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A Preliminary Study of the MDH
Variability in Lemna minor/Lemna turionifera

by

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A Thesis

submitted to the Department of Biological Sciences
in partial fulfilment of the requirements for the degree
of Master of Science

Brock University
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ABSTRACT

Lemna minor is a small aquatic polyploid angiosperm which reproduces apomictically and has a worldwide distribution. This study was undertaken to characterize the extent and nature of phenotypic variability.

The techniques of starch gel electrophoresis were used in this investigation and MDH phenotypes of several populations from Ontario, USA and Africa were examined and compared. Heat stability, molecular weight and cell fractionation analyses were also done to identify locus specific MDH bands.

The results of the population surveys suggest that there is little genetic variability present both within and between Lemna minor/Lemna turionifera. Evidence of correlation of physiological and seasonal variation patterns was found.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Dr. Martin L. Tracey Jr. for his guidance throughout this endeavour. Under his guidance this thesis became an important learning experience. The intrinsic value of creativity and pencil and paper in the field of scientific research became clear.

I would also like to thank Dr. Krystyna Urbanska-Worytkiewicz, of the Geobotanical Institute, Swiss Federal Institute of Technology, Zurich, Switzerland for her extreme kindness and also her interest in this project. Her aid in sending cytologically identified cultures and discussing the problems of speciation has been invaluable.

The assistance of Dr. William S. Hillman and Professor A.D. Bradshaw for valuable comments, Walter Giesbrecht for his skills in German translation, and Michael Cheek for Lemna specimens is also acknowledged.

The patience and stamina of Barbara Wilson, who typed this thesis is gratefully acknowledged.

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INTRODUCTION

BIOLOGY OF LEMNA MINOR

Taxonomy: Lemna minor is a small, colonial, floating-aquatic angiosperm of the Family Lemnaceae (Hegelmaier, 1868 in Hillman, 1961a; Fernald, 1950). Four genera are usually recognized within this family: Spirodela, Lemna, Wolffiella and Wolffia (Hillman, 1961d). Two genera, Spirodela and Lemna belong to the Tribe Lemneae, (Hegelmaier, 1868) Subfamily Lemnoidae, (Lawalree, 1945) whereas, the remaining two genera Wolffia and Wolffiella belong to the Tribe Wolffieae, (Hegelmaier, 1868) Subfamily Wolffioideae (Lawalree, 1945). The monocotyledonous Lemnaceae are a remarkably uniform family of plants all of which pass their life floating at or near the surface of the water (Arber, 1920; Jacobs, 1947; Hillman, 1961d). The members of this family show a high degree of morphological homogeneity and marked differences from other aquatic plants (Arber, 1920; Grant, 1975). For this reason and because only recently have thorough investigations into the species relationships been undertaken (Blazey and McClure, 1968; De Lange and Pieterse, 1973; Pieterse, 1974; De Lange, 1975; Den Hartog, 1975; Landolt, 1975; Kandeler, 1975; Urbanska-Worytkiewicz, 1975), the species delimitation within the Family Lemnaceae is still not clear. The most poorly described species are Lemna gibba, Lemna minor, and Lemna turionifera (Blazey and McClure, 1968; De Lange

and Pieterse, 1973; Pieterse, 1974; De Lange, 1975; Den Hartog, 1975, Kandeler, 1975 for Lemna gibba/Lemna minor and Landolt, 1975; Urbanska-Worytkiewicz, 1975, 1977 pers comm. for Lemna minor/Lemna turionifera) Specific points of conflict will be discussed below; the following sections describe the biology of Lemna minor and outline the present delimitation of the species Lemna minor.

PLANT MORPHOLOGY

Fronde Morphology: The vegetative plant of Lemna minor consists of one or more leaf-like structures known as fronds which can bear one adventitious, chloroplast-containing root per frond (Figure 1). The bulk of each frond is composed of chlorenchymatous cells which are often separated by large intercellular spaces filled with air or gases (Hillman, 1961d). These spaces facilitate the bouyant habit of this plant group (Arber, 1920; Hillman, 1961d).

The adult frond of Lemna minor is quite flat and approximately pear-shaped with the acute end proximal to the mother frond (Figure 1 and 2). On either side of the acute end of the frond body are the reproductive pockets from which daughter fronds are produced (Caldwell, 1899; Ashby, Wangermann and Winter, 1949; Hillman, 1961d). These pockets are formed by outgrowths of the upper and lower surfaces of the frond (Caldwell, 1899; Jacobs, 1947) and contain within them a small amount of meristematic tissue (Hillman, 1961d) (Figure 2).

Daughter fronds do not simultaneously appear on both



Figure 1. Vegetative fronds of Lemna minor.

Figure 2. Frond of Lemna minor - Reproductive pockets exposed (From Ashby et al, 1949).

- A. First daughter - remaining stalk.
- B. Half grown second daughter with its own daughter B¹.
- C. Third daughter just beginning to enlarge.
- D. Fourth daughter - primordium.
- E. Fifth daughter - primordium.

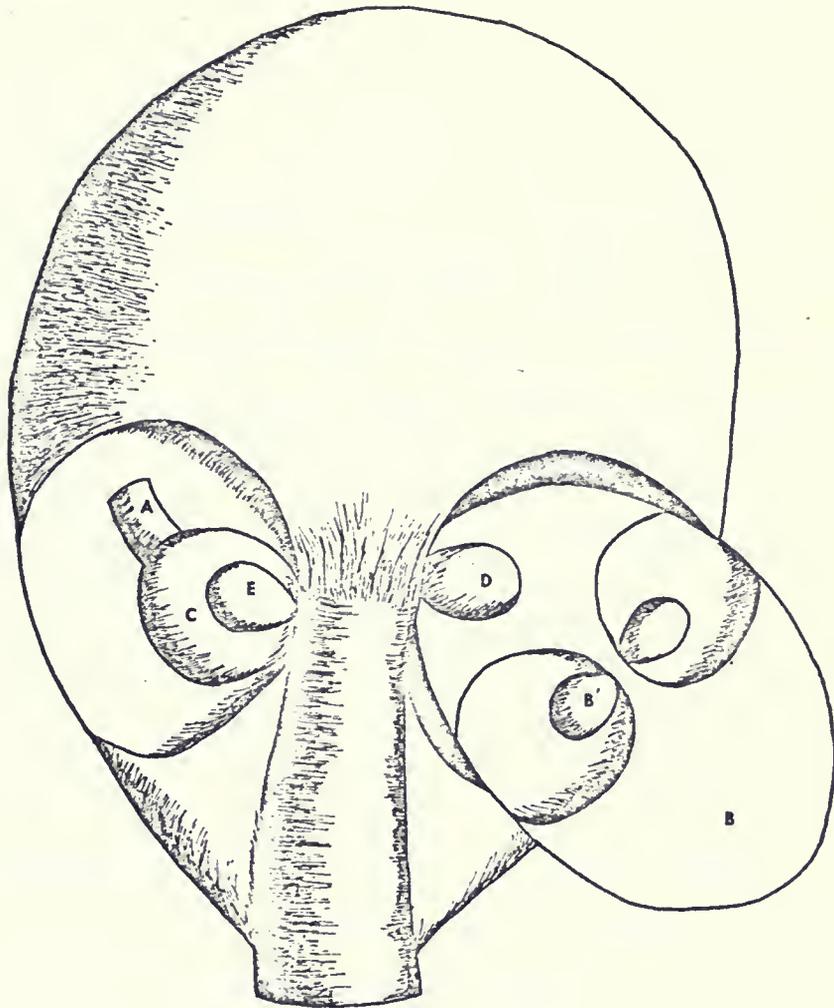


Figure 2. Fronds of Lemna minor - Reproductive pockets exposed (From Ashby et al, 1949).

sides of the mother plant (Caldwell, 1899; Ashby et al, 1949; Hillman, 1961d), and clones of Lemna minor appear to be consistent with respect to the side on which the first daughter is produced. Clones are either right-handed or left-handed (Ashby et al, 1949; Hillman, 1961d).

The actual size and shape of the adult frond is strongly influenced by external factors and is hence not a definitive diagnostic tool for distinguishing the various members of the genus Lemna (Figure 3). Length and width measurements usually exhibit a large amount of variation, particularly among strains (De Lange and Pieterse, 1973; Pieterse, 1974; De Lange, 1974, 1975); however, reasonable minimal, maximal and mean values (mm) for frond length and width are: Length; 3.21, 3.91, 3.58, Width; 2.18, 2.71, 2.68 (Calculated from Pieterse, 1974). Landolt (1975) and Kandeler (1975) have found that generally, flat individuals with a length/width ratio of greater than $3/2$ belong to Lemna minor. It is important to note, however, that the absolute size of fronds show a large degree of variability which is most likely correlated with the specific environmental conditions of each local pond (Jacobs, 1947; Landolt, 1975; Kandeler, 1975).

As mentioned above, Lemna gibba and Lemna minor are often confused and identification using only the frond morphology is especially difficult as morphologically intermediate strains are known to exist (De

Figure 3. Frond size variability in Lemna minor.

Lemna minor colonies in this photograph are from collections used in this study-SB, BN-4, LA-7, GU.
(From Landolt, 1975)



Figure 3. Frond size variability in Lemna minor.

Lange and Pieterse, 1973; De Lange, 1975; Kandeler, 1975) which in the field are difficult to distinguish from Lemna minor. Until recently, it was not known whether the flat forms of Lemna gibba represented a distinct genetical race or an environmental modification of the normally gibbous form (Guppy, 1894; Bhalla, Pieterse and Sabharwal, 1973; De Lange and Pieterse, 1973). It has recently been shown, however, that certain flat forms of the Lemna gibba/minor complex become gibbous in the presence of the chelating agent called EDDHA (Bhalla et al, 1973; De Lange and Pieterse, 1973; Pieterse, 1974; De Lange, 1975; Kandeler, 1975; Pieterse and Muller, 1977). Thus some Lemna gibba/minor strains appear to be only morphological variants of a single species (De Lange and Pieterse, 1973). On the other hand, Lemna minor does not thicken in response to EDDHA (Pieterse, 1974) and has hence been described as possessing genetically fixed flat fronds (Landolt, 1975). Still, strains intermediate in their ability to respond to EDDHA exist (De Lange and Pieterse, 1973) and as things stand, a consistent diagnostic tool for distinguishing Lemna gibba from Lemna minor has not been found. For the present, it should suffice to define Lemna minor as those strains possessing genetically fixed flat fronds with a length/width ratio of greater than $3/2$ (Kandeler, 1975; Landolt, 1975), and Lemna gibba as those strains possessing potentially gibbous fronds with a length/width ratio of $1-3/2$ (Landolt, 1975).

The upper epidermis in the Lemneae is very different from the lower epidermis (Jacobs, 1947; Hillman, 1961d). The upper epidermis consists of small cells and numerous smaller guard cells with stomata. This upper layer is highly cutinized and unwettable (Jacobs, 1947; Hillman, 1961d). The lower epidermis contains cells similar in size to the upper epidermis but the cuticle is replaced by a thin mucilaginous layer and stomata are lacking (Jacobs, 1947). These traits tend to be common to all four genera of the Lemnaceae Family (Hillman, 1961d).

Spirodela and Lemna fronds have greatly reduced vascular systems composed of one xylem element above, and one seive element and two companion cells below (Caldwell, 1899; Arber, 1920; Hillman, 1961d). One large vascular bundle runs from the node toward the acute (proximal) end of the frond and at the node several bundles diverge into the distal portion of the fronds forming what have been called nerves (Hillman, 1961d; Landolt, 1975).

The number of nerves born^e by a particular member of the Lemnaceae have recently been considered one of the more useful and consistent diagnostic characters in determining the species delimitation of this family (Hegelmaier, 1868 in Kandeler, 1975; Kandeler, 1975; Landolt, 1975). Lemna minor typically bears three nerves all of which branch from the base of the node (Hegelmaier 1868 in Kandeler, 1975; Hillman, 1961d;

Landolt, 1975) in cases where Lemna minor contains four or five nerves, the fourth and fifth nerves always branch out at some distance from the node (Landolt, 1975) (Figure 4 and 5). On Lemna gibba, there are characteristically four to five nerves but these all branch out from the base of the node (Landolt, 1975) (Figure 4). In Spirodela polyrrhiza, these nerves vary in number from eight to twelve with ten being the most common form (Jacobs, 1947). No such vascular system is known in the Wolffieae except for occasional traces of vascular tissue (Hillman, 1961d). The only other species that needs to be mentioned here with regard to its nerve number is Lemna turionifera sp.nov. (Landolt, 1975). This species bears many characteristics that are essentially identical to those of Lemna minor and nerve number is no exception. One difference between Lemna minor and Lemna turionifera is that Lemna turionifera forms distinct turions (a specialized overwintering frond) (Landolt, 1975). This species has been characterized by some authors as a subgroup of Lemna minor (Landolt, 1975; Urbanska-Worytkiewicz, pers. comm., 1977). This problem will be discussed at greater length later.

Root Morphology: The roots of Lemna sp. are considered to be adventitious (Hillman, 1961d); they arise from the node just inside the lower epidermis (Caldwell, 1899; Hillman, 1961d). The young root pushes its way through the epidermal layer which forms a temporary root sheath

Figure 4. Comparison of innervation patterns in Lemna.
(From Landolt, 1975)

- a) Lemna minor - Four-nerved form.
- b) Lemna gibba - Five-nerved form.

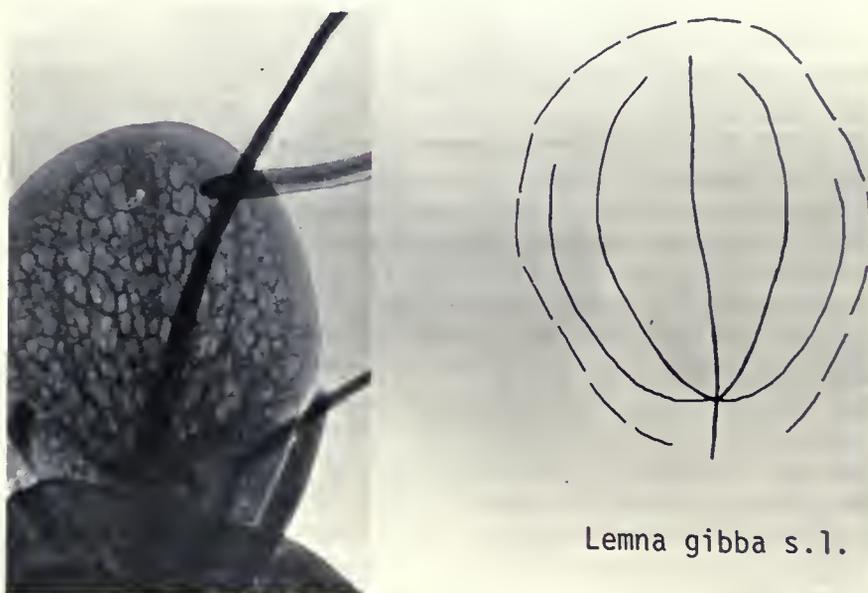
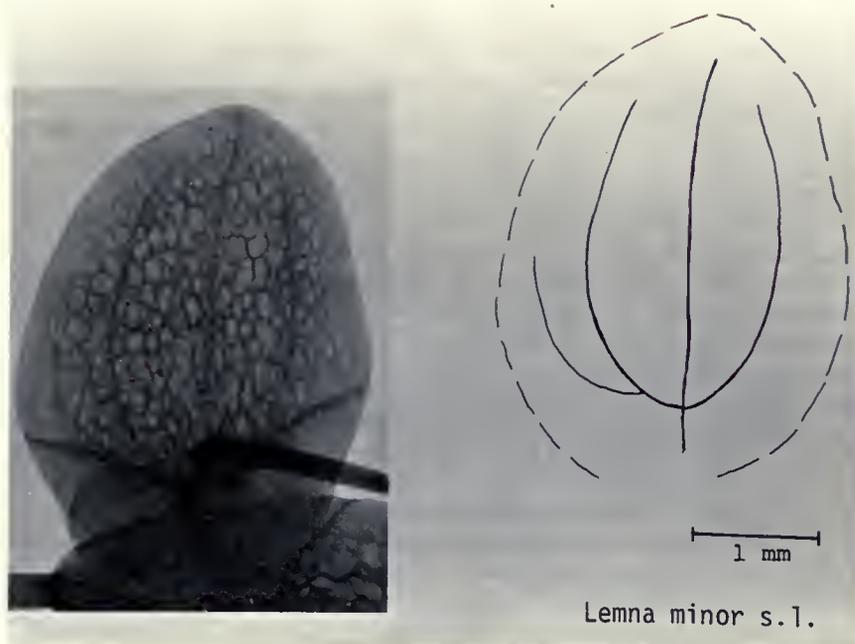


Figure 4. Comparison of innervation patterns in Lemna.
(From Landolt, 1975)



Figure 5. Innervation pattern of Lemna minor from Thorold - Three-nerved form. (magnification:15x)

around the top of the emergent root (Caldwell, 1899; Hillman, 1961d). Lemna minor bears only one unbranched, non-cutinized, hairless root per frond and a prominent root cap is usually developed (Caldwell, 1899; Jacobs, 1947; Hillman, 1961d; Kandeler, 1975; Landolt, 1975). The length of these roots is extremely variable and easily altered by external environmental factors, for example, temperature, light intensity, quality and concentration of nutrients in the water and season (Pirson and Gollner, 1953; Bornkamm, 1966; Hillman, 1961d; Landolt, 1975). Lemna generally grows well under conditions which entirely prevent root elongation (Hillman, 1955). The roots are characterized by an extremely reduced vascular system. The lower epidermal surface can however, absorb nutrients and the roots are actively photosynthetic (Arber, 1920; Pirson and Gollner, 1953). Consequently it has been suggested that the primary function of the root structure is one of equilibrium: they act to keep the fronds upright (Arber, 1920).

Size and shape of the root cap have also been studied for use as a potential diagnostic tool to distinguish Lemna minor from Lemna gibba (Figure 6). This was first attempted by Hegelmaier (1868 in Landolt, 1975). Hegelmaier reported that Lemna gibba typically has root cap tips which are acute while those of Lemna minor are obtuse. In several recent studies, this

Figure 6. Comparison of root caps in Lemna. (From Landolt, 1975)

a) Lemna minor

b) Lemna gibba

Fresh specimens appear on the left and dried specimens on the right.

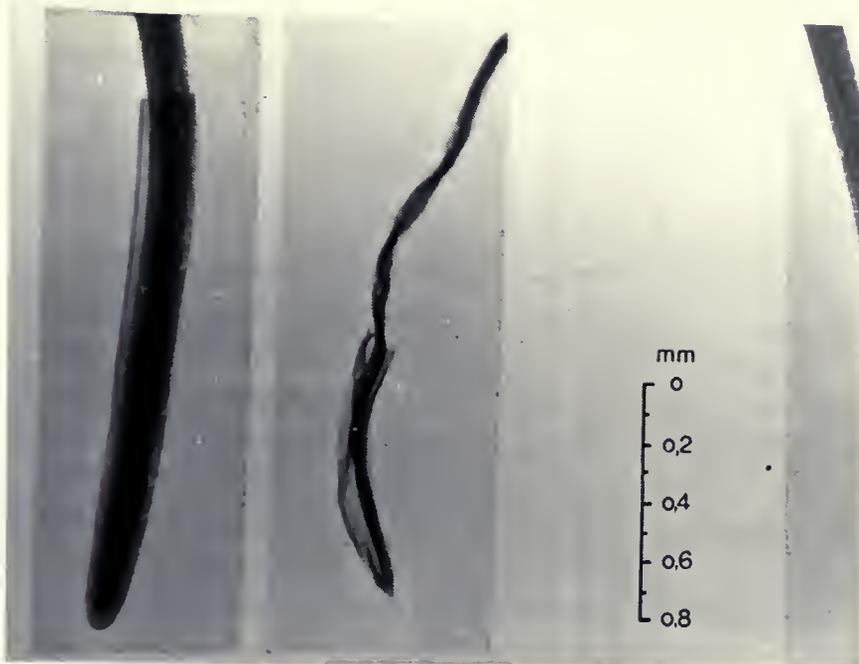


Figure 6. Comparison of root caps in Lemna. (From Landolt, 1975)

character has been shown to be non-taxon specific and therefore is now considered to have little diagnostic value (De Lange and Pieterse, 1973; De Lange, 1975; Kandiler, 1975; Landolt, 1975). Acute and obtuse tipped root caps occur in both species (De Lange, 1975; Kandeler, 1975). The length of the root cap also varies within strains on different fronds. It has been reported by Landolt (1975) however, that on the average, root caps are longer in Lemna gibba (0.6-1.8 mm.) than in Lemna minor (0.4-1.1 mm.). No specific root cap characteristics were given by any of these authors for Lemna turionifera.

