and TL preparations.

Octanol dehydrogenase (Long chain alcohol dehydrogenase: 1 ml octanol: ethanol solution (2:8), 1.5 ml NAD, 1 ml NBT (MTT), 0.5 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.5. Incubate in dark at 37°C until blue bands appear (modified from Schaal and Anderson, 1974). These systems have not been extensively investigated. There are at least two loci, possibly three. Buffer system H, 6 hours is best to date. Liver shows two areas of activity but kidney and gonad look promising as well.

Peptidase: 10 mg snake venom (*Crotalus atrox* or *C. adamanteus*, supplies L-amino acid oxidase activity), 10 mg O-dianisidine, 20 mg peroxidase, 35 mg peptide, 0.5 ml 0.25 M $MnCl_2 \cdot 4 H_2O$, 50 ml 0.05 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until orange bands appear, pour off stain, fix in a solution of glacial acetic acid: methanol: water (1:5:5).

PEP-1 (use D,L-leucyl-glycyl-glycine):

H/E, 4/3, RW10/A/P/2-3/M.

PEP-2 (use glycyl-L-leucine):

TP, TL, E, S/A, 3-4/3 or 1/A/P/2/D. Wick varies with TP samples.

Phosphoglucomutase: 250 mg disodium D-glucose-1-phosphate, 25 mg Na_2EDTA, 40 units G-6-PDH (Glucose-6-phosphate dehydrogenase), 0.5 ml NADP, 1 ml NBT (MTT), 0.1 ml PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (modified from Schaal and Anderson, 1974).

PGM-1: TP, TL, All Adult/B, 3-4/Any/A/M/1/?.

PGM-2: TP, TL, E/B, 3/3/A/P/2/M.

There is an unresolved area between PGM-1 and PGM-2.

Phosphoglucose isomerase: 4 ml 18 mM disodium D-fructose-6-phosphate, 1 ml NADP, 30 units G-6-PDH, 1.5 ml MTT (not NBT), 0.5 ml PMS, 10 ml of 0.1 M $MgCl_2 \cdot 6\ H_2O$, 30 ml 0.2 M Tris-HCl buffer, pH 8.0. Incubate in dark until blue bands appear (modified from Selander et al., 1971).

PGI-1: TP, TL/D, 5/1, RW05/S/P/3/?.

K/D, 5/3, RW05/S/P/3/?.

A very confusing pattern. Probably a dimer with much sub-banding but with Mendelian inheritance pattern. Genotypes easily discriminated but not

easily interpreted on molecular basis. Possible fourth allele.

PGI-2: Confusing anodal pattern in TP, TL, probably resolvable with work.

Sorbitol dehydrogenase: 250 mg D-sorbitol, 0.5 ml NAD, 0.5 ml NBT, 0.2 ml MTT, 0.1 ml PMS, 50 ml 0.2 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (S. Y. Yang, personal communication).

SDH-1: L/H, 6/3, RW10/S/P/4-5/?. Difficult to interpret. Needs more work.

SDH-2: L/H, 6/3, RW10/A/M/1/?.

Tetrazolium oxidase: 1 ml 0.1 M $MgCl_2 \cdot 6\ H_2O$, 1 ml MTT, 0.25 PMS, 50 ml 0.1 M Tris-HCl buffer, pH 8.0. Expose to bright light at room temperature until white bands appear on a blue background.

TO-1: L/B, 4/3, RW10/A/P/2/?.

Homozygotes of alternate alleles are easily discriminated but heterozygotes are revealed by merger of both slow and fast bands. Thus, a clear two or three band heterozygote pattern cannot be seen. Other TO activities are seen with other buffer systems and tissues but are not resolved.

"Dopa oxidase": 250 mg D,L-Dopa, 50 ml 0.05 M Tris-HCl, pH 8.0. Incubate in dark at 37°C until dark bands appear (U. Nur, personal communication).

DO-1: L, K, H/K, 2/3/A/M/1/?. Not well studied, needs more work.

<u>Xanthine dehydrogenase</u>: 25 mg hypoxanthine, 1 ml NAD, 1 ml NBT, 1 ml MTT, 0.5 ml PMS, 50 ml 0.05 M Tris-HCl buffer, pH 8.0. Incubate in dark at 37°C until blue bands appear (Selander et al., 1971).

XDH-1: L/E or K, 2-4?/3/A/?/?/?. Promising locus but not well studied.

Gels may be fixed for storage by either of two methods.

a). Cover gel with glycerine. After 2 hours, gel may be handled easily. Gel remains clear.

b). Cover gel with wash solution of glacial acetic acid: methanol: water (1:5:5). Let stand several hours. Gel becomes opaque white and is easily handled.