LIFE CYCLE OF LEMNA MINOR

Vegetative Reproduction: The primary mode of replication in Lemna minor is vegetative, the rapidity of which is quite remarkable (Guppy, 1894; Caldwell, 1899; Arber, 1920; Hicks, 1934; Jacobs, 1947; Ashby et al, 1949; Hillman, 1961d; Bornkamm, 1966; Urbanska-Worytkiewicz, 1975). As mentioned above, daughter fronds are produced from the reproductive pockets situated on either side of the acute end of the parental frond. These daughter cells are produced mitotically from the meristematic tissue situated at the base of these pockets (Ashby et al, 1949). By the time a daughter plant has extended half the length of the reproductive pouch in which it grows, it has itself developed reproductive pouches and begun the formation of its own daughter (Caldwell, 1899). It

is this mode of growth that permits the colonial habit of Lemna minor and it is not uncommon to see four to six generations of individual plants attached to one another (Caldwell, 1899; Hillman, 1961d).

The rate of growth of colonies in Lemna minor varies considerably with external conditions and from clone to clone (Hicks, 1934; Ashby et al, 1949; Hillman, 1961d). Several experimenters have studied these growth rates under constant conditions (full nutrient medium, 400 foot candles illumination, 25° C temperature) (Hicks, 1934; Winter, 1937; Ashby et al, 1949; Keddy, 1976). In these circumstances, growth rate tends to be exponential (Hicks, 1934; Winter, 1937; Ashby et al, 1949; Ashby and Wangermann, 1951; Hillman, 1961d; Keddy, 1976). It should be mentioned here, however, that this exponential growth rate reflects the colony and population growth. It does not quantify the rate of production of the daughter fronds and it does not address itself to the mortality of individual fronds (Winter, 1937).

Ashby et al (1949) have measured the growth rates and followed the fates of individual fronds while attempting to answer the following questions: (i) How long does a frond live? (ii) How many daughter fronds does each mother frond produce? (iii) What differences are there between successive daughter fronds produced by one mother frond? Their results showed that Lemna minor fronds do not remain alive for more than five to six weeks (Table 1) and that during this time period,

Table 1. Mean length of life of fronds, with standard errors (S.E.), and mean intervals between successive daughter fronds (From Ashby et al, 1949).

Clone ...	I (1937), mean		II (1948)		III (1948)	
	L.	S.E.	L.	S.E.	R.	S.E.
Mean length of life of mother fronds (days)	42	0.93	41	0.93	36	1.30
Position of first daughter frond	L.		L.		R.	
Mean interval (days) between daughter fronds:						
0-1	5	0.3	5.0	0.3	5.0	0.1
1-2	2	0.5	6.2	0.5	6.0	0.3
2-3	2.5		13.2	1.4	17.3	0.6
3-4	3		8.4	2.2	4.3	1.4
4-5	3		12.5	2.5		
5-6	3					
6-7	3					
7-8	3.5					
8-9	3.5					
9-10	4.5					
10-11	4.5					
11-12	5.0					

each frond gives rise to a limited number of offspring, the number of which is characteristic of each clone (Table 1 and 2). Each daughter frond, of course, if conditions are amenable to continued growth, repeats its mother's history and so this process continues, generation after generation. It has been found however, that the areas of successive daughter fronds produced by a single parent progressively diminish (Ashby et al, 1949; Hillman, 1961d). This reduction in size is due, not to a decrease in cell size but to a decrease in cell number in later formed daughters (Ashby et al, 1949). The average life span of these later daughters is also reduced (Ashby and Wangermann, 1951). Clones of Lemna minor do not, however, grow until they disappear, rather they exhibit cycles of senescence and rejuvenation (Hillman, 1961d). It appears that the smaller, short-lived, later daughters of the initial parental fronds themselves produce first daughters which are much larger than themselves. This process continues, through successive production of larger first daughters until the original frond area is restored (Ashby and Wangermann, 1951; Hillman, 1961d).

The classical concept of generation time in Lemna minor is not applicable due to this vegetative mode of replication (Hillman, 1961d). The rationale is the following (Figure 7): A culture is started with an inoculum frond O. O produces two daughter cells called A and B. A and B then produce their own daughters, A1, B1, A2

Table 2. Distribution of number of daughters from mother fronds.

Percentage of fronds producing	Clone II	Clone III
Only 1 daughter	0	0
Only 2 daughters	0	30.3
Only 3 daughters	11.1	42.4
Only 4 daughters	66.7	27.3
Only 5 daughters	22.2	0

Figure 7. Vegetative reproduction with generation identifications in Lemna minor.

The classical concept of generation time is not, strictly, applicable to Lemna minor because second generation fronds produced by the same parent may be older than first generation fronds.



O - Zero generation

O

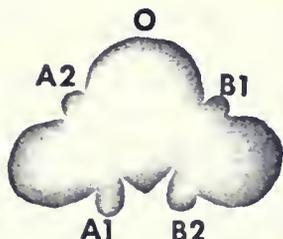
innoculum frond



A,B - First generation

A B

O

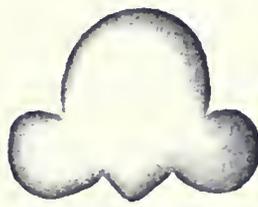


A₁A₂B₁B₂ - Second generation

A A₁ B₂ B



A₁ A A₂



C O D



B₁ B B₂

C,D - First generation

Figure 7. Vegetative reproduction with generation identifications in Lemna minor.

and B2. Therefore, O is a zero generation frond, A and B are first generation fronds and A1, A2, B1 and B2 are second generation fronds. Now if A, bearing A1 and A2, becomes detached from O during the growth of the colony, O can then produce a first generation frond C which is, of course, younger than both the first generation fronds A and B and the second generation fronds A1, A2, B1 and B2. Even though all fronds produced by the inoculum frond O are of identical genetic composition, the development of each frond could potentially take place under quite different environmental conditions and hence could possibly show the effects of these new conditions. This phenomenon also takes place simultaneously to the senescence-rejuvenation cycles shown by each frond as discussed above. Despite these complications, however, Mendiola (1919) was unable to alter the characteristics of a clone by selecting and propagating fronds in different stages. The aforementioned effects, then, are physiological in nature.

Some measurements are available which quantify the interactive effects of light and temperature on the growth of Lemna (Hicks, 1934). Using frond number, area and dry weight to measure growth, Hicks (1934) found that growth was exponential between 15-30 C and 350-1,400 foot candles light intensity. The optimal relative growth rate of Lemna occurs at 30° C and 1,000 foot candles light. Chlorophyll content, however, was maximal at 350 foot candles and at all temperatures, decreased slightly with increasing light intensity.

Flowering and Seed Set: Lemna minor has in the past, been noted to flower (Guppy 1894; Caldwell, 1899; Arber, 1920; Gilbert, 1937; Jacobs, 1947; Hillman, 1961d); however, despite some reports to the contrary (Guppy, 1894; Jacobs, 1947) flowering in this species appears to be a rare event and successful seed set is even rarer (Caldwell, 1899; Arber, 1920; Jacobs, 1947; Hillman, 1961d, 1977 pers. comm.; Bhalla et al, 1973; Kandeler, 1975; Landolt, 1975, Urbanska-Worytkiewicz, 1975). The massive and rapid vegetative reproduction is thus the primary and perhaps only mode of vegetative reproduction available to Lemna minor presently. There appears to be little doubt that Lemna minor is extremely successful using an apomictic vegetative form of reproduction (Caldwell, 1899; Arber, 1920; Jacobs, 1947; Stebbins, 1950; Hillman, 1961d; Kandeler, 1975; Landolt, 1975; Urbanska-Worytkiewicz, 1975).

When flowers are formed, they are simple and quite reduced. They consist of one pistil and usually two stamens surrounded by a sac-like spathe during their development (Caldwell, 1899; Arber, 1920; Hillman, 1961d; Bhalla et al, 1973). The basic form of the flower is shown in Figure 8. The pistil usually matures earlier than the stamens and one stamen earlier than the other. Both stamens and pistil turn up above the water surface as they emerge (Hillman, 1961d). One frond is capable of producing only one flower during its lifetime, as opposed to many vegetative



Figure 8. Typical flower form in the Lemnaceae (From Arber, 1920).

The figured flower is Spirodela polyrrhiza.

ST₁ and ST₂ - Male flowers (stamens).

C - Female flower (gynaecium).

SP - Spathe.

L - Lateral shoot.

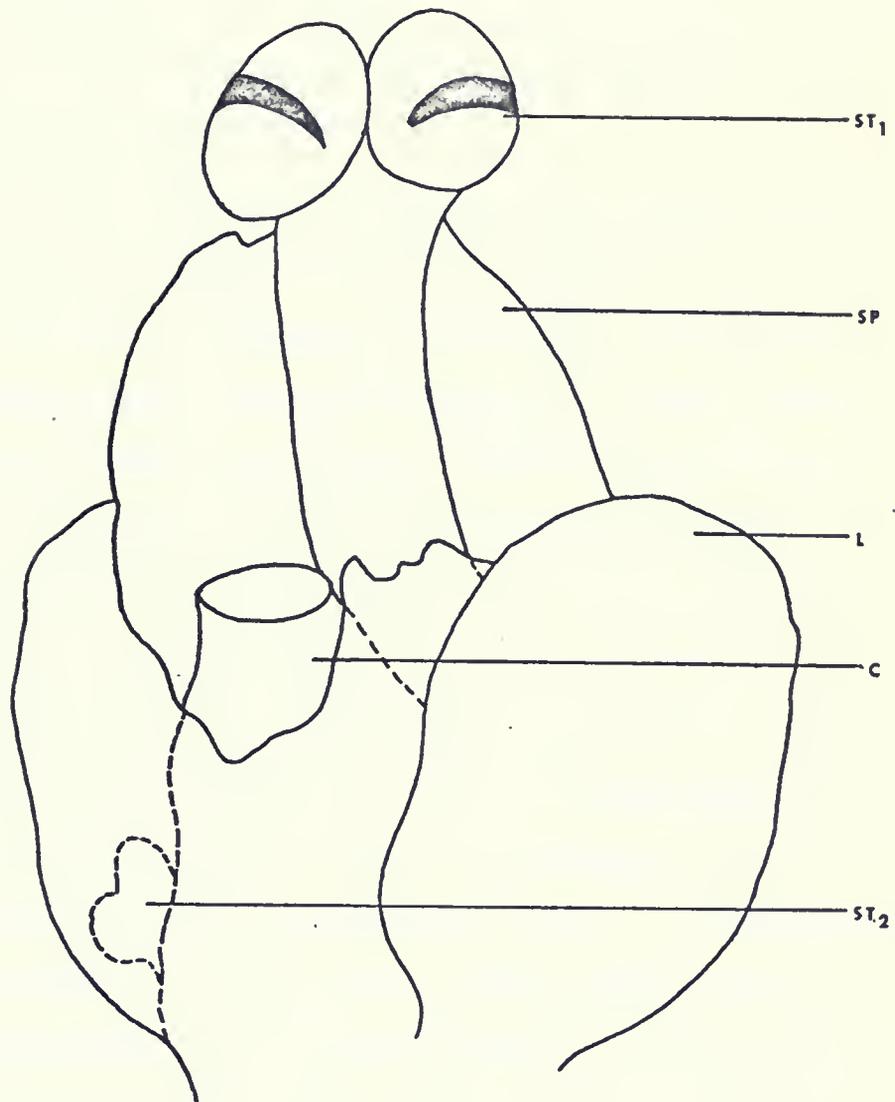


Figure 8. Typical flower form in the Lemnaceae (From Arber, 1920).

fronds. The flower is produced from the same meristematic regions from which daughter fronds arise (Hillman, 1961d). The mechanism of pollination is not well defined. Caldwell (1899) reported a water pollination mechanism and Hillman (1961d) suggested that either wind, water or insect pollination is possible. Arber (1920) suggested that insect pollination (entomophily) is important in this species.

One of the primary characteristics used by Hegelmaier (1868 in Kandeler, 1975) to separate the various species of the genus Lemna particularly to differentiate Lemna gibba from Lemna minor, were those associated with flowers and seeds. Number and position of ovules, fruit shape, fruit seed number and number of endosperm layers in each seed were primary characteristics used in identifying the species (Kandeler, 1975). Apparently, these characteristics are the only dependable ones for species delimitation and Lemna gibba and Lemna minor are indeed distinct species (Kandeler, 1975). This strong differentiation disappears when only vegetative characters can be used in identifying Lemna species.

Other members of the genus Lemna, Lemna gibba and Lemna perpusilla, are frequently found in flower in nature and are easily induced to flower under laboratory conditions (Hillman, 1961d; 1972; 1975; 1976 a,b; 1977 pers. comm.). Lemna minor, however, is next to impossible to induce or to find in flower in the field (Hillman, 1977, pers. comm.). On the other hand,

reports have appeared which suggest that Lemna minor is quite able to flower (Guppy, 1894; Gilbert, 1937; Jacobs, 1947; Stebbins, 1950), but with the controversy raging over distinct separation of Lemna gibba and Lemna minor, particularly in Europe (Guppy, 1894; Arber, 1920; Bhalla et al, 1973; De Lange and Pieterse, 1973; Pieterse, 1974; De Lange, 1974; Veen, 1975; Kandeler, 1975; Landolt, 1975); it is difficult to say anything definite about the sexual abilities of Lemna minor. A very interesting paper and perhaps an alternative explanation to the above conflict (Caldwell, 1899), may shed some light on this quandry. In August 1897, Lemna minor was found (in USA) flowering in abundance and a thorough investigation of the gametophytic structures and functional processes were undertaken. He found: (1) Quite often the flower would emerge from the reproductive pocket in accompaniment with a young vegetative plant. This plant often grew rapidly taking the place of the flower. He also noted that in some cases, the floral parts had broken down before this encroachment occurred. (2) The development of the embryo sac proceeded normally to the one-celled stage. It was only in exceptional cases that sacs beyond the one-celled stage did not show evidence of disorganization. The disorganization first affected the antipodal end of the sac; if development then continued normally, the endosperm disintegrated next and lastly the egg apparatus failed. A very small per

centage of the female gametophytes persisted until fertilization occurred. (3) Very few seeds were successfully formed.

Evidence such as this, suggests that Lemna minor may well be able to flower in a number of rare cases; however, sexual reproduction of the plant is less than successful. Lemna minor has a very successful mode of reproduction, one that is capable of producing larger numbers of individuals in a given time period than seed production. In plants with efficient means of vegetative reproduction, which the Lemnaceae have, seed sterility or lack of seed production is no detriment to the immediate fitness of the individual or the population (Stebbins, 1950). It is also possible that strictly vegetative forms, under the "right" conditions, completely out-compete their fertile relatives (Stebbins, 1950). Sex serves not so much to maintain and increase a species as to produce genetic variation (Stebbins, 1950; Williams, 1975). For these reasons it is, perhaps, best to define the habits of Lemna minor, not as an organism unable to flower, but as one incapable of reproducing itself successfully by sexual means. Lemna minor could be called an apomict utilizing vegetative reproduction.

Overwintering: To complete the life cycle picture of Lemna minor, a discussion of the overwintering mechanism utilized by this plant is necessary. When conditions, such as the approach of winter, become unfavourable

to growth, many species of the Lemnaceae form resistant fronds called turions (Biscoe, 1873; Guppy, 1894; Arber, 1920; Jacobs, 1947; Hillman, 1961d; Bhalla et al, 1973; Landolt, 1975). The turions of Spirodela polyrrhiza are the most specialized and best known and are called true turions (Biscoe, 1973; Jacobs, 1947; Landolt, 1975). These turions are smaller and thicker than the normal vegetative fronds, have very reduced air spaces and are heavily packed with starch grains (Guppy, 1894; Arber, 1920; Jacobs, 1947; Bhalla et al, 1973). Because of these modifications the fronds are more dense than water and when they detach themselves from the parental fronds they sink to the bottom where they spend the winter. This process reverses itself in the spring; the starch is broken down and the plants rise to the surface.

Jacobs (1947) studied growth and formation of the turions of Spirodela polyrrhiza under many combinations of laboratory conditions. He concluded that turions form under any combination of environmental conditions which maintain the rate of photosynthesis at a level well above that required for growth and respiration; for example, high light energy and/or high CO₂ concentration or, under conditions which limit the rates of growth and the respiration; for example, low temperature or nitrogen deficiency. Both of these processes would increase the amount of photosynthetic product stored as starch. Turions were induced between 10-35°C and were tolerant to temperature extremes of -8°C and 50°C. In the spring,

the minimum temperature for germination was 15°C and light intensities of 5-10 foot candles were required. Turions do not necessarily germinate the same season they are formed. Turions never give rise to other turions and at least two to four vegetative generations must intervene between one round of turion formation and the next. Vegetative reproduction continues as long as conditions allow it.

Jacobs (1947) also reported the formation of true turions in Lemna minor for the first time. Usually, under unfavourable but non-severe winter conditions, Lemna gibba and most strains of Lemna minor develop small specialized fronds that are very rich in starch. These however, do not sink to the bottom (Guppy, 1894; Arber, 1920; Landolt, 1975) and are not considered true turions relative to those produced by Spirodela polyrrhiza (Landolt, 1975). It is therefore interesting to note Jacob's (1947) observation of true turions in Lemna minor, as Landolt (1975) classifies Lemna minor as not forming characteristic turions similar to those of Spirodela polyrrhiza. Lemna turionifera, however, is nearly identical to Lemna minor except that it is often pigmented by anthocyanins on the lower surface, often has distinct papules on the median nerve, and does form characteristic turions similar to those of Spirodela polyrrhiza and Lemna turionifera although classified by Landolt (1975) as sp. nov., has also been placed in the Lemna minor group (Landolt, 1975; Urbanska-Worytkiewicz,

1977, pers. comm.-re strain 6735). Lemna turionifera overlaps some of the Lemna minor distribution range and is also the only species from the Lemna minor group that occurs in the northern part, as well as the high mountains of North America (Northern USA and Canada) (Landolt, 1975). It is in these areas that survival of the vegetative plant would be impossible, due to the severity of the winters, without turion formation (Jacobs, 1947).

In order to conclude this section on the overwintering of Lemna minor, the following generalizations are listed: (1) Lemna minor in non-severe winter conditions do not form true turions but are able, by starch packing to survive cold non-optimal periods. (2) In the northern distribution range of Lemna minor (in North America and Asia) true turion formation is a necessary survival adaptation and there is a strong likelihood that the species predominating in these regions is Lemna turionifera of the Lemna minor group. This statement should not preclude the possibility however, that true turion formation in Lemna minor "proper" is not possible. (3) No matter which specific adaptation to stress periods is used, Lemna minor can successfully and continuously survive all seasons vegetatively without need to resort to the sexual production of seeds. (4) One genotype is thus likely to be able to survive and propagate itself indefinitely (Stebbins, 1950; Grant, 1975; Urbanska-Worytkiewicz, 1975; Janzen, 1977). One

genotype of Lemna minor can be called a single evolutionary individual possessing a distinctive reproductive fitness (which may vary with habitat) (Janzen, 1977). This evolutionary individual is merely subdivided into several pieces but due to the genetic identity (barring chance mutation) of all the pieces, selection acts on this collection of pieces as a single evolutionary unit. Given the successful year round vegetative life cycle of Lemna minor, genotypes or evolutionary individuals are similar to long-lived perennial plants (Stebbins, 1950; Janzen, 1977).

Cytology: Until 1975 (Urbanska-Worytkiewicz, 1975), cytological investigations of the Lemnaceae were rare (Blackburn, 1933; Brooks, 1940 in Hillman, 1961d). The Lemnaceae are extremely difficult materials for cytological investigations as their chromosomes are exceedingly small (Urbanska-Worytkiewicz, 1975), and Lemna minor karyotypes are complex. Urbanska-Worytkiewicz (1975) undertook the cytological study of various Lemna L populations from 711 localities from around the world; 346 of which were Lemna minor (Figure 9). Although most populations of Lemna minor throughout the world demonstrate intra-population uniformity, (Table 3a) (334/346), one population exhibited aneusomaty, (The number of chromosomes varies between somatic cells of a plant but does so such that one or more chromosomes are represented more often than the rest), four exhi-

Figure 9. Geographical distribution of kartotypes of Lemna minor s.l. (From Urbanska-Worytkiewicz, 1975)

△ 2n=20

▲ 2n=30

○ 2n=40

● 2n=42

* 2n=50

■ 2n=80

(Some tetraploid and hypertetraploid stations not included).