APPENDIX IV

GENETIC ANALYSIS OF ELECTROPHORETIC VARIATION

Verification of mendelian inheritance patterns for eight loci examined in the main body of this paper was performed by means of laboratory crosses. Adult Bufo boreas were induced to spawn "naturally" in the laboratory by following the procedure modified from Dorsch's (1967) scheme for induced spawning of the species: Place pair in a plastic pan (dishpan) filled to a depth of five to seven cm with dechlorinated (pond) water. Cover pan to reduce light and prevent escape of animals. Keep chamber at 18-20°C. Inject animals with either human chorionic gonadotropin (H.C.G.) or Bufo boreas pituitary glands in amphibian ringers solution, as indicated:

DAY	TIME	FEMALE	MALE	COMMENT
0	Mid P.M.	None	750 u H.C.G.	Omit if amplexus has occurred.
1	Late P.M.	750 u H.C.G.	None	Amplexus should have occurred by this time
2	Mid A.M.	750 u H.C.G.	None	
3	Late P.M.	750 u H.C.G.	500 u H.C.G.	Male dose may be omitted if amplexus is strong
5	Mid P.M.	500 u H.C.G. 4 pituitaries	None	
6	——— Oviposition should have occurred ———			

Fertilization rate generally exceeds 95-98%.

Since spawns were very large, several samples of 40 zygotes were separated from an "egg" mass and placed into small plastic pans (11.1 x 11.1 x 5.4 cm: length x width x height) containing about 500 ml of dechlorinated water. When tadpoles became free swimming (about 7 days at 20°C), they were placed, in groups of 25, into large pans (25.4 x 45.8 x 15.24 cm) filled with dechlorinated water. At 10-14 days post-oviposition, nourishment in the form of pelleted rabbit food was added sparingly to each pan. Growth was rapid and mortality virtually nil if the water was kept clean by frequent (daily) changes.

The genetic bases of enzyme variation were investigated for eight of the loci utilized in this study: APH-2, EST-3, GOT-2, LDH-1, LDH-2, PEP-2, PGI-1 and PGM-2. EST-1 could not be studied genetically because its activity was not detectable in adults or tadpoles. Formal genetic analysis schemes involving extensive test-crossing and F_2 production were not practical in this study. The basic technique employed was a simple parent/offspring comparison. Parental genotypes could not be determined before breeding since electrophoretic analysis required dissection of the animals. Instead, random pairs of adults were crossed to produce an F_1, then sacrificed. Genotypes were determined to see if the pair had provided an appropriate cross. If so, the F_1 were later sacrificed and analyzed.

The data gathered through the method described are consistent with the idea that variation at the several loci mentioned above is

inherited in a mendelian fashion (Table AIV.1). Naturally, due to the haphazard mating scheme, not every possible combination of crosses was realized. However, in no cross did I encounter unexpected genotypes among the F_1, nor a lack of expected ones.

Table AIV.1. Results of genetic analyses of electrophoretic variation in *Bufo boreas*: Phenotypic proportions in F_1 progeny of known parents. Chi-squares are for goodness-of-fit of observed proportions to those expected assuming Mendelian inheritance.

APH-2

Female		Male	F_1 Phenotype (N)	χ^2
M/M	X	S/M	M/M (19)	0.38 ns
			S/M (23)	
M/F	X	M/M	M/M (2)	Consistent
			M/F (2)	

EST-3

Female		Male	F_1 Phenotype (N)	χ^2
1.00/1.06	X	1.06/1.09	1.00/1.06 (7)	0.48 ns
			1.00/1.09 (5)	
			1.06/1.06 (6)	
			1.06/1.09 (5)	
1.06/1.15[1]	X	1.06/1.09	1.06/1.09 (14)	4.18 ns
			1.06/1.15 (12)	
			1.09/1.15 (17)	
			1.06/1.06 (23)	

Table AIV.1 continued.

GOT-2

Female		Male	F_1 Phenotype (N)	χ^2
M/M	X	S/M	M/M (22)	0.09 ns
			S/M (24)	
M/M	X	M/M	M/M (30)	Consistent

LDH-1

Female		Male	F_1 Phenotype (N)	χ^2
F/F	X	S/F	F/F (11)	0.04 ns
			S/F (12)	
S/F	X	F/F	F/F (9)	1.14 ns
			S/F (5)	
COMBINED			F/F (20)	0.24 ns
			S/F (17)	

LDH-2

Female		Male	F_1 Phenotype (N)	χ^2
S/F	X	S/S	S/S (16)	3.52 ns
			S/F (7)	

Table AIV.1 continued.

LDH-2 continued.

Female		Male	F_1 Phenotype (N)		χ^2
S/M	X	S/M	S/S	(5)	2.57 ns
			S/M	(4)	
			M/M	(5)	
M/F	X	S/F	M/M	(9)	6.48 ns
			S/M	(15)	
			S/F	(23)	
			M/F	(19)	

PEP-2

Female		Male	F_1 Phenotype (N)		χ^2
F/F	X	S/F	F/F	(10)	0.00 ns
			S/F	(10)	
F/F	X	S/S	S/F	(23)	Consistent
S/S	X	S/S	S/S	(4)	Consistent

Table AIV.1 continued.