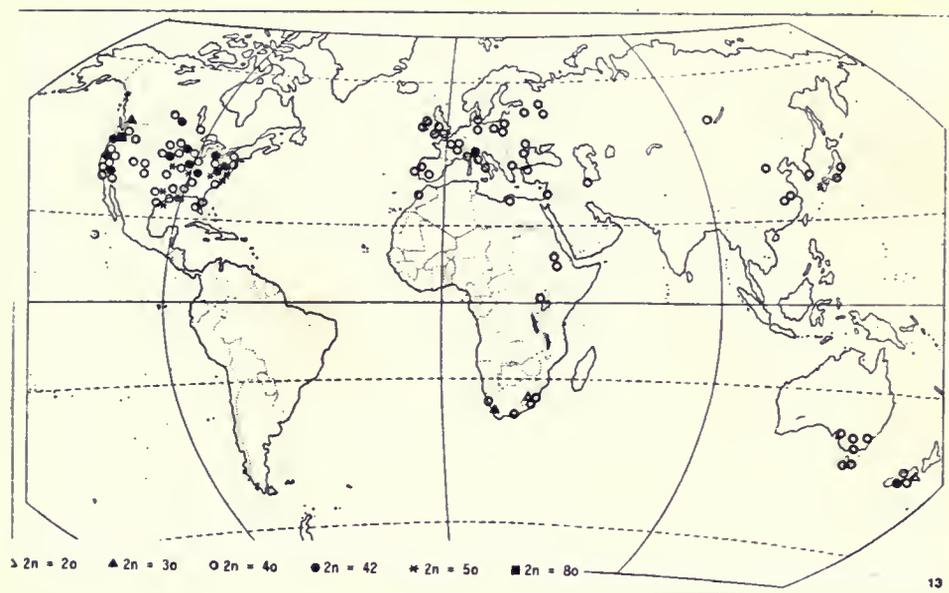


Figure 9. Geographical distribution of karyotypes of Lemna minor s.l. (From urbanska-Worytkiewicz, 1975)

Table 3. Cytological variation in Lemna minor populations (From Urbanska-Worytkiewicz, 1975).

(a) Total variable forms encountered.

Uniform Populations	Aberrant Populations			Total
	Aneusomaty	Mixoploidy	Mixed Populations	
334	1	4	7	346

(b) Frequency of each karyotype found in the uniform populations.

Number of Populations	20	30	40	42	50	80
334	1	2	290	32	8	1

Figure 10. Somatic metaphase chromosomes in Lemna minor s.l. (From Urbanska-Worytkiewicz, 1975).

- 6) 2n=20 (7789)
- 7) 2n=30 (7244)
- 8) 2n=38 (8166/3-aberrant form)
- 9) 2n=40 (7530)
- 10) 2n=42 (7572)
- 11) 2n=50 (6742)
- 12) 2n=80 (6735)



Figure 10. Somatic metaphase chromosomes in Lemna minor s.l. (From Urbanska-Worytkiewicz, 1975).



bited mixoploidy (The number of chromosomes varies between somatic cells of a plant but does so in multiples of whole chromosome sets, in this case by multiples of the base number 10) and seven mixed populations were found. Six karyotypic forms of Lemna minor were found: $2n=20, 30, 40, 42, 50$ or 80 (Figure 10). The base chromosome number was 10 and the tetraploid and hypertetraploid forms ($2n=40$ and 42 respectively) were the most common (Table 3). Only $1/334$ diploid population was found. Despite the complexity of ploidy forms in Lemna minor, from the data shown in Table 3, it is probable that most populations will represent some form of the polyploid (Urbanska-Worytkiewicz, 1975, 1977 pers. comm.).

Habitat: Despite the large amount of work done on various aspects of the Lemnaceae, there are few investigations yielding precise quantitative information about the habitat of Lemna minor (Hillman, 1961d). Generally, it occurs in still to slightly moving permanent bodies of water such as low-lying roadside ditches, shallow ponds or sheltered bays (Guppy, 1894; Arber, 1920; Jacobs, 1947; Hillman, 1961d; Keddy, 1976) (Figure 11). Vegetative growth can continue on wet mud (Guppy, 1894; Arber, 1920; Jacobs, 1947) as long as severe desiccation does not occur (Jacobs, 1947; Keddy, 1976). The best growth occurs in organically rich waters (Arber, 1920; Jacobs, 1947; Hillman, 1961d).

Figure 11. Typical habitat sites of Lemna minor.

- a) BN-5, Bridgenorth, Ontario.
- b) GU, Guelph, Ontario.



Figure 11. Typical habitat sites of Lemna minor.



The range of pH tolerance in this species is fairly broad (McLay, 1976). McLay (1976) in laboratory studies showed that the lower, optimal and upper limits for Lemna minor were 4-6.2-10. Table 4 shows the average range and mean values for pH in field habitats in Lemna minor (6.8-7.7, mean 7.3) as found by Landolt (1975). These values are somewhat different. Care must be taken, then, in extrapolation of the laboratory results to field situations, because specific nutrient form may have a large effect on the ambient pH experienced by the plant (McLay, 1976).

Growth in salt water is not possible and fronds do not survive more than one day in salt conditions. Lemna sp. is strictly a fresh water aquatic organism (Arber, 1920).

Although there has not been a great deal of work which gives detailed descriptions of the Lemna minor habitat, Landolt (1975) included a table (Table 4) which does describe some of the chemical conditions found in his North American collection sites. As can be seen in Table 4, Lemna minor does tolerate a rather large range of habitat conditions in the field.

Lemna minor grows and reproduces at all light intensities from full sunlight to below 50 foot candles (Hicks, 1934; Hillman, 1961d) and can grow heterotrophically if all necessary nutrients are supplied (Hillman, 1958). In general, 20°-30°C delimits the

Table 4. Some chemical properties of the water samples from different locations of Lemnaceae in North America. Ions in mg/l; mean values of the different samples, total variation in parentheses (From Landolt, 1975).

Taxon	Samples	pH	Conductivity	Ca	Mg	K	Na	N (as NO ₃ ⁻ and NH ₄ ⁺) ³	P (as PO ₄ ⁻³)
gibba	15	8.2 (7.4-9.8)	930 (200-2,600)	40 (6.5-115)	66 (7.5-145)	15 (3.4-30)	250 (28-850)	2.1 (0.2-10.6)	1.24 (0.02-9.9)
minor	9	7.3 (6.8-7.7)	320 (70-700)	13 (3.0-32)	23 (2.5-85)	12 (2.2-53)	40 (10-115)	1.4 (0.4-3.0)	0.05 (0.01-0.1)
turionifera	11	7.7 (7.1-9.8)	470 (185-1,300)	22 (4.0-73)	19 (3.9-95)	15 (3.3-18)	80 (17-225)	1.0 (0.2-2.9)	0.08 (0.00-0.3)
obscura	11	7.2 (6.6-7.9)	510 (120-2,900)	22 (4.8-68)	32 (3.6-100)	16 (5.3-35)	110 (9-800)	1.4 (0.2-11.2)	0.18 (0.01-0.5)

optimal temperature for vegetative growth (Guppy, 1894; Hillman, 1961d).

Geographically, the distribution of Lemna minor is limited by the 15 C° isotherm of the three coolest months in warm regions and by the -10° January isotherm in cold regions (Landolt, 1975) (Figure 12a). Lemna minor does not tolerate arid climates whereas Lemna gibba can and does (Landolt, 1975). Lemna turionifera is the only species of the Lemna minor group that occurs in the Northern part of North America and endures median January temperatures of -20°C (Landolt, 1975).

In contrast to the distribution of Lemna minor/Lemna turionifera, is that of Lemna gibba. Its occurrence is limited by the 18°C isotherm of the three coolest months in warm regions and by the -1°C January isotherm in cool regions (Landolt, 1975) (Figure 12b). This species also prefers drier regions than Lemna minor and is usually found in areas with an average yearly precipitation of less than 90mm. These areas are too dry for Lemna minor (Landolt, 1975). According to this information, overlap and confusion of Lemna gibba with Lemna minor, although a severe problem in Europe, is not likely to be a problem in the areas where the present investigation was undertaken (Figure 12a and b). Overlap and confusion of Lemna minor with Lemna turionifera (if in fact they are distinct species) is however, a potential danger in the areas studied.

Figure 12. Geographical distributions of Lemna gibba and Lemna minor (From Landolt, 1975).

- a) Distribution of Lemna minor.
- b) Distribution of Lemna gibba.



Figure 12. Geographical distributions of *Lemna gibba* and *Lemna minor* (From Landolt, 1975).

Dispersal: Some accounts exist which claim that dispersal of Lemna minor in the vegetative form is easily accomplished (Guppy, 1894; Arber, 1920; Jacobs, 1947; Grant, 1975; Keddy, 1976). The primary agents of dispersal are certain water birds, for example ducks and moorhens (Guppy, 1894; Arber, 1920; Jacobs, 1947; Keddy, 1976); dispersal by muscrats has also been reported (Jacobs, 1947). The main problem in dispersal of the vegetative form is, of course, tolerance to drying. Keddy (1976) reports a fifty percent survival of Lemna minor fronds exposed to air for one hour, after which time, survival rapidly decreases. It is thus theoretically possible that Lemna minor, attached to the wet feathers of waterfowl or to the wet fur of muskrat, could be dispersed around a limited range from a source population, subject to the drying agencies present on any given occasion. This would create a radially expanding dispersal pattern; survival occurring in appropriate habitats if dessication does not occur prior to introduction. Salt water also provides a barrier to dispersal (Arber, 1920) in two ways. The first is that if the body of salt water is large, drying is likely to occur before the next fresh water habitat is reached and secondly, if the dispersing agent contacts salt water prior to fresh water, even a non-dessicated Lemna minor individual does not have much chance of survival.

ADAPTATION

Adaptation, Evolution and Fitness: Evolution may be defined as changes in the genetic diversity and adaptive levels of populations of organisms. All populations are, then, adapted; they exist. Clearly however, not all organisms are equivalently adapted. Those individuals well adapted to a particular niche leave more progeny than their poorly adapted counterparts, and the well adapted individuals are said to be more fit. This leads to genetic changes and hence evolution in the population. In other words, genetic diversity produces adaptational diversity through differential survival which accounts for differential reproduction and evolution within a species group. Those populations well adapted to current environments and capable of coping with environmental change will continue to exist, while poorly adapted populations become extinct.

If it can be said from the study of organisms in their environments, that the structural and functional characteristics often appear (retrospectively) to be well suited for success and survival in that environment (Hochachka and Somero, 1973; Somero, 1975a; Somero and Low, 1977) and that environmental changes in the habitat of that organism are often successfully handled in ways that maintain the internal homeostasis of that organism (Hochachka and Somero, 1973; Somero, 1975a; Moon, 1975; Somero and Low, 1977); then it can be said

that adaptation, whatever the strategy, has been or is the process of selecting organismic properties which allow optimal or at least best possible homeostatic biological function under all environmental demands that the organism or population is likely to meet. By meeting these demands, successful survival and reproduction occurs.

The basic evolutionary strategy used by species to survive and reproduce in a range of environments is maintenance and production of genetic variability and hence response flexibility (Mather, 1943, 1973; Clarke, 1975; Ayala, Powell and Tracey, 1972). In other words, "the evolutionary potential, at a given time, of a population or a species, is a function of how much genetic variation the population or species has" (Ayala, Powell and Tracey, 1972). This is Fisher's Fundamental Theorem of Natural Selection (Fisher, 1930). The maintenance and production of variable forms must be balanced by the adaptive demands to replicate the already successful genotypes or phenotypes (Mather, 1943, 1973; Stebbins, 1950; Grant, 1975). Out-crossing organisms easily maintain this variability while inbreeding leads to a reduction of this potential variability (Inbreeding creates excesses of homozygotes (Mather, 1973); potential variability is the hidden or latent variability carried in the heterozygote.). On the other hand, free variability (Free variability is the expressed form of the homozygote (Mather, 1975)) is higher in inbreeding organ-

isms. Vegetative reproducers, if fitnesses are equal, do not necessarily reduce the already present free variability, nor do they convert potential variability into free variability. There are modes of adaptation other than production of genetic variability and these shall be discussed shortly. These other modes are dependent on the level of treatment of the adaptation question, for example, at the population or the individual level. Whatever strategy is used for adaptation, the key is flexibility.

Before discussing strategies or modes of adaptation, it should be mentioned here that a technique exists which allows an investigator to score genetic variability: gel electrophoresis. Electrophoresis detects differences in enzymes or proteins on the basis of net electrostatic charges, size or any other character which affects the mobility of the substance in question in an electric field (Gottlieb, 1973; Clarke, 1975; Brewer, 1970). The usefulness of this technique is that the variation in the resulting enzyme band patterns can be directly equated to variation in the genes coding for the proteins (Gottlieb, 1973; Clarke, 1975).

Essentially, two modes of adaptation can be envisaged: (1) genetic; (2) physiological.

Genetic adaptation occurs at the population level. A population is a collection of individuals made up of a set of genotype frequencies, each of which possess an ability to react to environmental change. Adaptation,

in these circumstances occurs through the selective death, in the genetic sense of genotypes. Individual genotypes are differentially able to maintain their internal homeostatic activities and reproduce. This leads to genotype frequency changes (Fisher, 1930; Wright, 1970; Haldane, 1932). This is a long term evolutionary process and involves generations of time (Hochachka and Somero, 1973). Genetic variability is very important in this process (Fisher, 1930; Ayala et al, 1971).

Physiological adaptation occurs primarily at the individual level and two basic enzymatic strategies are possible: (1) polymorphic stenotolerant enzymes (2) monomorphic eurytolerant enzymes.

An organism may utilize two or more isozymes to buffer its metabolic functions against the effects of environmental change. This has been called the multiple variant protein strategy (Somero, 1975a). Here no single isozyme is functional over the entire range of environmental conditions encountered but the presence of two or more stenotolerant isozymes, each of which functions in a different part of the range of conditions, allows the organism to function over the entire range of environmental conditions met by an organism (Somero, 1975a; Somero and Low, 1977).

In the eurytolerant protein strategy (Somero, 1975a), on the other hand, a single protein form is capable of maintaining its structural and functional characteristics over the entire range of environmental conditions met by an organism (Somero, 1975a; Somero and Low, 1977).

The physiological and genetic modes of adaptation just outlined above are, of course, not mutually exclusive. Generations of time are required for an organism to acquire information for the production of either polymorphic stenotolerant enzymes or monomorphic eurytolerant enzymes. Many adaptive genetic changes, in transition or equilibrium may encode eurytolerant enzymes which are part of an isozymic system.

These modes of adaptation are, however, identifiable. Using gel electrophoresis as the scoring technique; manipulation of the environmental conditions about an individual will not immediately change the banding patterns on the gel if the mode of adaptation is genetic. Genetic adaptation can be scored by following the trend of frequency changes of genotypes in the population over several generations under constant conditions. If the mode of adaptation is physiological, however, manipulation of the environmental conditions, either artificially or naturally, may cause immediate changes in band patterns within individuals. If these changes involve the appearance of new proteins they are isozymic in nature; if conformational they represent eurytolerant shifts.

Lemna minor is an organism whose primary or sole mode of reproduction is one of apomixis. It is also a polyploid and has a wealth of genetic material for potential use. Lemna minor is also widely distributed

throughout the world and certainly shows toleration to a fairly high degree of variable conditions (Table 4). This particular study of Lemna minor was undertaken to try and ascertain what mode or modes of adaptation are used by such an organism. In order to do this, a survey electrophoretic study of some enzymes in Lemna minor was undertaken.

Before proceeding three descriptive terms will be encountered and need to be defined. These are:

- (1) Colony: Individual fronds that still remain attached, a family unit.
- (2) Clone: Colonies known to be directly related to each other and to have descended from one colony.
- (3) Population: The sum series of colonies occupying a single definable habitat space, for example a pond. If this area was large and diverse, sub-populations were collected.

MATERIALS AND METHODS

Collections: Populations of Lemna minor were sampled between June and November 1976 and between May and August 1977. During both years of the study, collections were cultured in glass containers using water from the sample sites and kept on a 16:8 L:D photoperiod regime under Westinghouse 40 watt cool white fluorescent light banks. Evaporated water was replaced with distilled water. In 1977, populations were also cultured axenically in slant tubes on Hillman's M medium (1961a) supplemented with 1% sucrose and solidified with 3% agar (Table 5). Fronds were sterilized prior to inoculation into the slant cultures by dipping into a 10% by volume solution of Javex for 45 seconds (Hillman, 1961d). These cultures were kept in a 25°C incubator on a 12:12 L:D lighting regime. Axenically cultured Lemna minor represented cloned materials as the tubes were started with single colonies. During the 1976 sampling season, Lemna minor was kept in outdoor holding facilities and Lemna minor from local areas was collected from the field. These culturing regimes allowed comparison of the culturing effects. Randomly chosen colonies from these collections were used in all experiments.

The populations sampled were:

- 1) BN-1 Bridgenorth, Ontario

Table 5. Recipe for Hillmans M Medium (Hillman, 1961d). Quantities represent mg/l.

KH_2PO_4	680
KNO_3	155
H_3BO_3	2.86
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.22
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.12
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.08
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3.62
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	1180
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	492
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	5.40

pH adjusted to 4.6 with 1M KH_2PO_4 or 1M KOH. 1% sucrose and 3% agar added to solution and boiled prior to autoclaving. Slant tubes poured, autoclaved and allowed to sit overnight prior to sample inoculation.

- | | |
|---------|----------------------|
| 2) BN-2 | Bridgenorth, Ontario |
| 3) BN-3 | Bridgenorth, Ontario |
| 4) BN-4 | Bridgenorth, Ontario |
| 5) BN-5 | Bridgenorth, Ontario |

All of the above are sub-populations of one continuous pond area approximately one and a half kilometers in length.

- | | |
|---------------------|-----------------------------------|
| 6) LA-6 | Lamable, Ontario |
| 7) LA-7 | Lamable, Ontario |
| 8) GA | Glen Alda, Ontario |
| 9) TH | Thorold, Ontario |
| 10) GU | Guelph, Ontario |
| 11) SB | Stinking Barn, Wainfleet, Ontario |
| 12) No. 7789, 2n=20 | Natal, Africa |
| 13) No. 7744, 2n=30 | South Africa |
| 14) No. 7102, 2n=40 | Kansas City, Kansas, USA |
| 15) No. 7210, 2n=50 | Grahamstown, South Africa |
| 16) No. 6570, 2n=42 | Davenport, Washington, USA |
| 17) No. 6580, 2n=42 | Harrington, New Jersey, USA |
| 18) No. 6728, 2n=50 | Baltimore, Maryland, USA |
| 19) No. 6742, 2n=50 | Spring Creek, Pennsylvania, USA |
| 20) No. 6735, 2n=80 | Washington, USA |

Sample # 12-20 inclusive were kindly sent to the author by Dr. Krystyna Urbanska-Worytkiewicz, Geobotanical Institute, Zurich, Switzerland from her collection used for the cytological analysis discussed in the Introduction (Urbanska-Worytkiewicz, 1975).

Habitat descriptions for the above collections are to be found in Table 6.

Table 6. Habitat descriptions for field populations surveyed 1976-1977.