PGI-1

Female		Male	F_1 Phenotype (N)	χ^2
S/S	X	S/S	S/S (19)	Consistent
S/S	X	S/F	S/S (24)	2.05 ns
			S/F (35)	
S/S	X	S/M	S/S (20)	0.00 ns
			S/M (20)	

PGM-2

Female		Male	F_1 Phenotype (N)	χ^2
S/S	X	S/F	S/S (25)	0.08 ns
			S/F (23)	
S/F	X	S/F	S/S (10)	3.18 ns
			S/F (20)	
			F/F (4)	
S/S	X	S/S	S/S (10)	Consistent

[1] The genotype of this female is in question. A check of parental genotypes after two and one half years of extract storage at

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-76°C showed this female to be genotype 1.06/1.06 and not 1.06/1.15. The discrepancy may be due to enzyme degredation but may also be the result of a mix-up of samples. If the true genotype is 1.06/1.06, then the F_1 is inconsistent with Mendelian inheritance of the variation. Further testing is necessary but data as recorded are not inconsistent with the Mendelian hypothesis.

APPENDIX V

GENOTYPE FREQUENCIES IN ADULT *BUFO BOREAS* FROM LOST LAKE: 1973 AND 1975.

Chi-squares are for goodness-of-fit of observed genotype distributions to those computed (expected) from observed allele frequencies by Levene's (1949) formula for small samples. Genotypic classes with expected numbers less than 1.0 were pooled. Where necessary, such classes were pooled with classes which had expected numbers greater than 1.0. Asterisks indicate probabilities as in previous tables. D is also as previously described.

Locus	Genotype	1973	1975
APH-2	Sample size	94	105
	S/S	.000	.000
	M/M	.596	.591
	F/F	.021	.019
	S/M	.149	.143
	S/F	.032	.019
	M/F	.202	.229
	Chi-square	0.30	0.05
	df	2	2
	D	+.008	+.049

Locus	Genotype	1973	1975
EST-3	Sample size	94	104
	1.06/1.06	.160	.135
	1.09/1.09	.298	.250
	1.00/1.06	.053	.058
	1.00/1.09	.138	.106
	1.06/1.09	.128	.212
	1.06/1.15	.096	.106
	1.09/1.15	.053	.067
	Others[1]	.074	.067
	Chi-square	21.92***	8.02
	df	4	4
	D	-.276	-.118
GOT-2	Sample size	94	105
	S/S	.011	.000
	M/M	.872	.867
	F/F	.000	.000
	S/M	.085	.067
	S/F	.000	.000
	M/F	.032	.067
	Chi-square	1.01	0.49
	df	1	1
	D	-.108	+.049
LDH-1	Sample size	94	105
	S/S	.000	.010
	F/F	.840	.914
	S/F	.160	.076
	Chi-square	0.65	3.03
	df	1	1
	D	+.081	-.164

Locus	Genotype	1973	1975
LDH-2	Sample size	94	104
	S/S	.245	.212
	M/M	.106	.096
	F/F	.011	.077
	S/M	.298	.279
	S/F	.234	.135
	M/F	.106	.202
	Chi-square	2.88	5.04
	df	3	3
	D	+.039	-.060
PEP-2	Sample size	94	105
	S/S	.277	.333
	F/F	.223	.191
	S/F	.500	.476
	Chi-square	0.00	0.11
	df	1	1
	D	-.003	-.032
PGI-1	Sample size	94	104
	S/S	.436	.519
	M/M	.043	.058
	F/F	.011	.010
	S/M	.255	.240
	S/F	.202	.144
	M/F	.053	.029
	Chi-square	0.20	1.86
	df	2	2
	D	-.022	-.080

Locus	Genotype	1973	1975
PGM-2	Sample size	94	105
	S/S	.819	.695
	F/F	.011	.010
	S/F	.170	.295
	Chi-square	0.05	1.29
	df	1	1
	D	-.022	+.109

[1] Pooled 1.00/1.00, 1.15/1.15 and 1.00/1.15.

APPENDIX VI

ALLELE FREQUENCIES IN ADULT MALE AND FEMALE *BUFO BOREAS* FROM LOST LAKE: POOLED DATA, 1973 AND 1975.