Popula- tion	Location	Habitat Description	Other <u>Lemnaceae</u> Present	% Cover <u>Lemna</u> <u>minor</u>	Year Collected	Culture Method	Sample Size in Survey Gels
BN-1	Bridgenorth, Ontario 44°22'N/78°15' W Off Canadian Shield. Figure 13	Flooded forest swamp, 1.5km long, 4km wide, 460m from the road. Samples stranded on black mud.	none	100	September 26, 1976. June 18, 1977.	water axenic	141
BN-2	As above. Figure 14.	Same as above except BN-2 col- lected 2m from swamp edge. <u>Lemna</u> <u>minor</u> floating.	<u>Wolffia</u> sp.	90	September 26, 1976 June 18, 1977.	water axenic	96
BN-3	As above. Figure 15.	500m W of BN-1. Sample taken at edge. Floating.	none	100	September 26, 1976. June 18, 1977.	water axenic	68
BN-4	As above. Figure 16.	Same as above, 1 meter farther from edge than BN-3. Floating.	<u>Wolffia</u> sp.	10 (90% <u>Wolffia</u>)	September 26, 1976. June 18, 1977.	water axenic	152
BN-5	As above. Figure 17.	1000m W of BN-1. Sample taken 1m from edge.	<u>Wolffia</u> sp.	90	September 26, 1976. June 18, 1977.	water axenic	145

tion	Description	Lemnaceae Present	Lemna minor	Collected	Method	Size
LA-6	Lamable, Ontario 45° N/77°45'N Canadian Shield.	none	5 (sparse population)	September 26, 1976. June 18, 1977	water axenic	149
LA-7	As above.	none	100	September 26, 1976. June 18, 1977.	water axenic	175
GU	Guelph, Ontario 43° 36'N/80°15'W. Off Canadian Shield. Figure 18.	<u>Wolffia</u> sp. Lemna <u>trisulca</u>	95	June 17, 1977.	water axenic	102
GA	Glen Alda, Ontario Canadian Shield. 45°N/77°W.	<u>Wolffia</u> sp. <u>Spirodela polyrrhiza</u>	98	June 17, 1977.	water axenic	104
SB	Stinking Barn, Wainfleet, Ontario. 43° 8'N/79°15'W. Off Canadian Shield. Figure 19.	<u>Wolffia</u> sp. Lemna <u>trisula</u>	100	once a month during 1976 and 1977 collection seasons.	water axenic outdoor holding facilities.	120
TH	Thorold, Ontario.	none	100	September 1976 did not succeed in 1977. Overwintered in Lab.	water	150

Locality	Locality	Description	Other Lemnaceae Present	% Cover <u>Lemna</u> <u>minor</u>	Year Collected	Culture Method	Sample Size
7789 (2n=20)	Natal sprint olei Alberton, South Africa. 26 18'S/ 28 10'E.		unknown	unknown	unknown	axenic	14
7244 (2n=30)	Stellenbosch, South Africa.	pond in Nature Con- servation area, De- partment of Botony, University at Jonk- ersveh.	unknown	unknown	unknown	axenic	14
7210 (2n=40)	Grahamstown, South, Africa. Eastern Cape	Fish pond in a far- mers garden; Eleva- tion - 1,500 ft.	unknown	loose, floating clear green colonies.	unknown	axenic	20
7102 (2n=40)	Kansas City, Kansas.	unknown, McClures collection.	unknown	unknown	unknown	axenic	42
6742 (2n=50)	Spring Creek Pennsylvania	Creek, Elevation- 1,000 ft.	unknown	unknown	unknown	axenic	14
6728 (2n=50)	Baltimore, Maryland.	pond on campus of John Hopkins Univer- sity.	unknown	unknown	unknown	axenic	14
6570 (2n=42)	12 miles W of Davenport, Wash- ington.	volcanic sink.	unknown	unknown	unknown	axenic	14

Popula- tion	Location	Habitat Description	Other Lemnaceae Present	% Cover <u>Lemna</u> <u>minor</u>	Year Collected	Culture Method	Sample Size
6580 (2n=42)	Harrington, New Jersey.	Mill pond.	unknown	unknown	unknown	axenic	14
6735 (2n=80)	Touchet, West of Walla Walla Washington.	slow moving creek.	unknown	unknown	unknown	axenic	42



Figure 13. Collection site BN-1.



Figure 14. Collection site BN-2.



Figure 15. Collection site BN-3.



Figure 16. Collection site BN-4.



Figure 17. Collection site BN-5.



Figure 18. Collection site GU.



Figure 19. Collection site SB.

Electrophoresis: For the purposes of electrophoresis, colonies were randomly chosen that consisted of at least two fronds. Each individual was thoroughly washed in distilled water and blotted dry prior to grinding. Grinding was accomplished by placing each individual, with one drop of gel buffer (4°C) onto a pre-cooled, specially constructed glass spotting plate (Figure 20). The plants were then ground with a lid that had carborundum powder-roughed glass balls fitted onto it. The resulting homogenate was then soaked onto 7 mm x 4 mm Whatman #1 filter paper wicks which were then inserted into starch gel blocks for electrophoresis. Controls were treated in the same manner. These controls consisted of one of the following: 1) a mass-grind of several individuals from each population used in the study. 2) individuals of known ploidy number.

The gel recipe used in all these studies consisted of 40 grams of Electrostarch (Electrostarch Co., Madison, Wisconsin) and 330 ml of Tris-Boric acid Buffer for Dehydrogenase, pH 9.0 (DH Buffer) (Ayala, Hedgcock, Zumwalt, and Valentine, 1973). Gels were left to solidify overnight and were then cooled in the refrigerator for approximately one hour before insertion of the sample. Starch gel blocks were run in the same container in which they were formed. These were plexiglass, rectangular troughs with side slats that slid up and down on screw mounts to expose the starch con-

Figure 20. Grinding apparatus.

Bottom plate is a glass spotting plate with surfaces roughed with carborundum powder. Top plate is a glass plate with carborundum powder roughed glass balls fixed to match impressions on the lower plate.

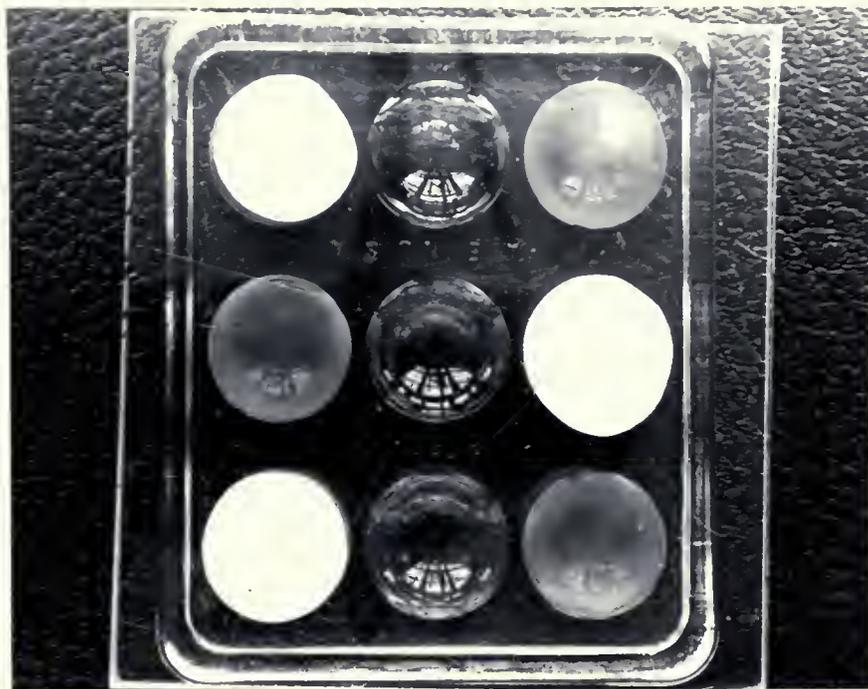


Figure 20. Grinding apparatus.

tacts to the current flowing through the bridge trays (Figure 21). The dimensions of the starch block were 17.3cm x 13cm x .7cm (L x W x H). Contact starch was 1.8cm deep. Each block held 24 samples and at least two slices could be cut from each gel yielding a potential of 48 data pieces/gel. Electrophoresis was carried out in a cold room at 80mA for 4 hours. At the end of this time, gels were sliced and assayed for NAD-dependent Malate Dehydrogenase (1.1.1-37) and Tetrazolium Oxidase. On occasion NADP-Malic enzyme and Catalase were assayed.

Most of the results in this investigation are based on the malate dehydrogenase system. Malate dehydrogenase catalyzes the reaction: $\text{Malate} + \text{DPN} \rightleftharpoons \text{oxalacetate} + \text{DPNH}$. Also the malate dehydrogenase, malic enzyme and tetrazolium oxidase systems were studied using a tetrazolium staining system. In these reactions, the studied enzyme reduces a pyridine nucleotide which in turn reduces an electron carrier such as phenazine methosulfate. This in its turn reduces the tetrazolium dye forming a formazan precipitate which produces a blue band (Brewer, 1970). Tetrazolium oxidase oxidizes the mixture and yields clear bands.

The specific assays were as follows:

(1) Malate Dehydrogenase (MDH): 50 ml Tris-HCl, pH 8.6 DH strain buffer (Ayala et al, 1973), 50 ml distilled water, 25 mg NAD, 25 mg NBT, 100 mg L-malic acid, 5mg PMS (modified from Scandalios, 1969).

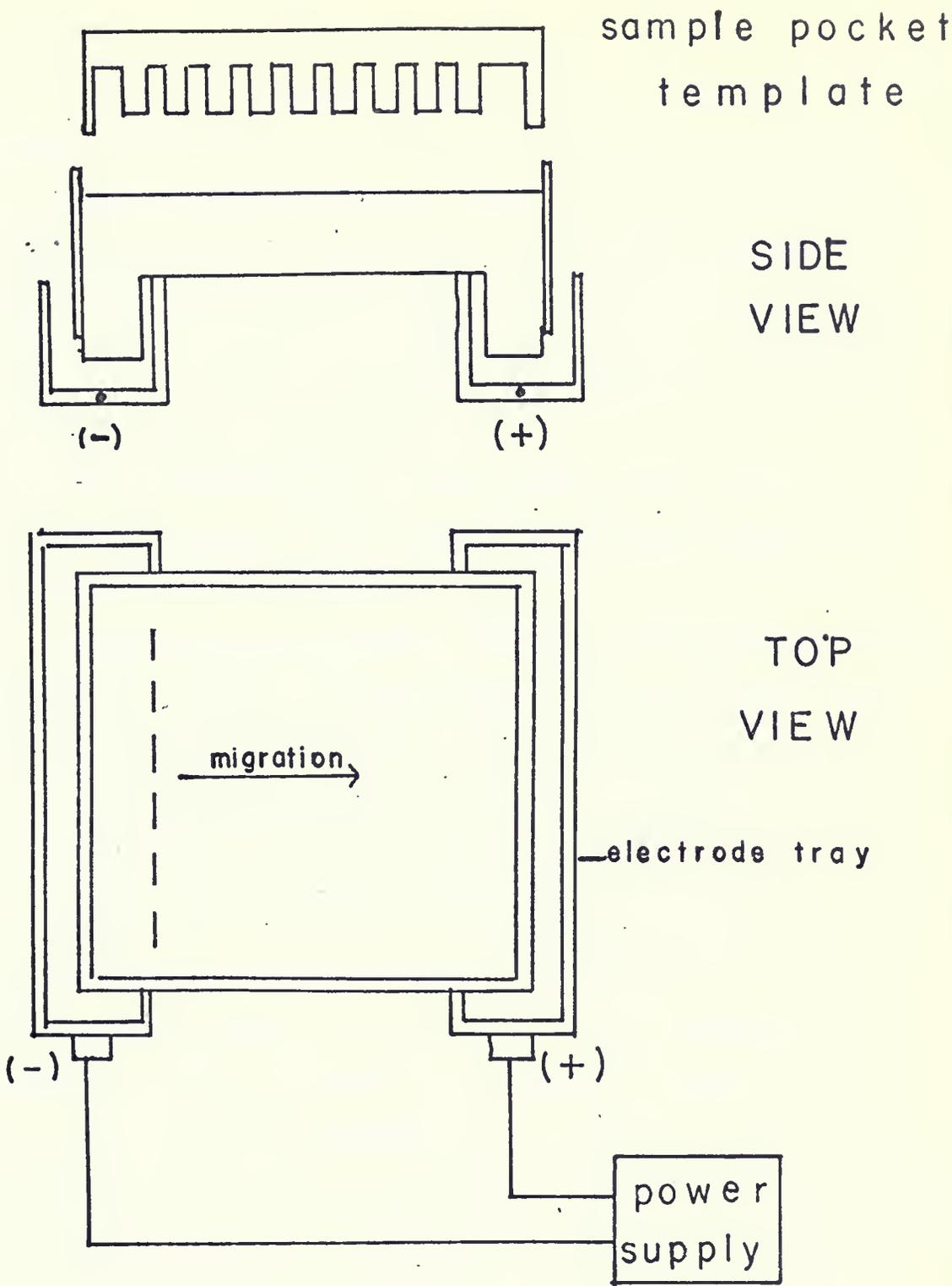


Figure 21. Gel form used for Electrophoresis.

(2) Tetrazolium Oxidase (TO): recipe identical to MDH except the substrate, L-malic acid, was not included and 50 mg NBT was used. The tetrazolium system was mainly used as a test system to check the MDH system for Tetrazolium Reductase activity (Tracey et al, 1975) and to show that MDH band patterns did not occur in the absence of substrate.

(3) Malic Enzyme (ME): when assayed the recipe was identical to that of MDH except that 50mg of NADP was substituted for the NAD.

(4) Catalase (CAT): 100ml 0.5% H₂O₂ poured over the cut surface of the gel for 30 seconds, this was rinsed off with distilled water, 100ml 1.5% KI acidified with 3ml glacial acetic acid poured over gel. Staining reaction occurred within 2 minutes (Scandalios, 1969).

Stains for MDH, TO and ME were left overnight and full stain reaction was usually completed in 16 hours. MDH usually began to initiate the stain reaction within 15 minutes of stain contact with the gel slice.

Time was spent attempting to stain for other enzymes. These were: Alkaline Phosphatase, Acid Phosphatase, Esterases, Leucine Aminopeptidase, Fumarase, Peroxidase, Aldehyde Oxidase, Alcohol Dehydrogenase, Lactate Dehydrogenase, Glutamate Dehydrogenase, Isocitrate Dehydrogenase, Glucose 6-Phosphate Dehydrogenase, Sorbitol Dehydrogenase, Catalase, Malic Enzyme and General Protein. Several recipes on several buffer systems were tried and all

attempts were either unsuccessful or they did not stain at all times. Lithium Borate-Tris-citrate (discontinuous) pH 8.3 (Scandalios, 1969), Histidine-Sodium Citrate (discontinuous) pH 8.0 (Brewer, 1970 and Poulik (discontinuous) pH 9.1 (Poulik 1957) buffering systems were also tested. The DH continuous buffer system mentioned above, gave the best resolution for the MDH and TO enzyme systems in Lemna minor and hence, use of the others was discontinued. The addition of various concentrations of NAD and NADP to the molten gels was also tested but since it did not change the gel patterns or improve the resolution of MDH or TO, this practice was also discontinued.

At the end of the staining reaction gels were fixed in a 60% ethanol : 40% distilled water mixture and then wrapped in Saran Wrap for future reference. It was also found early in the assay trial period, that a great deal of gel to gel variation, probably attributable to power pac variation, was present. This did not occur within each gel. Samples that were to be compared were set up and run on the same gel and all experiments were designed to allow this. Gel to gel comparisons, if needed, were made on the basis of mobilities relative to a common control.

Heat Stability: In order to clarify which MDH bands were allelic and which represented separate loci, heat stability studies were carried out on the MDH system.

The rationale behind this study was based on the premise that alleles should have more similar heat stabilities than separate loci. The Thorold population was used in this investigation. Four gels containing twenty-four individuals each, four of which were controls (untreated individuals), were run representing four replicates of the experiment. Groups of washed Lemna minor colonies were placed in distilled water in a 60°C water bath for 1, 5, 15 or 30 minutes. At the end of these treatments, colonies were immediately ground, electrophoresed and stained in the usual manner.

Molecular Weight: In addition to the heat stability determinations, crude molecular weight separations were done to identify locus specific bands. For this four Minicon TM Concentrations (Amicon Corporation, Lexington, Massachusetts) were used. These are multi-chambered devices bound by a membrane of selective permeability and backed by absorbant pads which absorb water and permeating species (Figure 22). The retained species are concentrated in the chambers (10-20 X concentration factor). For this experiment, Molecular weight cut-off sizes of 15,000MW, 25,000MW, 75,000MW and 125,000MW were used.

Large quantities of Lemna minor were cleaned and then ground in DH buffer with a mortar and pestle. The resulting homogenate was centrifuged at top speed in an IEC International Clinical Centrifuge (CL model)



Figure 22. Minicon TM Concentrators used in molecular weight separation. (15,000MW shown, Amicon Corporation, Lexington, Mass.)

for 5 minutes to remove the debris. The MiniconTM Concentrator chambers were then filled with the supernatant. Concentration was complete in one hour and the end products of this process were electrophoresed in the usual manner. All stages of this experiment were carried out in the cold room. Two replicate gels, containing controls, were run for this analysis. The Thorold population, once again, composed the test population.

Cell Fractionation: To determine which MDH band sets were associated with which cellular organelles, a cell fractionation was done utilizing differential centrifugation techniques, (Axelrod, 1955; Gorham, 1955; Webster, 1962; Ting, 1975; Nicholls, 1977, pers. comm.) 10 grams (blotted dry weight) of Lemna minor from the Thorold population was cleaned and ground in a Virtis "45" Homogenizer (The Virtis Company Inc., Gardiner, New York) in cold 0.5M sucrose buffer. 3ml buffer/gram tissue was used. The homogenate was then centrifuged at 250 x g for 90 seconds in an IEC International Clinical Centrifuge (Needham Mts., Massachusetts) to remove the cellular debris. The resulting supernatant was removed and centrifuged at 3,000 x g for fifteen minutes to pellet the intact organelles. This supernatant was discarded and the pellet was resuspended in fresh sucrose buffer. The rest of the experiment was carried out in a Sorvall RC-5 Superspeed Refrigerated Centrifuge. An SS-34 head

was used with Corex 8445 30ml Centrifuge tubes with Corning 8445 rubber adaptors.

To pellet the chloroplasts, the resuspended mixture was centrifuged at 1,478 x g (3,500 rpm) for ten minutes. The supernatant was poured off and reserved for the next step and the pellet was resuspended and respun for further purification. To separate the mitochondrial fraction, the supernatant remaining from the first chloroplast separation was centrifuged at 10,000 x g (9,500 rpm) for fifteen minutes. This was done to remove the heavy mitochondria and a second purification run was done on this pellet. The light mitochondria and microbodies were separated by centrifuging at 17,893 x g (12,000 rpm) for fifteen minutes. This pellet was also purified. The final supernatant was then spun at 25,000 x g, the final pellet discarded and this supernatant used for the cytosol fraction.

Each of the above pellets was treated with .25% Triton detergent to lyse the organelles and release the enzyme contained. Samples representing the chloroplast, mitochondrial, microbody and cytosol fractions, along with controls, were soaked onto filter paper wicks and electrophoresed as usual. All steps were carried out at less than 4°C. Five gels containing controls were run and stained for MDH and TO. ME and CAT were also assayed to identify the chloroplast and microbody fractions respectively.

Ploidy Analysis: Due to the complexity of both the MDH banding patterns yielded in Lemna minor and the large number of karyotypic forms, an attempt was made to study the amount of electrophoretically scorable gene duplication which has occurred and to identify the ploidy-forms of the survey populations collected for study. Axenic cultures representing each ploidy type ($2n=20, 30, 40, 50$ and 80) were obtained from Dr. Urbanska-Worytkiewicz (see population list) and electrophoresed in the usual manner. Four gels were run specifically comparing each karyotype to each karyotype. Also from the time the cultures were received (May 14, 1977) the octoploid (No. 6735) and the tetraploid (No. 7102) were used as controls on all survey gels.

Survey gels: These experiments made up the bulk of the investigation. As stated in the description of the electrophoretic method used, randomly chosen colonies from each population were treated as described above and gels containing 22 population members and one of each control were run every two to three weeks of the testing period. In 1976, before the ploidy controls were available, a mass-grind consisting of all populations available was used as a control. The ploidy control system yields much more information.

Mixing Experiment: During the 1977 seasonal survey, the appearance of an intermediate form in one of the MDH band systems as compared to the ploidy controls suggested a

mixing experiment (later found to be a second tetraploid form). Four non-variable populations (LA-6, LA-7, GU, SB) plus the 6735 and 7102 cultures were used. A member of each population was run in pure form plus the fifteen unique pairwise combinations that could be made out of the six populations, were electrophoresed in the usual manner. Two replicates of this test were done. Also, to yield further information, two more similar gels were run except a set of combinations of each population mixed with boiled tetraploid 7102 colonies were added.

Induced Variation: As part of the aims of this investigation were to distinguish genetic and physiological variation, several attempts were made to manipulate the band patterns. Several colonies of the SB population in 1976, were subcultured in distilled water supplemented with Hillman's M medium without the agar or sucrose supplement (Table 5). Four such cultures were made and each of these was placed in an extreme condition. One flask was placed in a dark cupboard, another in a refrigerator (8°C), a third under a 24 hour light regime and a fourth on top of an incubator (40°C). From a suggestion that calcium concentration had significant effects on MDH subunit associations (Jefferies, pers. comm.) three cultures of Lemna minor from SB were grown in .01M, .1M and 1M CaCl₂ in distilled water. Four replicate gels containing individuals from each of the seven conditions described above plus SB populations from the field,

the outdoor holding facility and the lab water cultures were electrophoresed as usual every five days for a period of 2.5 weeks.

Seasonal Variation: Evidence was found during the 1976 season which suggested putative seasonal variation manifest by a change in the banding patterns for MDH. The pattern changes were dramatic and occurred in all culture and field conditions simultaneously. When these changes occurred, attempts were made to cross check all techniques to ensure that the changes were not due to technique variation. Also, predictions of band pattern time changes as evidenced by the 1976 season were made for 1977 (Figure 23).

Species Identification: Due to the controversy surrounding species identification, discussed in the Introduction, a morphological investigation of all populations used was deemed necessary. The primary parameter used in these checks was the innervation pattern as this has been considered one of the most useful diagnostic tools (Landolt, 1975; Kandeler, 1975). To do this, forty colonies were randomly selected from each population. These were boiled in ethanol and then dipped into an iodine stain (IKI-10gms K₂ in 1 litre, H₂O 2.5gms iodine). The colonies were then examined under a dissecting microscope and nerve number counted. The presence or absence of anthocyanin pigmentation was determined during survey electrophoresis. Root cap form and presence of papules on the median nerve were also considered.

Figure 23. Seasonal changes in MDH pattern - Results of 1976 survey and pattern prediction for 1977.

- a) Band patterns occurring between June 25, 1976 and July 20, 1976.
 - b) Band patterns observed between July 20, 1976 and August 15, 1976.
 - c) Band patterns observed between August 15, 1976 and December 9, 1976.
- solid well resolved bands. 
 - poorly resolved bands. 
 - very poorly resolved bands. 
 - smear connects bands A and C. 

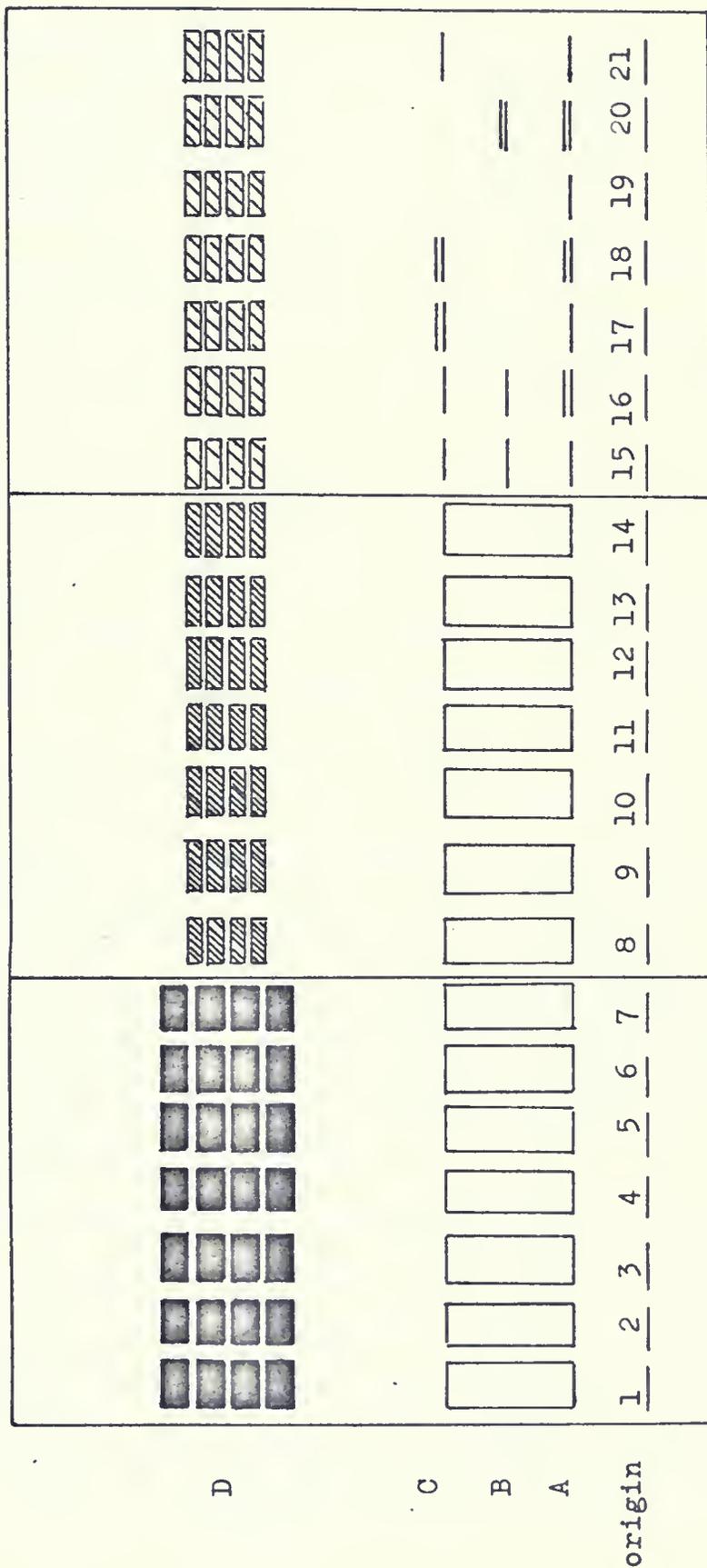
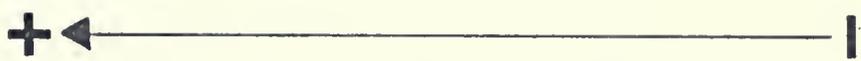


Figure 23. Seasonal changes in MDH pattern - Results of 1976 survey and pattern prediction for 1977.

Species Identification: Due to the controversy over the identification of species in the genus Lemna, a detailed morphological investigation was undertaken. Table 7 presents Landolt's (1975) characterization of the species in the Lemna minor/Lemna gibba group. As mentioned in the Introduction, confusion between Lemna gibba and Lemna minor is not likely to be a problem in the areas studied in this investigation; however, confusion between Lemna minor and Lemna turionifera is a strong possibility. As can be seen in Table 7, the main characteristics which distinguish Lemna minor from Lemna turionifera are (1) Formation of turions - Lemna turionifera does form turions, Lemna minor does not. (2) Pigmentation of the lower surface - Pigmentation of the lower surface does not occur in Lemna minor but does infrequently occur in Lemna turionifera. (3) Papules along the medium nerve - Papules occur frequently and are pronounced in Lemna turionifera but occur infrequently and are not pronounced in Lemna minor. Even though these are the most distinct characteristics, a good deal of overlap is apparent.

The results of the identification of all field populations used in this study; BN-1, BN-2, BN-3, BN-4, BN-5, LA-6, LA-7, SB, TH, GA and GU, appear in Table 8. Results shown for the characters "Nerve number" and "Papules along medium nerve" were generated from an investigation of forty randomly chosen individuals from each population

Table 7. Morphological characters of the *Lemna gibba*-L. minor group (From Landolt, 1975).

Taxon	Gibbosity at apex of papilla	Papules along medium nerve	Four to five nerves	Turions pigmented	Lower surface	Pigment spots near the apex of upper surface	Number of ovules ^a
<i>L. gibba</i>	++	+	(+)	+++	-	+	1-6
<i>L. parodiana</i>	++	+	(+)	+	-	+	1-3
<i>L. disperma</i>	+	++	(+)	(+)*	-	-	1-3
<i>L. obscura</i>	+	++	+	(+)**	-	++	1
<i>L. turionifera</i>	-	+	++	(+)**	++	+	1
<i>L. minor</i>	-	+	+	+	-	-	1

+++ Frequently occurring and pronounced.

++ Infrequently occurring and not pronounced.

+) Only rarely occurring and not pronounced.

+) Not occurring.

* When four to five nerves occur, all nerves remain branched from the base.

**When four to five nerves occur, the outermost nerves are connected with the innermost nerves at some distance from the base.

^a Only preliminary counts, partly completed from indications of Hegelmaier (1896) and Giardelli (1937).

Table 8. Morphological characters of Lemna populations used in this study.

Population	Gibbosity	Papules along medium nerve	Nerve* number	Turions	Lower surface pigmented	Flowers
BN-1	-	distinct 4-7	3	+	xx	-
BN-2	-	distinct 4-7	3	?	xx	-
BN-3	-	distinct 4-7	3	?	xx	-
BN-4	-	distinct 4-7	3	?	xx	-
BN-5	-	distinct 4-7	3	+	xx	-
LA-6	-	distinct 4-5	3	?	x	-
LA-7	-	distinct 4-5	3	+	x	-
SB	-	distinct 5	3	?	-	-
TH	-	distinct 5	3	+	-	-
GA	-	distinct 4-5	3	?	x	-
GU	-	indistinct 3	3	?	-	-

- not occurring
- ? not known
- + known to occur
- xx occurring at very low frequencies
- x occurring at low frequencies
- * no other innervation patterns than the three-nerved forms were observed.

studied. The remaining characters listed in Table 8 were studied throughout the normal course of the investigation. Neither flowers nor gibbous forms were observed. All individuals examined possessed three distinct nerves. The four to five nerved forms of Lemna minor, as described in the Introduction and in Table 7 were not observed. All populations except GU possessed four to seven distinct papules along the medium nerve. Individuals from the GU population possessed three indistinct papules. There were only three populations in which pigmentation of the lower surface was not observed, all the rest showed very low frequencies of pigmented individuals.

For two years running populations cultured in the laboratory "died" over a two day period around December 9. In 1975, the culture jars were cleaned and put away until the following collecting season. In 1976, culture jars were left full of water, and the lights were turned off; everything was just left. In May, 1977, the lights were turned on and, in jars which still contained water, Lemna appeared within four days and entered its vegetative reproductive phase. Some small green bodies resembling turions were found in the bottom of these jars (Jacobs, 1947; Landolt, 1975). It thus appears that these laboratory cultures underwent an overwintering phase and are capable of forming true turions (Jacobs, 1947; Landolt, 1975). Unfortunately, results for turion formation are only available for four populations, BN-1, BN-5, LA-7 and TH. GA and GU were not collected until June 1977 and therefore

were not available for overwintering observations. The water in the jars containing BN-2, BN-3, BN-4, LA-6 and SB evaporated; therefore, the turion forming abilities of these populations are unknown.

When all the characteristics listed in Table 8 are considered, there is a strong possibility that this investigation, represents a study of Lemna turionifera rather than Lemna minor; however, it is not possible to conclude this with certainty. For this reason, the plant used to produce the results to be given in this section will be called Lemna minor/Lemna turionifera.

Zymogram Patterns: Typical MDH banding patterns are presented in Figure 24 and 25. The individuals on this gel were from clones subjected to karyotypic analysis (Urbanska-Worytkiewicz, 1975) and the ploidy of each clone determined. Nine different collection sites from around the world are represented. Four replicates of this test have been run and results are repeatable. Band systems have been labelled A, C and D as independent systems.

It can be seen in Figures 24 and 25, that there is very little variability between samples in banding patterns or in staining intensity. No detectable difference in staining intensity was observed between individuals possessing different amounts of gene duplication. Also, all individuals initiated the staining reaction within fifteen minutes. The A and C systems are present and invariable in all individuals tested; they all migrate approximately 2 mm and 5 mm respectively, from the origin and here, both systems

Figure 24. Typical zymogram of MDH in Lemna minor/Lemna turionifera - Results from Ploidy analysis.

Material on this gel represents samples of Lemna minor sent by Dr. Urbanska-Worytkiewicz.

1,2	:	6570,	2n=42
3,4	:	6580,	2n=42
5,6	:	6728,	2n=50
7,8	:	6742,	2n=50
9,10	:	7102,	2n=40
11,12	:	7210,	2n=40
13,14	:	7244,	2n=30
15,16	:	7789,	2n=20
17,18	:	6735,	2n=80

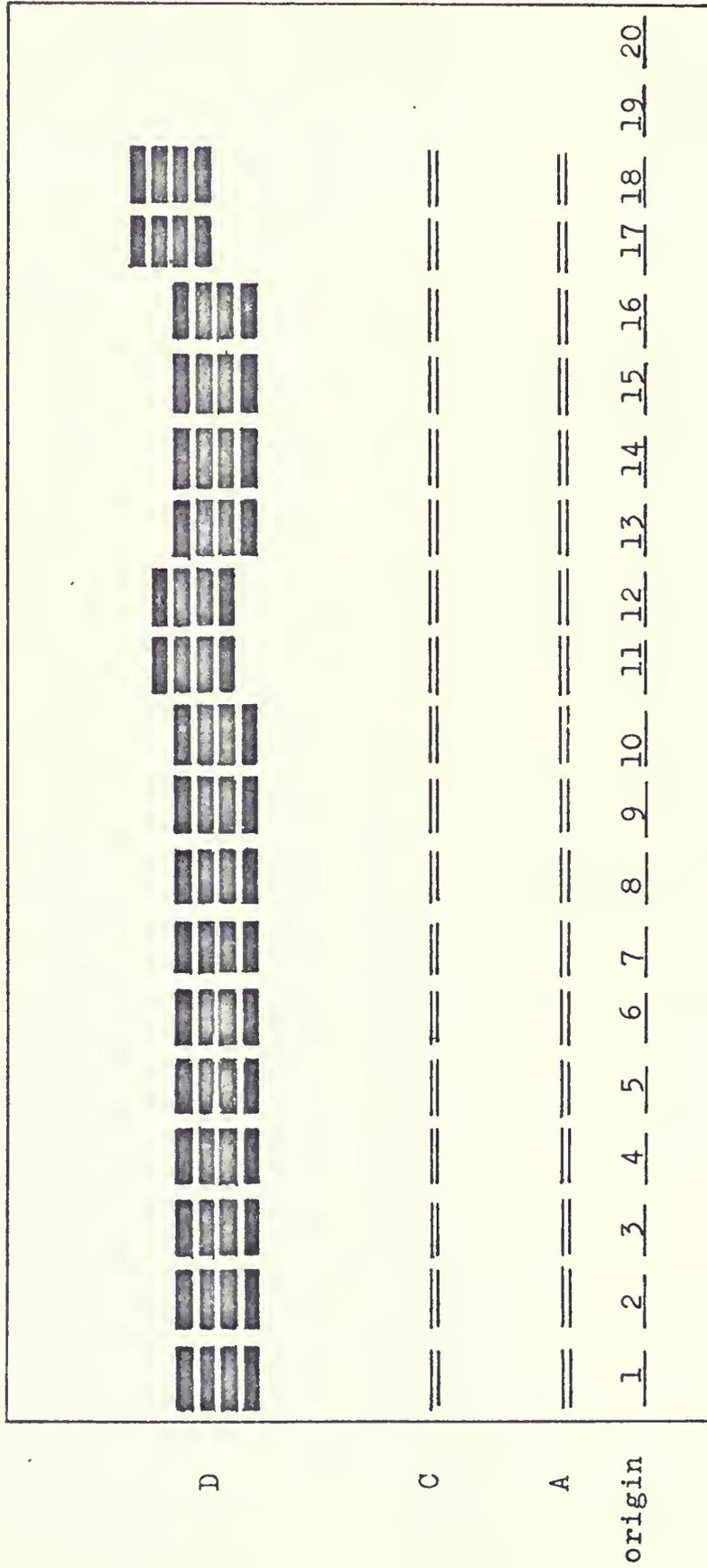


Figure 24. Typical zymogram of MDH in Lemna minor/Lemna turionifera - Results from Ploidy analysis.

Figure 25. Photograph of MDH gel from Lemna minor.
Results from ploidy analysis. Samples as
in Figure 23. 20-24:SB.



Figure 25. Photograph of MDH gel from Lemna minor.

appear to contain double bands. There are, however, observable differences in the D system(s). These differences are best shown in Figure 24 which is a diagrammatic interpretation of all four replicates. In terms of the mobility of the D system from the origin, 6570 (2n=42), 6580 (2n=42), 6728 (2n=50), 6742 (2n=50), 7102 (2n=40), 7244 (2n=30) and 7789 (2n=20) are identical; all move approximately 50mm in a four hour run. 7210 (2n=40) and 6735 (2n=80) however, do show variable patterns with respect to D system mobility. 7210 runs approximately 2.5mm further than the previously listed samples and 6735 runs 5.0mm further than these same samples. This relative pattern difference occurs whenever these samples are run. The results depicted in Figure 24 have been chosen to represent "typical" banding patterns because the individuals run have such a variable background of habitat and chromosome number and yet show both low amounts of variability and patterns identical to those encountered in the study of Ontario populations.

It should also be stated here that all band patterns obtained when the gels were subjected to the MDH stain were MDH bands. For every MDH gel run in this study, one slice was stained for MDH and a second slice of the same gel was stained for TO. The stain recipe used for TO (Materials and Methods) in this study is the MDH stain recipe without the L-malic acid substrate. No blue stain reactions were observed in the absence of substrate. This finding alleviates the possibility of Tetrazalium Reductase staining (Tracey, 1975) which would appear similar to the MDH stains.

As discussed, the band systems have been named as independent systems A, C and D. Since an electrophoretic study has not been attempted on Lemna minor prior to this investigation, identification of locus-specific bands was undertaken. Breeding tests are by far the best way to do this as genotypes may be assigned on the basis of progeny ratios and segregation patterns (Oxford, 1973; Li, 1976). As this method does not represent a viable possibility in Lemna minor, other test methods were employed.

Heat Stability: The results of the heat stability investigation are presented in Figure 26 and Table 9. All four replicates of this test yielded identical results. Controls on these gels were untreated individuals from the same population as the treated individuals. The staining process from stain initiation (Table 9) through to reaction completion (Figure 26) was recorded in this test and it was found that treated individuals took different lengths of time to initiate the staining reaction relative to controls on the same gel. In addition, treatment differences in the band patterns at reaction completion, sixteen hours later were recorded.

After one minute of exposure at 60°C, the D system initiated staining 5 minutes after the controls. The same final staining intensity and band pattern was, however, achieved 16 hours later. The A system stained at the same time and to the same intensity as the controls. The C system, in treated individuals showed a 30 minute delay in stain initiation plus these bands were fainter than control

Figure 26. Heat stability investigation - MDH band patterns after different exposure times to 60 C.

1 : control
2-6 : One minute treatment
7 : control
8-12 : Five minute treatment
13 : control
14-18 : Fifteen minute treatment
19 : control
20-24 : Thirty minute treatment

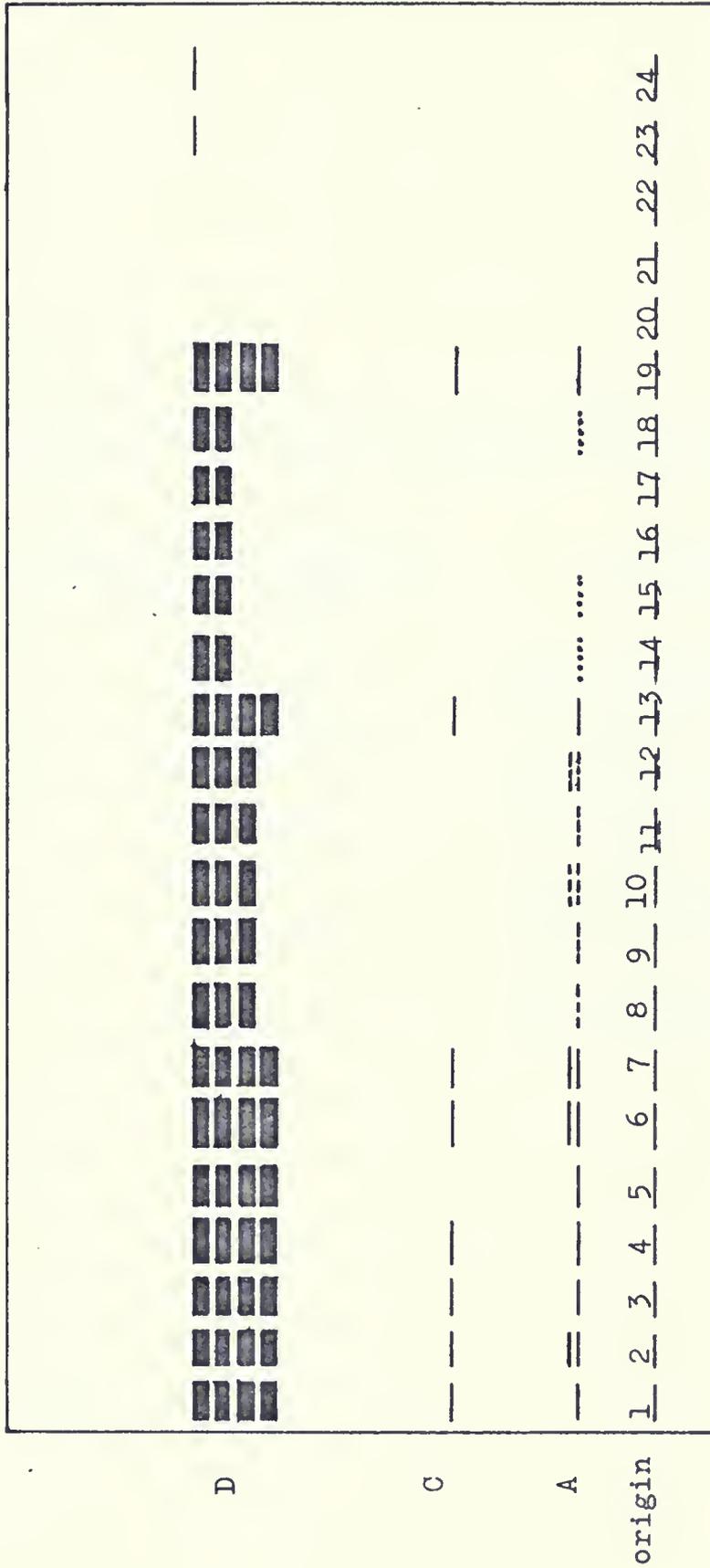
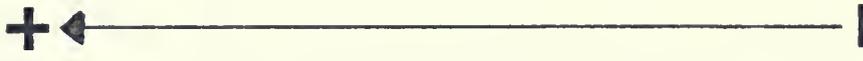


Figure 26. Heat stability investigation - MDH Band patterns after different exposure times to 60 C.