Locus	Allele	Males	Females	Homogeneity Chi-square
APH-2	Number of Alleles	322	76	
	S	.081	.105	1.19 (ns)
	M	.773	.790	
	F	.146	.105	
EST-3	Number of Alleles	320	76	
	1.00	.109	.118	2.41 (ns)
	1.06	.313	.303	
	1.09	.437	.500	
	1.15	.141	.079	
GOT-2	Number of Alleles	322	76	
	S	.040	.053	1.05 (ns)
	M	.938	.908	
	F	.022	.039	
LDH-1	Number of Alleles	322	76	
	S	.059	.079	0.42 (ns)
	F	.941	.921	
LDH-2	Number of Alleles	320	76	
	S	.475	.408	1.11 (ns)
	M	.316	.355	
	F	.209	.237	

Locus	Allele	Males	Females	Homogeneity Chi-square
PEP-2	Number of Alleles	322	76	
	S	.547	.566	0.09 (ns)
	F	.453	.434	
PGI-1	Number of Alleles	320	76	
	S	.694	.645	2.19 (ns)
	M	.197	.184	
	F	.109	.171	
PGM-2	Number of Alleles	322	76	
	S	.870	.908	0.84 (ns)
	F	.130	.092	

APPENDIX VII

INDIVIDUAL SAMPLE CHI-SQUARES FOR ADULT/PRE-REPRODUCTIVE COMPARISONS: ALLELES AND GENOTYPES

The following is a tabulation of chi-squares for goodness-of-fit of pre-reproductive (offspring) sample allele and genotype frequencies to those distributions expected based on the assumption of random mating among parents. The expected distributions were computed from pooled 1973 and 1975 adult ("parental") allelic frequencies. Asterisks indicate probabilities as in previous tables.

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
APH-2							
1973	14	2	3.14	ns	3	5.68	ns
	12	2	8.39	*	3	14.11	**
	17	2	5.63	ns	3	6.25	ns
	18	2	1.57	ns	3	1.68	ns
	21	2	1.81	ns	3	5.13	ns
	22	2	1.55	ns	3	2.87	ns
	26	2	14.74	***	3	6.55	ns
	32	2	0.20	ns	3	0.88	ns
1974	27	2	5.41	ns	3	5.87	ns
	46	2	5.23	ns	3	6.63	ns
	30	2	6.86	*	3	11.32	*
	37	2	3.17	ns	3	5.11	ns
	39	2	4.86	ns	3	10.16	*
1975	40	2	16.28	***	3	20.81	***
	41	2	2.19	ns	3	3.84	ns
	42	2	4.74	ns	3	5.30	ns
	43	2	0.96	ns	3	2.04	ns
	47	2	9.33	**	3	10.48	*
	50	2	0.05	ns	3	7.38	ns
EST-1							
1973	17	(No Parental Estimates)			3	4.51	*
	18				3	4.28	*
	21				3	0.12	ns
	22				3	5.96	*
	26				3	1.83	ns
	32				3	1.03	ns
1974	46	(No Parental Estimates)			3	1.22	ns
	30				3	23.39	***
	37				3	0.85	ns
	39				3	1.68	ns
1975	42	(No Parental Estimates)			3	22.79	**
	47				3	21.83	**
	50				3	10.10	**

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
EST-3							
1973	14	3	0.30	ns	7	8.28	ns
	12	3	54.46	***	7	49.48	***
	17	3	9.84	*	7	15.86	*
	18	3	6.85	ns	7	14.41	*
	21	3	5.79	ns	7	12.87	ns
	22	3	15.20	**	7	19.36	**
	26	3	11.94	*	7	12.37	ns
	32	3	12.58	*	7	13.94	ns
1974	27	3	1.80	ns	7	9.82	ns
	46	3	6.32	*	7	14.80	*
	30	3	8.24	*	7	12.44	ns
	37	3	14.67	**	7	14.90	*
	39	3	15.65	**	7	16.62	*
1975	40	3	13.36	**	7	13.40	ns
	41	3	18.26	***	7	19.26	**
	42	3	17.37	***	7	31.55	***
	43	3	18.77	***	7	22.34	**
	47	3	10.78	*	7	15.71	*
	50	3	24.90	***	7	30.16	***
GOT-2							
1973	13	2	4.42	ns	2	23.39	***
	14	2	11.26	**	2	9.64	**
	12	2	2.85	ns	2	2.29	ns
	17	2	10.13	**	2	13.13	**
	18	2	1.76	ns	2	2.56	ns
	21	2	7.17	*	2	6.42	*
	22	2	2.39	ns	2	2.82	ns
	26	2	9.77	**	2	9.94	**
	32	2	11.63	**	2	15.52	***
1974	27	2	29.64	**	2	28.36	**
	28	2	2.81	ns	2	3.12	ns
	46	2	3.02	ns	2	4.32	ns
	30	2	1.14	ns	2	2.03	ns
	37	2	1.10	ns	2	1.65	ns
	39	2	0.92	ns	2	1.18	ns

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
EST-3 continued.							
1975	40	2	3.90	ns	2	4.27	ns
	41	2	20.20	***	2	16.29	***
	42	2	1.96	ns	2	2.84	ns
	43	2	1.95	ns	2	0.87	ns
	47	2	2.40	ns	2	1.95	ns
	50	2	0.43	ns	2	0.79	ns
LDH-1							
1973	13	1	9.40	**	1	8.18	**
	14	1	7.31	**	1	7.39	**
	12	1	0.51	ns	1	0.81	ns
	17	1	13.54	***	1	10.51	**
	18	1	2.09	ns	1	2.78	ns
	21	1	3.46	ns	1	3.40	ns
	22	1	1.29	ns	1	1.16	ns
	26	1	0.51	ns	1	0.77	ns
	32	1	2.20	ns	1	2.74	ns
1974	27	1	6.25	*	1	5.09	*
	28	1	0.93	ns	1	0.17	ns
	46	1	2.09	ns	1	1.41	ns
	30	1	0.00	ns	1	0.01	ns
	37	1	12.92	***	1	8.41	**
	39	1	2.20	ns	1	2.90	ns
1975	40	1	6.89	**	1	5.72	*
	41	1	8.44	**	1	10.24	**
	42	1	4.58	*	1	5.72	*
	43	1	0.51	ns	1	0.42	ns
	47	1	16.52	***	1	11.62	**
	50	1	3.32	ns	1	4.25	**
LDH-2							
1973	13	2	7.67	*	5	9.57	ns
	14	2	5.82	ns	5	11.59	*
	12	2	11.48	**	5	15.60	**
	17	2	11.83	**	5	12.00	*
	18	2	1.49	ns	5	4.27	ns

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
LDH-2 continued.							
	21	2	5.61	ns	5	6.18	ns
	22	2	7.12	*	5	9.12	ns
	26	2	3.34	ns	5	4.95	ns
	32	2	1.72	ns	5	2.12	ns
1974	27	2	13.32	**	5	16.84	**
	46	2	9.81	**	5	20.63	***
	30	2	0.48	ns	5	4.88	ns
	37	2	0.94	ns	5	1.52	ns
	39	2	4.51	ns	5	15.98	**
1975	40	2	7.54	*	5	7.99	ns
	41	2	9.98	**	5	12.13	*
	42	2	6.17	*	5	7.99	ns
	43	2	1.73	ns	5	1.95	ns
	47	2	12.00	**	5	13.12	*
	50	2	3.24	ns	5	6.23	ns
PEP-2							
1973	13	1	0.20	ns	2	5.90	ns
	14	1	0.70	ns	2	0.42	ns
	12	1	2.00	ns	2	1.52	ns
	17	1	0.62	ns	2	1.32	ns
	18	1	2.17	ns	2	3.44	ns
	21	1	0.59	ns	2	1.75	ns
	22	1	0.81	ns	2	1.50	ns
	26	1	3.63	ns	2	0.87	ns
	32	1	5.98	*	2	6.08	*
1974	27	1	1.37	ns	2	2.14	ns
	28	1	0.26	ns	2	0.53	ns
	46	1	6.00	*	2	6.64	*
	30	1	7.23	**	2	7.57	*
	37	1	0.09	ns	2	0.20	ns
	39	1	4.42	*	2	5.68	ns

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
PEP-2 continued.							
1975	40	1	0.09	ns	2	0.44	ns
	41	1	0.02	ns	2	1.84	ns
	42	1	3.51	ns	2	3.56	ns
	43	1	0.87	ns	2	1.12	ns
	47	1	4.00	*	2	4.73	ns
	50	1	1.30	ns	2	5.49	ns
<u>PGI-1</u>							
1973	13	2	2.56	ns	3	5.54	ns
	12	2	1.76	ns	3	0.59	ns
	17	2	2.62	ns	3	6.05	ns
	21	2	6.17	*	3	7.92	*
	22	2	3.96	ns	3	3.18	ns
	26	2	4.98	ns	3	9.25	*
	32	2	2.73	ns	3	0.93	ns
1974	27	2	1.76	ns	3	1.97	ns
	28	2	14.51	***	3	17.03	***
	46	2	7.12	*	3	11.18	*
	30	2	0.11	ns	3	2.49	ns
	37	2	2.36	ns	3	1.50	ns
	39	2	3.73	ns	3	0.34	ns
1975	40	2	13.35	**	3	12.79	**
	41	2	17.33	***	3	19.88	***
	42	2	2.97	ns	3	10.34	*
	43	2	14.45	***	3	21.09	***
	47	2	9.97	**	3	10.08	*
	50	2	15.35	***	3	19.58	***
<u>PGM-2</u>							
1973	13	1	1.04	ns	1	0.78	ns
	14	1	0.55	ns	1	0.23	ns
	12	1	0.35	ns	1	0.11	ns
	17	1	1.27	ns	1	0.85	ns
	18	1	0.41	ns	1	0.06	ns
	21	1	1.06	ns	1	1.03	ns