Table 9. Reaction initiation times and final reaction form in heat stability studies. Results given relative to controls.

Treatment time at 60°C (min.)		Time delay in reaction initiation (minutes after control)	Final reaction (16 hours after start)
1	A	0	same as control
	C	30 minutes	much fainter
	D	5 minutes	same as control
5	A	180 minutes	fainter
	C		did not stain
	D	45 minutes	no stain in bottom $\frac{1}{4}$
15	A	240 minutes	very faint
	C		did not stain
	D	60 minutes	no stain on bottom $\frac{1}{2}$
30	A		did not stain
	C		did not stain
	D	16 hours	very faint trace at top

C bands after the staining reaction was completed.

In the five minute treatment, the D system took 45 minutes longer than controls to initiate staining. After 16 hours, the bottom quarter (one thick band) did not stain. The A system in the treated individuals, took 3 hours longer than controls to initiate staining and these bands were fainter at reaction completion. The C system did not show any staining activity.

After a one hour exposure of the gel to the stain mixture, the D system showed hints of staining in the fifteen minute treatment set. When the reaction was completed, the total length of the D system was reduced by approximately half (the bottom two thick bands did not stain). The A system did not begin to stain until 4 hours after the control samples and A was extremely faint when the reaction was completed. Only 50% of the individuals subjected to the fifteen minute treatment (over the four replicates) showed A staining.

After the thirty minute treatment, only a very small band at the top of the D system stained in 40% of the individuals tested. No other system stained after a thirty minute exposure to 60°C.

It appears from these results, that the C system is quite heat labile while the A system is fairly heat stable. The D system shows a differentiation of heat stability within it; some parts of which are extremely heat stable.

Molecular Weight: The molecular weight analysis did not yield decisive results, probably due to the insensitivity of the technique, however, rough estimates of the molecular weights of MDH in Lemna minor/Lemna turionifera were produced. The results of this separation are shown in Figure 27. After all molecules of less than 15,000MW, and 75,000MW were selectively removed from the plant preparation, all MDH band systems stained on all gels. When all molecules smaller than 125,000MW were absorbed, however, only the A and C systems stained as before. The dark band appearing to connect the A and C systems in Figure 27 will be discussed later. As stated, these results are crude; they do indicate, however, that the molecular weights of the MDH enzymes composing the D system are between 75,000MW and 125,000MW. The molecular weights of the A and C systems are over 125,000MW. Not all MDH band systems in Lemna minor/Lemna turionifera have the same molecular weight.

Cell Fractionation: The results of the cell fractionation are presented in Figure 28. This figure shows the MDH bands found in each fraction. From Figure 28, it can be seen that all bands except the middle two bands of the D system were absent in the cytosol fraction. Only the A and C systems stain strongly in the microbody fraction and only the outer two bands of the D system in the heavy mitochondrial fraction stain. All bands appeared in the controls and the first supernatant. All bands also appeared to stain in the chloroplast fraction representing a good deal of impurity in the

Figure 27. Molecular weight separation - MDH band pattern remaining after separation at 15,000MW, 25,000MW, 75,000MW and 125,000MW.

1 : control
2-6 : 15,000MW
7 : control
8-12 : 25,000MW
13 : control
14-18 : 75,000MW
19 : control
20-24 : 125,000MW

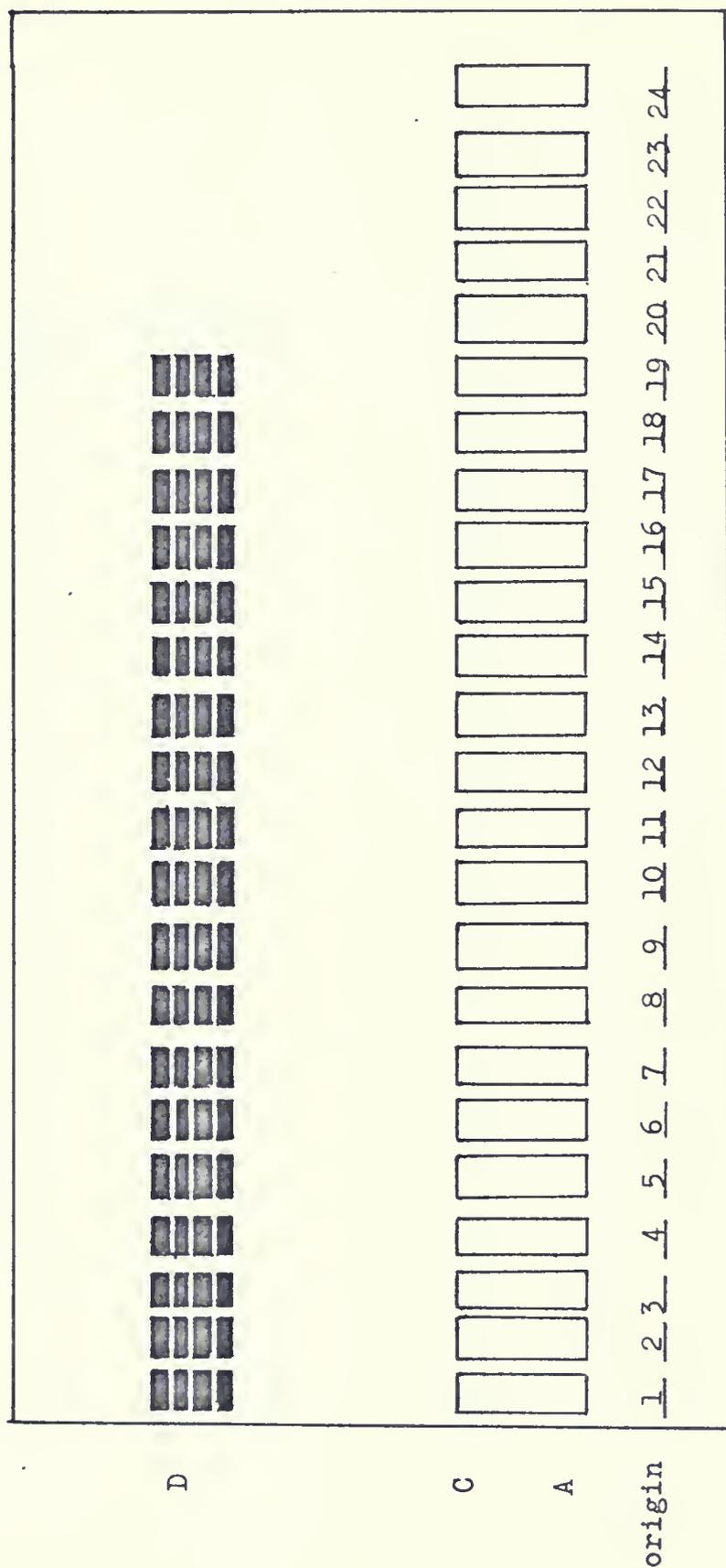


Figure 27. Molecular weight separation - MDH band pattern remaining after separation at 15,000MW, 25,000MW, 75,000MW, and 125,000MW.

Figure 28. Cell fraction.

- 1-2 : control
- 3-4 : First supernatant; debris removed
- 5-9 : Chloroplast fraction
- 10-14 : Heavy mitochondria fraction
- 15-19 : Light mitochondria and microbody fraction
- 20-24 : Cytosol fraction

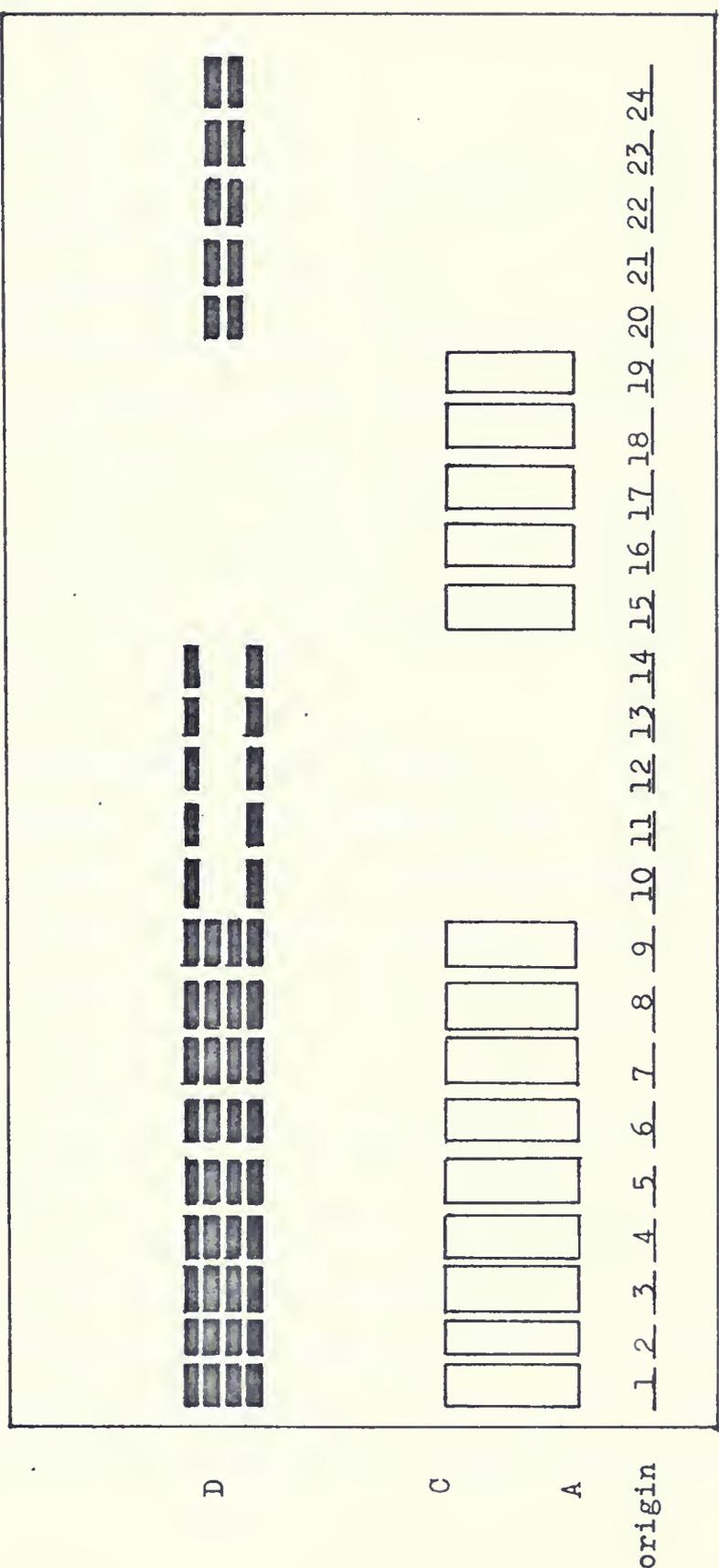
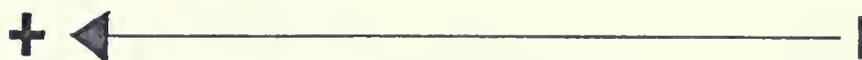


Figure 28. Cell fractionation.

separation of the chloroplasts. Unfortunately, the ME and CAT systems used as reference stains did not stain. This was not surprising in that ME and CAT, as discussed in the Materials and Methods section, did not stain at all times in whole individuals of Lemna minor/Lemna turionifera. Fraction identification in this study is thus solely based on the centrifugation parameters.

The results generated by the heat stability investigation, the molecular weight separation and the cell fractionation are suggestive of the independence of the A, C and D systems. Each band set appeared or disappeared differentially depending on treatment. It is thus possible that A and C are separate loci and the D system may be composed of two loci.

Population Survey: As mentioned, one of the main aims of this endeavour was the surveying of the various field populations listed in Table 6. Zymograms generated from the study of these populations generated patterns similar to those in Figure 24. Sample sizes of scorable individuals are given in Table 6.

The variation in MDH phenotype, found in these survey gels was very similar to that shown in Figures 24 and 25. During the 1976 survey season, very little band pattern variation was observed; in general, all samples on all gels looked the same. The impetus, however, to collect the Bridgenorth sites came from the fact that a small sample, collected in late July, 1976, yielded individual band

patterns with differing mobilities in the D system. When a detailed collection was taken at this site and electrophoresed in late September, however, this variable pattern had disappeared. Also in the 1976 survey season, controls consisted of a mass-grind of individuals taken from each population studied. At that time this represented BN-1 to BN-5, LA-6, LA-7, SB and TH. Using this kind of control, there did not appear to be any real gel to gel differences in zymogram patterns. In the 1977 survey season, however, the ploidy controls (which arrived May 14, 1977) were used on all survey gels. The mobility differences in the D system, observed the previous summer in the Bridgenorth samples were again evident in gels run on the Bridgenorth subpopulations collected on June 18, 1977. When the ploidy controls were first used on these gels, it was found that the mobility differences, in the D system, observed in Bridgenorth field individuals corresponded to those found between the octoploid (6735) and the tetraploid (7102). When these same controls were run on gels which were generated by uniform populations (LA-6, LA-7, SB, GU, GA and TH), it was found that in fact, these populations were composed of individuals possessing either form - a difference that was not detectable unless the 6735 and 7102 cultures were used as controls. After further study, a D system mobility pattern similar to the 7210 tetraploid culture was found in LA-7 and in some BN individuals.

Because these D system comparisons are electrophoretically correlated with ploidy type, the descriptions

Octoploid-Type, Tetraploid-Type I (7102 pattern) and Tetraploid-Type II (7210) will be used to identify the gel patterns. Electrophoretic identification of ploidy level is, in theory, possible (see Appendix A). These identifications are, however, descriptive of gel pattern and do not imply identification of ploidy level.

Table 10 characterizes each field population studied by D system variability using the above three descriptions. The numbers in the table represent frequencies of each pattern type found in each population. Briefly summarized, it can be seen that two uniform Octoploid-Type populations (SB and TH), three uniform Tetraploid-Type I populations (LA-6, GU and GA) and one uniform Tetraploid-Type II population (LA-7) were studied. BN-1 to BN-5 were mixed populations. All three pattern types were observed in the BN samples although not all three appeared in equal frequencies in each subpopulation. The frequencies found in each subpopulation varied considerably.

Mixing Experiment I: During the 1977 survey, the appearance in the BN and LA-7 samples, of an intermediate mobility form of the D system prompted a mixing experiment to characterize this form. The form turned out to be the Tetraploid-Type II pattern when the appropriate control was run; however, the results gained by mixing individuals from uniform populations (6735, 7102, SB, GU, LA-6 and LA-7) were surprising and quite interesting. The results are shown in Figure 29. It was found that by mixing homogenates of different individuals together that, in some cases, the band patterns yielded were composed of the combination of patterns yielded

Table 10. D system variability found in 1977 survey season. Results represent frequencies of each form found in each population.

Population	Octoploid-Type	Tetraploid-Type I	Tetraploid Type II
BN-1	0.0563	0.6760	0.2676
BN-2	0.5532	0.2553	0.1915
BN-3	0.1957	0.6957	0.1087
BN-4	0.1064	0.8936	0.0000
BN-5	0.3438	0.6563	0.0000
LA-6	0.0000	1.0000	0.0000
LA-7	0.0000	0.0000	1.0000
GU	0.0000	1.0000	0.0000
GA	0.0000	1.0000	0.0000
SB	1.0000	0.0000	0.0000
TH	1.0000	0.0000	0.0000

Figure 29. Mixing experiment I.

1. Octoploid 6735
2. Tetraploid 7102
3. SB
4. GU
5. LA-6
6. LA-7
7. Octoploid + Tetraploid
8. SB + Tetraploid
9. GU + Tetraploid
10. LA-6 + Tetraploid
11. LA-7 + Tetraploid
12. SB + Octoploid
13. GU + Octoploid
14. LA-6 + Octoploid
15. LA-7 + Octoploid
16. GU + SB
17. LA-6 + SB
18. LA-7 + SB
19. LA-6 + GU
20. LA-7 + GU
21. LA-7 + LA-6.

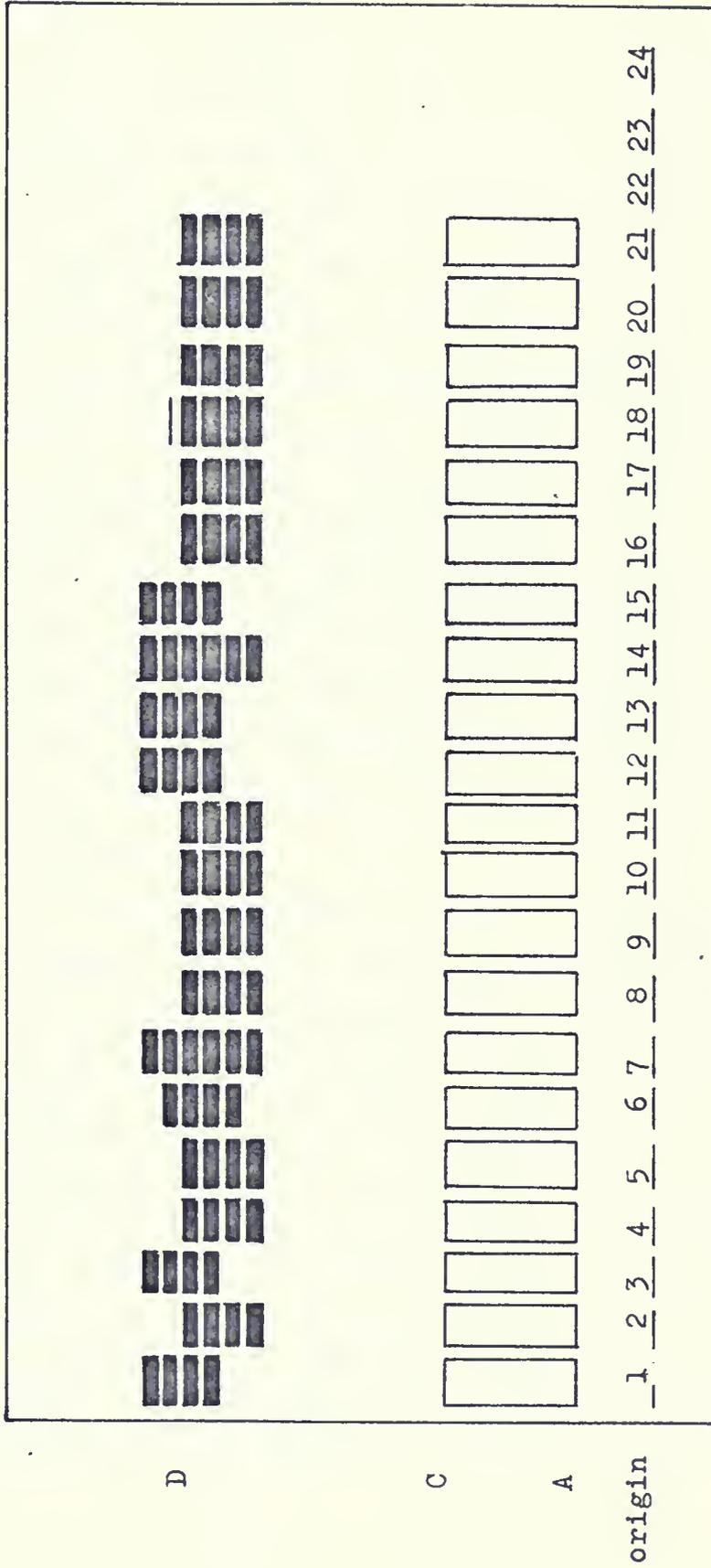


Figure 29. Mixing experiment I.

by each individual run separately. In other cases, the band pattern normally generated by an individual run separately was completely overridden by the pattern of the other individual in the homogenate. This occurred to the point where bands actually disappeared. Populations also showed variable abilities to maintain their own patterns on mixing.