Year	Collection	Alleles df	Alleles χ^2	Alleles P	Genotypes df	Genotypes χ^2	Genotypes P
PGM-2 continued.							
	22	1	0.01	ns	1	0.38	ns
	26	1	0.31	ns	1	0.13	ns
	32	1	0.05	ns	1	0.31	ns
1974	27	1	3.11	ns	1	2.96	ns
	28	1	1.20	ns	1	1.24	ns
	46	1	5.00	*	1	4.89	*
	30	1	0.17	ns	1	0.11	ns
	37	1	15.20	***	1	10.33	**
	39	1	5.85	*	1	7.52	**
1975	40	1	13.85	***	1	10.33	**
	41	1	1.02	ns	1	1.41	ns
	42	1	0.22	ns	1	0.20	ns
	43	1	4.04	*	1	2.28	ns
	47	1	2.58	ns	1	2.13	ns
	50	1	0.65	ns	1	1.84	ns

APPENDIX VIII

MICROGEOGRAPHIC GENETIC DIFFERENTIATION IN PRE-REPRODUCTIVE *BUFO BOREAS*

In recent years, a great deal of theoretical consideration has been given to the conditions fostering genetic polymorphism in populations inhabiting ecologically diverse environments (reviewed by Christiansen and Feldman, 1975; Hedrick, 1976). Recently, Lewontin, Ginzburg and Tuljapurkar (1978) examined the potential of overdominance as an explanation for the maintenance of multiple-allele polymorphism. They found little. In their view, polymorphism is much more likely to result from spatial and/or temporal variations in selection pressure; the so-called multiple-niche selection model. Gillespie concluded (Gillespie and Langley, 1974; Gillespie, 1976, 1977), for a wide range of conditions, that differences in relative fitness rankings of homozygotes in stochastically varying environments are sufficient to maintain many alleles at a locus. Several earlier works analyzed the likelihood of multiple-niche polymorphism under more predictable (deterministic) environmental conditions (e.g., Levene, 1953; Levins and MacArthur, 1966; Levins, 1968; Prout, 1968; Christiansen, 1974; Gillespie, 1974a, 1974b). The conditions fostering genetic polymorphism have sometimes proven stringent but the maintenance of genetic diversity via spatially or temporally varying selection pressures remains a theoretically viable and intuitively appealing concept.

Intriguing evidence of genetic diversity among microgeographic subdividions of physically continuous natural populations has been

found in plants; _Avena barbata_ (Clegg, Allard and Kahler, 1972; Hamrick and Allard, 1975), _Veronica perigrina_ (Linhart, 1974), numerous grass species (Antonvics, Bradshaw and Turner, 1971) and animals of such sessile habit but large neighborhood size as _Modiolus demissus_ (Koehn, Turano and Mitton, 1973) and _Mylilus edulus_ (Koehn, Milkman and Mitton, 1976), as well as in more vagile ones such as _Colias meadii_ (Johnson, 1976), _Drosophila persimilis_ (Taylor and Powell, 1977), and _Mus musculus_ (Selander, 1970). Furthermore Powell (1971) and MacDonald and Ayala(1974) found evidence of correlations between the levels of genetic variation in laboratory populations of _Drosophila_ and their adaptedness to varying levels of environmental heterogeneity.

In a previous section, (Appendix I) the periphery of Lost Lake was subjectively described as consisting of two qualitatively distinct habitat types. In consideration of microgeographical variation in temperature, sunlight, wind exposure, limnological and edaphic conditions, vegetation, food quality and abundance and myriad other factors, the area must be divisible into many more quantitatively different spatial and temporal "patches". Perhaps the differences among patches are subtle or even irrelevent to young toads, but if certain genotypic combinations survive better under one set of conditions while others are superior under an alternate set, the potential exists for genetic divergence at the sub-population level. If "survivorship patches" are spatially distinct and temporally stable for relatively long periods (weeks or months) it might be possible to detect genetic differences developing among young toads living in different areas.

Another way in which differentiation among sub-groups might arise is through the active selection of different habitats by the bearers of different genotypes. Although such habitat selection behavior could lead to detectable genetic differentiation among population sub-groups, it would have little effect in altering the genetic structure of the population unless accompanied by differential survivorship as well (Christiansen, 1974).

Temporal patchiness in ecological factors relevant to survivorship has already been shown in the distinct temporal genetic changes taking place within cohorts collected in a single locality. In the present section, I will document evidence of spatial differentiation among young toads inhabiting different areas of the lake periphery. At this time, a somewhat more detailed description of these areas is in order. Area 5 is the site from which the samples for the main study (temporal heterogeneity) were taken. Although it is classified "forest", each year as the season progresses and the water level of the lake receeds, a large expanse of gradually sloping shoreline is exposed. It is upon this and similar "emergence slopes" (ES) that virtually all of the transformation and emerging activities of young toads take place. Area 3 is classified as generally "marshy" but, like area 5, includes a grassy and shallow ES. However, the aquatic vegetation of the ES in this area is much denser and slope more gradual than in area 5. Area Ø, is part of the large, continuous "marshy" area at the south-east corner of the lake. It's shoreline area has been poorly studied as a direct consequence of the "impenetrable" nature of the dense shrubby

vegetation present but one successful excursion to the water's edge revealed that metamorphic activities took place upon a gradually sloping substrate similar to those previously described but almost totally lacking the lush, grassy vegetation. That present was very patchily distributed. Newly transformed individuals from this area must successfully traverse at least 50-80 m before they encounter abundant low vegetation and its associated fauna.

Preliminary examination of population differentiation on a microgeographic scale was conducted by comparing the allele and heterozygote frequencies in samples collected contemporaneously from the different areas. C44 and C42 were collected at area 3 and area 5 respectively on August 24, 1975. The collections consisted of transforming individuals in the later (semi-terrestrial) stages of metamorphosis. On October 18, 1975, collections of dispersing toadlets were made at area 5 (C51) and area Ø (C52).

Contingency chi-squares were computed to test homogeneity among the pairs of collections (C42 versus C44 and C51 versus C52). Six of the eight loci tested (PGI-1 has not been studied in this regard) show evidence of significant differentiation over the small geographic distances (Figure AVIII.1). Two of the loci, EST-1 and PEP-2 discriminate the collections in both sets of comparisions. Among the "metamorphic" pair of samples there are differences in allele and/or heterozygote proportions at the EST-3 and GOT-2 loci as well as EST-1 and PEP-2. The "dispersing" pair of samples may be considered different for some aspect of genetic content at the EST-1, LDH-1, PEP-2 and PGM-2 loci. It seems

especially surprising to find such striking differentiation among the "metamorphic" sub-populations so recently derived from the same pool of tadpoles. Since selective mortality is the presumed cause of these microgeographic differences, the relationship between sample allele frequencies and genotype proportions might be expected to show some departure from that anticipated under Hardy-Weinberg conditions. Considering all eight loci, 50% (8 of 16) of the samples from pairwise comparison showing microgeographic differentiation also show poor Hardy-Weinberg agreement while among the samples of pairs in which no microgeographic differences are manifest, only 6.25% (1 of 16) fail to agree with the Hardy-Weinberg expectations of genotype distribution (Table AVIII.1 and Figure AVIII.1).

Of course, with so few data, one may draw few conclusions regarding pattern or cause of these genetic differences other than to say that distinct genetic differentiation may develop very quickly over relatively short physical distances. By extrapolation, the potential for genetic differentiation must indeed be great during the considerably longer total pre-reproductive period (two to three years) and over what must surely be a broader range of ecological diversity than is represented in these two pairwise comparisons. These findings and those of the main study increase the likelihood that some sort of multiple-niche selection may be supporting polymorphism among the toads at this lake through the operation of spatio-temporally stochastic variation in the relative fitnesses of genetically divergent individuals.

Table AVIII.1. Genotypic variation among larval and juvenile _Bufo boreas_ from Lost Lake in 1975: samples illustrating microgeographic differentiation. Information regarding chi-square computation, probability codes (asterisks) and D may be found in Table 3.

		Collection			
Locus	Genotype	42	44	51	52
APH-2	Sample size	129	153	91	144
	S/S	.000	.007	.022	.014
	M/M	.698	.719	.648	.688
	F/F	.016	.000	.022	.028
	S/M	.093	.124	.143	.090
	S/F	.023	.007	.044	.007
	M/F	.171	.144	.121	.174
	Chi-square	1.25	0.77	5.19	6.81*
	df	2	2	2	2
	D	-.035	+.046	-.166	-.131
EST-1	Sample size	125	145	91	144
	S/S	.000	.000	.011	.014
	M/M	.696	.538	.516	.403
	F/F	.088	.131	.132	.056
	S/M	.032	.069	.066	.104
	S/F	.008	.007	.011	.035
	M/F	.176	.255	.264	.389
	Chi-square	18.55***	16.28***	7.88*	1.31
	df	2	2	2	2
	D	-.343	-.250	-.265	+.052

Table AVIII.1 continued.