Two replicates of this test were done. After all combinations had been scored it was found that: (1) SB, which normally yields an Octoploid-Type pattern, was overridden by everything it was mixed with. (2) 6735 (Octoploid-Type), 7102 (Tetraploid-Type I) and LA-6 (Tetraploid-Type I) maintained their normal patterns regardless of what they were mixed with. If an Octoploid-Type and a Tetraploid-Type I were mixed, both patterns appeared superimposed on one another. The individuals of these populations could also override the others. (3) GU overrides only SB and LA-7. (4) LA-7 overrides only SB.

When two more replicates of the experiment were done, this time using 7102 after several minutes of boiling, it was found that 7102 was incapable of overriding other patterns (Figure 30) indicating that this ability to override or maintain pattern is due to the presence of some heat labile substance which has an effect on the phenotype of the individual. These tests were not run on the Bridgenorth populations because the D system patterns are variable.

It should also be noted here that the results generated by these mixing experiments bear implications for the

Figure 30. Mixing experiment II.

1. Octoploid-6735
2. Tetraploid-7102
3. SB
4. GU
5. LA-6
6. LA-7
7. Octoploid + Tetraploid (live)
8. Octoploid + Tetraploid (boiled)
9. SB + Tetraploid (boiled)
10. GU + Tetraploid (boiled)
11. LA-6 + Tetraploid (boiled)
12. LA-7 + Tetraploid (boiled)
13. SB + Tetraploid (live)
14. GU + Tetraploid (live)
15. LA-7 + Tetraploid (live)
16. GU + SB
17. GU + LA-7
18. LA-6 + Octoploid
19. LA-7 + Octoploid
20. LA-6 + SB
21. LA-7 + SB

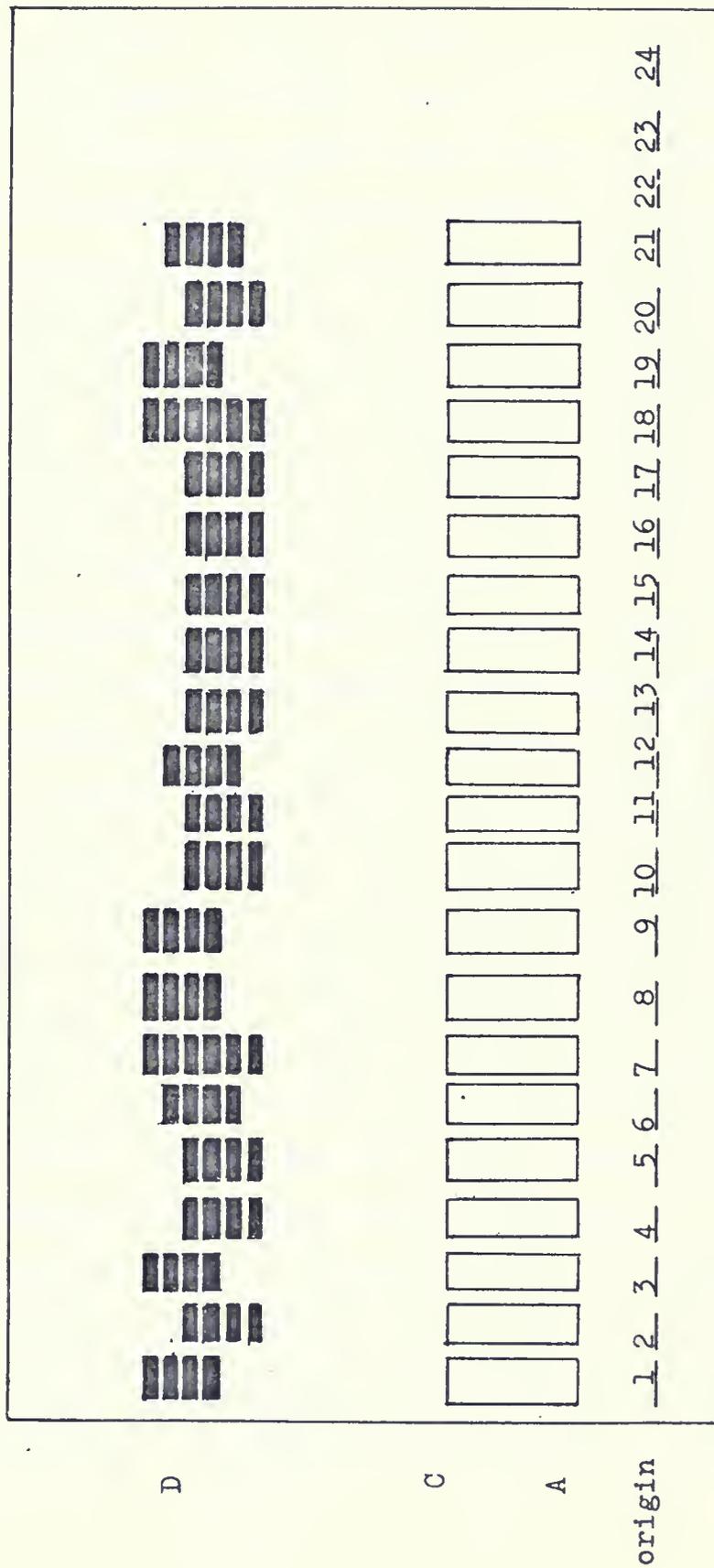


Figure 30. Mixing experiment II.

use of mass-grind controls such as those used in the 1976 survey season. The rationale for using the mass-grind control system in the first place was that all possible band systems should appear when the controls are run and stained. Test individuals could then be compared and scored for pattern relative to those generated by the control. Given the results of the two mixing experiments, together with the number of populations mixed to form the control sample, the resultant phenotype could be anything and it is no surprise that D system variability was not detected in the 1976 survey. In this case, use of a mass-grind control did not provide a useful reference.

Manipulation Study: Part of the aim of this endeavour was to distinguish between genetic and physiological adaptation, therefore attempts were made to manipulate the band patterns shown by treated individuals. A resultant phenotype change in the zymogram must be shown in order to suggest the presence of physiological flexibility in the gene system. As detailed in Materials and Methods, several colonies of the SB population were subcultured under seven different conditions (0.1, .1 and 1M CaCl_2 ; constant darkness and constant light, cold and hot). Normal field, outdoor holding facility and normal indoor culture conditions were also available for comparison. All ten conditions were compared electrophoretically every four days for a period of two and a half weeks. No differences in zymogram patterns were detected as a result of any of these manipulations. The addition

of various concentrations of NAD or NADP to the molten gel prior to electrophoresis was also ineffective in changing the band patterns of MDH in Lemna minor/Lemna turionifera.

Seasonal Variation: Despite the ineffectiveness of the manipulation studies, there were changes in the zymogram patterns across the survey seasons of 1976 and 1977 suggestive of a seasonal variation in the MDH of Lemna minor/Lemna turionifera. These pattern changes for 1976 are shown in Figure 23 (in the Materials and Methods section). Basically there were three abrupt changes in the gel patterns which occurred at distinct times during the season. When these changes occurred, they occurred simultaneously in all field populations being examined as well as in laboratory cultures and those kept outdoors that had been derived from field populations.

The three basic pattern changes which emerged were:

(1) Band patterns found between June 25, 1976 and July 20, 1976 - The D system, during this time, appeared to be composed of 3-4 closely apposed thick band sets with smears between each band. The length of the D system was approximately 25mm. The A and C systems were very indistinct and connected by a very dark smear. In fact, it was not until August, 1976 that A and C were recognized as distinct band systems. In Figures 23, 27, 28, 29 and 30 the boxes which appear to connect the A and C systems together represent this particular 'seasonal state' of the A and C systems. Very little if any genetic variability was observed during this

period. (2) Band patterns observed between July 20, 1976 and August, 1976 - This phase was represented essentially by a loss of resolution in the patterns described in (1). A gradual shrinking of the length of the D system was observed as well as a loss of resolution. The condition of the A and C systems remained unchanged. Little if any genetic variability was observed during this period. (3) Band patterns found between August 15 and December 9, 1976 (Electrophoresis done between November 1 and December 9, 1976 involved laboratory cultures due to fall freeze) - During this phase, three sets of bands appeared between the origin and the D system. These were the A and C systems plus one in between A and C which has been labelled B. The D system was very poorly resolved and approximately 7mm in length. No variability was observed in the D system during this period.

Variability was observed in the A, B and C systems during this time period. The variability observed was of two types: (1) Presence or absence of B and/or C - All individuals had A but not all individuals had B and/or C. The frequencies of individuals possessing each of these systems is shown in Table 11. (2) Within each band set composing the A, B and C systems, double or single banded forms occurred. These double and single banded forms do appear to represent homozygotic and heterozygotic forms of these systems as the frequencies of their occurrence agree with a predictive Hardy-Weinberg model for tetraploids and octoploids (Appendix A). Breeding tests are required,

Table 11. Band frequencies of A,B,C and D systems in each population - 1976 survey season. Sample sizes in parentheses.

Population:	SYSTEM			
	A	B	C	D
BN-1 (19)	1.00	0.68	0.68	1.00
BN-3 (19)	1.00	0.63	0.68	1.00
BN-4 (62)	1.00	0.13	0.86	1.00
BN-5 (37)	1.00	0.35	0.70	1.00
LA-6 (55)	1.00	0.66	0.55	1.00
LA-7 (51)	1.00	0.24	0.80	1.00
TH-1 (73)	1.00	0.26	0.78	1.00

however, to establish inheritance and ploidy accurately.

It should be noted here also, that these changes may not have occurred as abruptly as the dating sequence may indicate. The shrinkage of the D system was a gradual process. The loss of resolution of the D system as described for phase (2) and the appearance of the A, B and C systems was quite abrupt. Some of the abruptness, however, may be due to sampling error, for although electrophoresis was carried out on these populations for three or four days out of each week during the survey season, there were three to five day gaps during which electrophoresis was not done. Attempts were made, when these changes were observed, to bring back the previous pattern or to find cultures or populations in which the changes had not occurred. All techniques were checked to ensure that technique errors had not been committed. All results were negative; the band patterns remained unchanged despite all manipulations until the pattern, of itself, changed the following season.

During the 1977 survey season, these changes were watched for and to some extent, occurred again. Electrophoretic sampling was begun approximately one and a half months earlier in 1977. It was done on populations that had overwintered in the laboratory (BN-1, BN-5, LA-7, TH: Table 8) and on cultures sent by Dr. Urbanska-Worytkiewicz. Results from the runs carried out from May 19, 1977 to June 20, 1977 were very similar to those typical of the August 15, 1976 to December 9, 1976 patterns. The D system was approximately 7mm in length and the A and C systems were clear and

distinctive. No evidence of the B system was, however, found during this sample period. After June 20, 1977, the smeared band between A and C was again present and has remained until August 1, 1977 (Appendix B for August results). It is also interesting to note that the change in the A and C systems occurred in Dr. Urbanska-Worytkiewicz' samples which have been in these culture conditions for a number of years. The very dramatic changes in the D system have not yet occurred in the 1977 season. The D system increased in length after June 30, 1977 to 12mm but it never reached the 25mm length observed in 1976. The loss of resolution in the D system observed between July 20, 1976 and August 15, 1976 was also observed in 1977 and it occurred after July 15, 1977. Essentially, it appears that the zymogram changes which were observed in 1976 did, in fact, occur again in 1977. As in 1976, these changes occurred in all field populations and lab cultures (including axenically cultured Lemna minor) simultaneously.

Another occurrence noted from time to time during each season was the appearance of satellite bands. These were bands which appeared outside the positions of A, C and D systems and which stain very faintly. There does not appear to be any apparent pattern for their appearance or disappearance in a population. Positions of satellite bands are shown in Figure 31.

Also observed, but not studied, was the variability in the appearance and disappearance of field populations in

Figure 31. Position of MDH satellite bands.

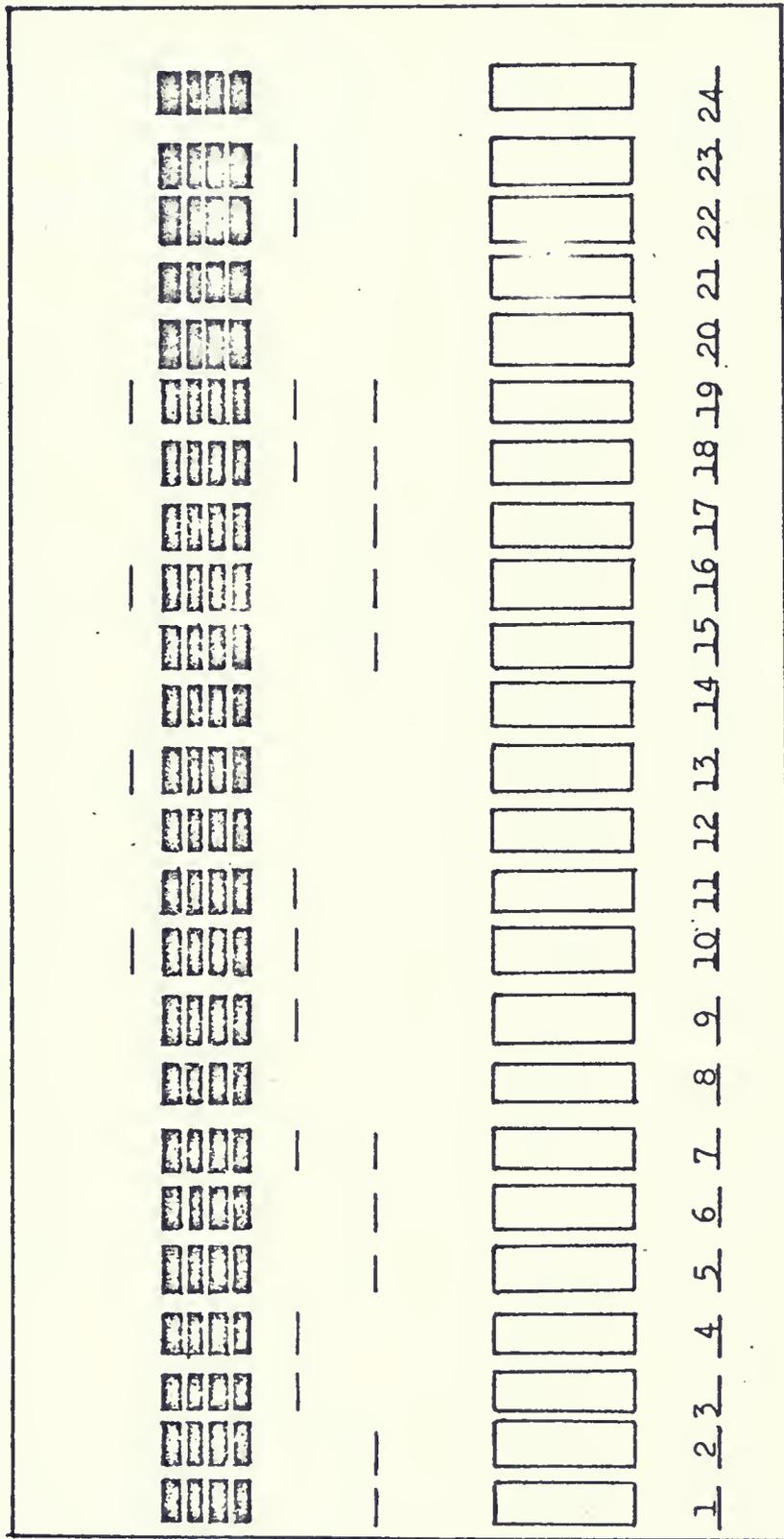


Figure 31. Position of MDH satellite bands.

origin

D

C

A

the spring and fall. Locally, Lemna minor populations were observed to start spring growth from April 10, 1977 to June 30, 1977. In 1976, disappearance of populations in the fall was observed to occur from mid-September until approximately October 30.

DISCUSSION

Identification of Loci: As mentioned in Materials and Methods, no electrophoretic studies of protein variability have been done on Lemna spp. One of the distinct advantages of electrophoresis in survey studies is the ease with which large sample numbers can be phenotyped. If the central dogma (DNA→RNA→protein) is assumed uncritically, one may, then identify DNA variation by sampling protein variation. In other words, protein variation reflects genetic variation, either allelic (allozymes) or interallelic (isozymes). Proteins do, however, vary electrophoretically even when the encoding DNA is invariant (Somero, 1969; Hochachka and Somero, 1973; Somero, 1975a; Somero and Low, 1977), moreover genetic interpretations of zymogram variation are not always straight forward (Hedgecock, Nelson, Shlessor and Tracey, 1975). For these reasons electrophoretic bands, putatively identified as allelic, should be carefully characterized. In sexually reproducing organisms, breeding tests of the Mendelian hypothesis are sufficient. In asexual organisms, other tests must be run. Several such tests were done in the course of this investigation which provide this information.

Figure 26 and Table 7 present the results from the heat stability investigation. These results show that the C system disappears after the one minute treatment and the A system disappears between the five and fifteen minute exposures to 60°C. The first part of the D system affected,

the bottom quarter of the system, did not stain after the five minute exposure and the bottom half of the D system did not stain after a fifteen minute exposure. The D system was not finally inactivated until after a thirty-minute exposure to 60°C.

Assuming that allelic reactions to treatment are usually more similar than those of homologous loci, the data suggest that A is genetically separate from C, and both are genetically separate from all bands composing D. The D system appears to contain up to three separate loci based on the heat stability results. Whether these heat stability differences are due to differences in amino acid sequences or in three-dimensional structure, is unknown for the MDH systems of Lemna minor/Lemna turionifera, however, that these differences exist, suggests that at least three and possibly five distinct MDH loci exist in this plant.

The results of the cell fractionation (Figure 26) also suggest a system of separate MDH loci. It should be noted here that even though fraction identification in this investigation, rests solely on centrifugation because the cell fraction marker systems (ME-chloroplast, CAT-peroxisomes) did not stain, band systems are separable by fraction. To recapitulate briefly, no D system staining occurred in the putative microbody fraction in which only A and C stained; only the uppermost and lowermost bands of the D system stained in the putative mitochondrial fraction; and only

the middle two D system bands stained in the cytosol fraction. These results suggest the presence of separate isozymes by virtue of compartmentalization, in other words, of organelle or fraction specific isozymes.

The presence of three distinct classes of NAD-MDH (E.C. 1.1.1.37); cytosol-MDH, microbody-MDH and mitochondrial-MDH has been observed in many vascular plants (Ting, Sherman and Dugger, 1966; Yue, 1966; Kuramitsu, 1968; Grimwood and McDaniel, 1970; Rocha and Ting, 1970; Zschoche and Ting, 1973; Scandalios, 1974; Ting, Fuhr, Curry and Zschoche, 1975). The three distinct forms tend to be common to green photosynthetic tissue, while the microbody forms show low activity or absence from non-photosynthetic root tissue (Rocha and Ting, 1970; Zschoche and Ting, 1973; Ting et al, 1975). Generally, in electrophoretic studies, the microbody form of MDH is the slowest anodally moving system, the second anodally moving form is mitochondrial-MDH and the fastest moving form is from the cytosol fraction (Ting et al, 1975); a few exceptions have been noted (Zschoche and Ting, 1973).

The results of the cell fractionation found in this investigation for the most part agree with the generalizations stated above. Three band set combinations of MDH were found associated with three fractions. Since all parts of a Lemna minor plant are photosynthetic (Arber, 1920; Pirson and Gollner, 1953; Hillman, 1961d) and they are vascular plants, all three forms were expected. The slowest moving band systems, A and C, were associated with

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the putative microbody fraction. The D system appeared to be split between the cytosol fraction and the putative mitochondrial fraction. Despite this minor discrepancy with the general case, the results yielded for the D system are probably accurate as there was no overlap of bands staining in each of the two fractions and after a final centrifugation of 25,000 x g, very little membraneous tissue likely to contain bound MDH would remain (P. Nicholls, pers. comm.).