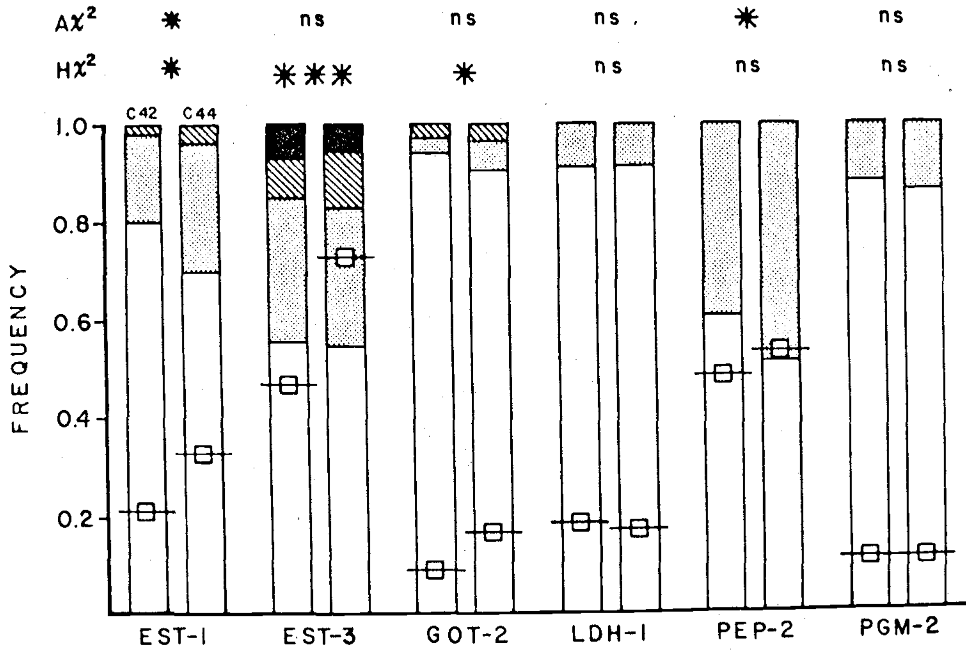
Locus	Genotype	Collection 42	44	51	52
EST-3	Sample size	144	149	82	64
	1.06/1.06	.139	.027	.037	.031
	1.09/1.09	.361	.228	.268	.203
	1.00/1.06	.042	.034	.024	.031
	1.00/1.09	.069	.067	.049	.047
	1.06/1.09	.229	.443	.244	.328
	1.06/1.15	.028	.034	.012	.016
	1.09/1.15	.097	.141	.293	.266
	Others	.035	.027	.073	.078
	Chi-square	13.07*	18.49***	7.23	9.19
	df	4	4	4	4
	D	-.206	+.217	+.076	+.125
GOT-2	Sample size	144	153	91	144
	S/S	.000	.006	.000	.000
	M/M	.903	.824	.868	.861
	F/F	.007	.000	.000	.000
	S/M	.042	.092	.099	.076
	S/F	.014	.013	.000	.000
	M/F	.035	.065	.033	.063
	Chi-square	15.04***	2.13	0.41	0.76
	df	1	1	1	1
	D	-.203	-.067	+.051	+.052
LDH-1	Sample size	144	153	91	144
	S/S	.000	.000	.033	.007
	F/F	.813	.824	.868	.764
	S/F	.187	.176	.099	.229
	Chi-square	1.48	1.37	11.86***	0.71
	df	1	1	1	1
	D	+.100	+.093	-.350	+.070

Table AVIII.1 continued.

Locus	Genotype	Collection 42	44	51	52
LDH-2	Sample size	144	153	91	144
	S/S	.201	.183	.242	.264
	M/M	.083	.092	.121	.118
	F/F	.076	.065	.055	.090
	S/M	.257	.294	.264	.257
	S/F	.264	.248	.165	.194
	M/F	.118	.118	.154	.076
	Chi-square	1.28	2.04	1.89	11.35**
	df	3	3	3	3
	D	-.008	+.022	-.091	-.163
PEP-2	Sample size	142	152	59	144
	S/S	.359	.250	.322	.250
	F/F	.148	.217	.102	.250
	S/F	.493	.533	.576	.500
	Chi-square	0.11	0.61	2.43	0.00
	df	1	1	1	1
	D	+.028	+.063	+.201	-.004
PGM-2	Sample size	143	153	91	144
	S/S	.776	.758	.802	.722
	F/F	.014	.033	.000	.056
	S/F	.210	.209	.198	.222
	Chi-square	0.00	2.24	0.21	6.03*
	df	1	1	1	1
	D	-.002	-.120	+.104	-.203

Figure AVIII.1. Microgeographic genetic variation among pre-reproductive Bufo boreas: Allele and heterozygote proportions at six loci for two pairs of contemporaneously collected samples from different sampling areas. A. Metamorphic pair; collected August 24, 1975 at area 5 (C42) and area 3 (C44). B. Dispersing pair; collected October 18, 1975 at area 5 (C51) and area Ø (C52). Probabilities of contingency chi-squares for heterogeneity among allele frequencies (AX^2) and heterozygote frequencies (HX^2) are indicated by asterisks as before. Allelic proportions in any particular collection are represented by segments of a unit rectangle. EST-1 and GOT-2; S = hatched, M = solid white, F = stippled. EST-3; 1.00 = solid black, 1.06 = stippled, 1.09 = solid white, 1.15 = hatched. LDH-1; S = stippled, F = solid white. PEP-2 and PGM-2; S = solid white, F = stippled. Heterozygote frequency is indicated by a square bisected by a horizontal line.

A



B