Whatever the case for other plants, it appears that in Lemna minor/Lemna turionifera there are two microbody-MDH systems (A and C), two cytosol-MDH bands (the middle two of D) and two mitochondrial-MDH bands (the outer two of D). Multiple forms of organelle specific isozymes have been reported in Zea mays (Longo and Scandalios, 1969; Scandalios, 1974) thus there exists a precedent for the observation of two distinct bands associated with each of the three organelle fractions. Two forms of microbodies are also known to exist in the photosynthetic tissue of some vascular plants. These are the glyoxysomes and peroxisomes, MDH specific to each has been found in some vascular plants (Ting et al, 1975). Studies to detect differences between glyoxysomal-MDH and peroxisomal-MDH have, however, failed to detect any (Ting et al, 1975). It is possible that the A and C systems represent MDH's from each of these two forms of the microbodies but this cannot be concluded with certainty. Evidence exists which suggests that each organelle specific MDH isozyme is synthesized by separate nuclear genes (Longo

and Scandalios, 1969; Zschoche and Ting, 1973; Scandalios,¹³⁸ 1974; Ting et al, 1975). It is, however, not known, whether the multiple forms are allozymic or isozymic. Assuming organelle specific MDH's, the results of this study suggest that there are three or more separate MDH loci in Lemna minor/Lemna turionifera.

The combination of the heat stability results with those of the cell fractionation permits further genetic definition. This indicates that the putative microbody bands, A and C, have two distinct sensitivities to heat; C being quite sensitive and A being fairly heat stable. The superimposition of the two result sets also splits the D system. One cytosol band and one mitochondrial band, the slowest in each case, are more heat sensitive than the faster moving bands in either fraction. These comparisons suggest that two separate gene loci code for MDH enzymes found in each fraction.

The results of the molecular weight separation, although crude, do deserve brief mention. First of all, they serve to further substantiate the separateness of the A and C systems from the D system (Figure 27). Also molecular weight estimates for a good number of the MDH forms in vascular plants range from about 66,000MW to about 70,000MW. The disappearance of the D system bands after the 75,000MW separation probably indicates a similarity in the weight of MDH from the D system of Lemna minor/Lemna turionifera to MDH's of other vascular plants. It has also been observed that microbody-MDH can be substantially heavier due to the

formation of complexes composed of these 70,000MW subunits (Ting et al, 1975). The constant intensity of the stain reaction in the A and C systems after the removal of molecules smaller than 125,000MW further suggests that the A and C systems may be microbody in origin.

Variability: On the whole, very little genetic variability was observed in the MDH systems of Lemna minor/Lemna turionifera during each seasonal phase (Figure 24). There was, however, limited variability and this will be discussed first for the A,B and C systems and then for the D system.

As described in Results, A, B and C are only visible as distinct bands during the late summer and fall. A and C are also present and scorable during the spring but during the summer, they are connected by a dark smear. A and C appear to sharply delineate this smear at each end. The delineations of this smear migrate to the same position as A and C, but it is usually impossible, during the summer, to define the number of bands contained in A or C. B is difficult to identify. As shown in Table 11, not all colonies possess C in the late summer and fall. During the summer, some colonies yielded smears which were sharply delineated by the A system on the bottom but which were not very sharply defined at the top. It is possible that such individuals do not possess C. As a general statement, however, it can be said that the band patterns of A,B and C did not change in response to investigative manipulations; changes were not affected by the three CaCl₂ concentrations, the two temperature or light extremes or by the addition of

NAD or NADP. The technique cross-checks done after each seasonal change were also unsuccessful in changing the band patterns yielded by A, B or C. The only changes observed in A, B or C across the 1976 and 1977 seasonal surveys then, were resolution changes in these systems. It is possible that these resolution changes represent physiological variability in the A and C systems but whether these changes are caused by a change in the expression of the MDH genes or by conformational changes in the proteins is unknown.

During the periods when A, B and C were distinct and the number of bands present in each system could be determined, the phenotypes were scored and subjected to statistical analysis to determine whether the double and single bands represented genetic variability and hence whether the ploidy level could be identified from these phenotypes. This analysis, along with the raw data, is found in Appendix A.

The model uses the Hardy-Weinberg distribution and assumes that single banded forms are homozygotes and double banded forms are heterozygotes. Expected Hardy-Weinberg frequencies for each genotype can be generated for any ploidy level by raising the allele vector to the power of the ploidy level, for example, in diploids with two alleles of frequencies p and q , the expected genotypic array is calculated by expansion of the binomial $(p+q)^2$. In octoploids and tetraploids, the genotypic arrays are calculated by expanding $(p+q)^8$ and $(p+q)^4$ respectively. This of course, assumes that there are only two alleles

at each locus. Given that the total of all frequencies of alleles in a population must sum to one, and each must therefore be equal to or less than one, the chances of detecting low frequency homozygotes is extremely slim unless very large sample sizes are employed. For example when the rare allele frequency equals 0.4, the expected tetraploid frequency of homozygotes is equal to $(0.4)^4$ or 0.0256 and less than three plants in one hundred will be homozygotes. (This model also assumes that all genotypic heterozygotes produce indistinguishable phenotypes.) The use of such large sample sizes can be compensated for, by use of homozygote/heterozygote ratios. In the calculations (Appendix A) done for this investigation, the predicted ratios for diploids, tetraploids and octoploids were compared to those observed. Goodness-of-Fit Chi-Square tests were run using probabilities combined over populations and systems (Fisher, 1954 in Sokal and Rohlf, 1969). The results are tabulated in Table 12. Clearly, only diploidy is eliminated (Chi-square = 58.27, $P < 0.001$, 28df). Tetraploidy (Chi-square = 5.47, $P = 0.999$, 28df) and octoploidy (Chi-square = 6.12, $P = 0.999$, 28df) are not statistically distinguishable. It is, however, worth noting that the tetraploid probability is higher than the octoploid probability for pooled system-pooled population calculations. If only the systems (A, B and C) are pooled for each population, and the mean probability taken, tetraploidy by a slightly larger amount, fits the model best.



Table 12.

Homozygote/Heterozygote Ratios and Goodness-of-Fit to Hardy-Weinberg Chi-Square Probabilities for Diploidy, Tetraploidy and Octoploidy. The ratios are tabulated in columns 3-6 and the probabilities in columns 8-10. Sample size (n) and the ploidy level of best fit (highest probability) are also tabulated. See Appendix A for computer programs and raw data.

Population	System	Homozygote/Heterozygote			Data	N	Chi-Square Probability			Best Fit
		Diploid	Tetraploid	Octoploid			Diploid	Tetraploid	Octoploid	
Bridgenorth	A	1.00	0.34	0.33	0.34	254	10 ⁵ **	0.954	0.827	
	B	4.70	4.14	4.14	4.14	36	0.958	0.833	0.833	
	C	1.00	.34	.33	.34	115	0.489	0.518	0.513	
LA-6	A	1.19	0.73	0.72	0.82	69	0.145	0.733	0.714	
	B	1.63	1.25	1.25	1.25	36	0.543	0.870	0.867	
	C	1.84	1.48	1.47	1.47	47	0.552	0.884	0.882	
LA-7	A	1.57	1.18	1.18	1.35	61	0.643	0.705	0.700	
	B	1.05	0.51	0.50	0.71	12	0.710	0.779	0.759	
	C	1.11	0.61	0.60	0.60	69	0.018*	0.931	0.901	
TH	A	1.27	0.83	0.83	0.87	84	0.099	0.928	0.913	
	B	2.58	2.25	2.25	2.25	26	0.919	0.832	0.832	
	C	1.70	1.32	1.32	1.32	72	0.349	0.909	0.905	
SB	A	1.22	0.77	0.76	0.85	37	0.348	0.881	0.868	
	A	1.02	0.27	0.25	0.25	20	0.012*	0.887	0.780	

* See Appendix D for assumptions used in this application of Hardy-Weinberg.

Identification of most populations as tetraploids is in accordance with the findings of Urbanska-Worytkiewicz, (1975) (Table 3). In fact, tetraploidy tends to be the most prevalent case for other taxa of the Lemna minor group and of the Lemnaceae family; cases of diploidy and octoploidy are exceptional and most studied samples tend to 40 or 42-chromosomic (Urbanska-Worytkiewicz, 1977, pers. comm.).

In the D systems, on the other hand, the number of bands do not appear to vary between the populations studied and observed variability occurs as resolution changes with season and as mobility jumps of the whole system. The resolution changes occurred across all populations and culture conditions in the A, C and D systems simultaneously as the season progressed. These changes appear to represent physiological variability.

The mobility variation observed in the D system (Table 10) is difficult to explain. The mobility differences correlate exactly with known differences in ploidy; however, a definitive statement to the effect that ploidy levels alter MDH cannot be made by gel pattern alone. Even if it could, it would not provide a cause-effect explanation for these jumps occurring between some gene duplications and not between others.

The D system mobility differences could be genetic as both uniform and mixed populations occur in nature (this investigation; Urbanska-Worytkiewicz, 1975, 1977, pers. comm.) and these forms are maintained through generations of

vegetative propagation. In other words, the D system mobility differences are passed from parent to offspring. The genetic events that must occur at MDH loci to explain the origin of one D from the other, however, have extremely low probabilities of occurrence. The explanations must account for shifts of the entire D system. If the bands composing the D system represent the phenotypic expression of heterozygotes, (in which case the four thick D bands represent two to four alleles of one to two loci) all alleles must have mutated simultaneously on the four or eight chromosomes which encode them. If, on the other hand, the D bands represent monomeric breakdown products of a large complex MDH, and each monomer has maintained an active site so that staining is still possible then, in order to account for such shifts, identical mutations must occur at four or eight gene sites for tetraploids or octoploids respectively. If the probability of a mutation in one gene on one chromosome is μ^{-s} , then the probability of this mutation occurring simultaneously on all four or eight chromosomes is $(\mu^{-s})^4$ or $(\mu^{-s})^8$. The probability of either of these events is extremely low. These explanations of the shifts in D system mobility argue against these differences representing MDH genetic variability.

The results of the two mixing experiments (Figure 29 and Figure 30) do offer some alternative possibilities. It was found, in these mixing experiments, that the phenotype normally yielded by one population, could be changed

to that of another by mixing. The degree to which this occurred varied in the populations studied. If it is possible for the phenotypes of the D system to be changed by a heat labile substance, other than MDH, contained within the individuals from other populations, then the MDH variability observed is a result of this compound. There is no necessity for claiming that the observed variability results from selection acting directly on the MDH of the D system (Gottlieb, 1976; P. Young, pers. comm.). It is quite possible that selection acts on genes coding for other patterns and the MDH is observed to change as a result of selectively directed change(s) at other loci. This MDH alteration has been documented and preliminary steps toward causal identification taken. The functional significance of the shift, if there is one, and the specific nature of its effector are open questions.

The changes in zymogram patterns observed during the 1976 and 1977 survey seasons (Figure 23) are highly suggestive of physiological variation. It is interesting that these changes occurred simultaneously in all populations including the axenically cultured specimens. Other evidence does exist, in the literature, for seasonal patterns in Lemna minor. Pirson and Gollner (1953) and Bornkamm (1966) subjected clones of Lemna minor to constant conditions for three year periods (constant nutrient, light intensity, 24:0 L:D, constant temperature). Pirson and Gollner (1953) measured growth of Lemna minor roots and found the rates of growth changed over yearly periods. Using the number of

hours required for roots to increase from 1 to 20mm as an indicator, they found that this process took twice as long in December as it did in June. Bornkamm (1966) used protein/carbohydrate (P/C) ratios and dry weight changes in Lemna minor for his studies of seasonal patterns and like Pirson and Gollner (1953), noted an increase in dry weight in the summer and a decrease in the winter. The P/C ratio increased in the spring, reached a maximum in the summer and decreased in the fall to a winter minimum. Alternatively, the amount of protein increased in the plants throughout the spring and summer and the amount of carbohydrate increased throughout the fall and winter. The increase and decrease of carbohydrate was connected to changes in starch content. Parallel studies done in the field, and therefore, under normally variable conditions, showed similar trends to those plants kept under constant conditions. Bornkamm (1966) found similar rhythmic trends to those found by Pirson and Gollner (1953).

Although it may not be possible to show that these rhythms are unequivocally circannular, as maintenance of constant conditions is difficult (Sweeney, 1969), the fact that such rhythms exist in parallel, in the field and in the laboratory, is certainly suggestive of circannular periodicity. For the present investigation, the cause is not important but, the existence of the rhythms is. Although the following correlation is made with extreme caution, it is nonetheless interesting that one MDH phenotype was found in the spring (May and June, 1977), another in early summer

(June 20 to July 20, 1976, 1977), another in mid-summer (July 20 to August 15, 1976, 1977) and finally another in late summer and fall; all of which represent periods which parallel the trends in the P/C ratios and dryweights described in Bornkamm's (1966) study. It is also interesting to note the December 9 "die-off", described for 1975 and 1976 in the Results section, representing an entry into the overwintering phase for Lemna kept indoors. The changes in P/C ratios found by Bornkamm (1966) were connected to changes in starch concentration. Starch buildup is important in the fall for overwintering and starch breakdown is important in the spring to begin the active growth phase (Arber, 1920; Jacobs, 1947; Hillman, 1961d). The pathways in which plant MDH participates have been described previously. Although MDH is not involved in pathways responsible for sugar or starch production it is not unlikely that MDH responds conformationally to the metabolic state of the plant. Observed seasonal changes in MDH may reflect initiation and conclusion of the overwintering stages. It should be stressed here that these suggestions are based merely on interesting correlations, no data was produced to test this hypothesis. What is important here, is that seasonal changes in MDH phenotype are real.

As mentioned in the Introduction, one of the aims of this investigation was to determine whether the mode of adaptation in Lemna minor was genetic or physiological. Determination of the mode was to be accomplished by an examination of the types of variability manifest by the

enzymes in Lemna minor. Since there are no grounds to expect that either, all organismic systems respond with similar strategies to all environmental parameters or that each response system must respond similarly to one environmental parameter (Valentine, 1976), it is possible to discuss, in the abstract, the mode of adaptation, not of Lemna minor/Lemna turionifera, but of the MDH system of Lemna minor/Lemna turionifera.

Two physiological enzyme strategies exist which allow for the homeostatic maintenance of organismic function; the eurytolerant protein strategy and the multiple variant protein strategy (Somero, 1969; Hochachka and Somero, 1973; Somero, 1975a; Somero and Low, 1977). Briefly, in the eurytolerant protein strategy, a single protein maintains its functional characteristics over a range of environmental conditions. In the multiple variant strategy, two or more isozymes are required to maintain organismic function over a range of environmental conditions (Somero, 1975a). These multiple isozymes may be expressed simultaneously or switched on or off as required.

The results of this study and most other studies do not allow a clear differentiation between the two strategies. Both strategies may be found in the MDH of Lemna minor/Lemna turionifera. Work done by Somero and co-workers (Somero and Hochachka, 1973; Somero, 1975a; Moon, 1975; Somero and Low, 1977) on temperature response in several species of fish, suggests that except for the tetraploid salmonids, which due to the amount of gene duplication

can afford, a multiple isozymic strategy, most fish species studied possess a eurytolerant protein strategy. Diploid organisms; through development of this strategy can exist with simpler genetic and protein systems and energy need not be expended synthesizing each newly required variant (Moon, 1975; Somero, 1975a). Use of this eurytolerant strategy also reduces segregational load (Somero, 1975a; Tracey and Ayala, 1974). McNaughton (1972) studied MLH responses to temperatures in Typha latifolia. His data indicate that conspicuously different forms of malate dehydrogenase were produced by the same population subjected to different conditions (McNaughton, 1972). This suggests use by Typha latifolia of the multiple variant strategy. Also, as regards this issue of adaptive strategy, it is interesting to note that quite often, polyploid forms of plant species occupy much larger geographic regions and seem to tolerate a larger degree of environmental variation than do their diploid relatives (Gustoffson, 1948; Stebbins, 1950, 1971). Lemna minor/Lemna turionifera shows variability consistent with a multiple variant strategy among organelles. If, on the other hand, the similarity of phenotype patterns over the large distribution range studied in this investigation are considered, and the resolution changes represent conformational changes (the enzyme is active during the entire season), the variation implies usage of a eurytolerant strategy.

It should also be stressed here that these variability patterns only imply the adaptive strategies used by Lemna

minor/Lemna turionifera. In the strict ^{→P.}sence of the word adaptation, an increased fitness potential must be demonstrated to justify its proper usage. This entails a comparison of survivorship and fecundity parameters that result from the possession of each of these variable strategies. This information is not presently available.

Speciation: One of the main problems identified during the course of this investigation and one that has run through the recent literature, is that of species identification. (It should perhaps be mentioned here, that when reading papers about the various aspects of the Lemnaceae, particularly about species identification, extreme care should be given to noting the country of origin of the paper. This saves a certain amount of confusion.) In the geographic area studied in the present investigation, the main problem of species identification arises between Lemna minor and Lemna turionifera (Table 7 and Table 8). Landolt (1975) has classified Lemna turionifera as a distinct species despite its great similarity to Lemna minor.

A general definition of species is difficult primarily because there is no genetically defined way in which speciation occurs (Dohzansky, 1976). Nonetheless, a workable definition of species is the following: "Species must consist of systems of populations that are separated from each other by complete or at least sharp discontinuities in the variation pattern, and these discontinuities must have

a genetic basis. That is, they must reflect the existence of isolating mechanisms which greatly hinder or completely prevent the transfer of genes from one system of populations to another" (Stebbins, 1950). In Dohzhansky's (1976) words; "Whenever the reproductive biology allows any form of exchange of genetic materials between populations to take place, it is a biological necessity that the field within which the exchange occurs must be circumscribed. ... Natural selection restricts gene exchange within tolerable limits" Within groups, such as the Lemna minor group (Landolt, 1975) which are primarily apomictic (Each separate and definable clone is reproductively isolated from the next), how much of a discontinuity in variation must exist to define a species?

Studies described by Gottlieb (1976) shed some light on this matter. This evidence he presents challenges the theory that species at their origin, possess distinctive adaptive features and differ genetically at many loci from their progenitors. Rather, species at their inception, tend to be electrophoretically, morphologically and/or ecologically similar to their progenitors or close relatives. They are, nonetheless, reproductively isolated and hence distinct species (Gottlieb, 1976). Some trait, and maybe not an obvious one, tends to separate the two species. Results generated from this study of Lemna minor/Lemna turionifera support the findings described by Gottlieb (1976); the two species are morphologically, electrophoretically and ecologically similar, one adaptive trait separates them.

Very little electrophoretically characterizable distinctness exists between Lemna minor and Lemna turionifera. It should be stated, at this time, that the octoploid (No. 6735) (Figure 24) is not Lemna minor but Lemna turionifera (Landolt through Urbanska-Worytkiewicz, 1977, pers. comm.); all the rest of the samples examined (Figure 24) are Lemna minor. The A and C systems are identical in the two species but note the D system mobility difference. It is possible that the mobility difference in the D system represents a genetic difference that characterizes Lemna turionifera despite the genetic arguments presented against this being the case at the MDH loci. Lemna minor and Lemna turionifera may differ genetically at another locus, x, which modifies MDH differentially in the two species; we have no direct evidence of this.

Further evidence, which supports the genetic argument and therefore denies use of the D system mobility as a species identification marker, exists. Table 7 and Table 8 show the results of morphological species identifications. The results in Table 7 (Landolt, 1975) show that Lemna turionifera differs from Lemna minor in the formation of anthocyanin pigments in some individuals, in the possession of distinct papules along the median nerve and most importantly, in the formation of turions. Results in Table 8 strongly suggest that Lemna turionifera was the species used in this investigation (presence of distinct papules and turion formation). This identification is supported by Landolt (pers. comm. through Urbanska-Worytkiewicz, 1977).

If this is indeed the case, then at least in the 1977 seasonal survey, Lemna minor (populations #12-19 in Materials and Methods) have been compared to Lemna turionifera (populations #1-11, 20). Thus the D system variability observed in Lemna minor is indistinguishable from that in Lemna turionifera and the two remain uncharacterized electrophoretically. Lemna turionifera also shows cytological variation (Urbanska-Worytkiewicz, 1977, pers. comm.) similar to that described for Lemna minor (Urbanska-Worytkiewicz, 1975).

Not only are these two species electrophoretically similar, they are morphologically very similar (Landolt, 1975) and in the field, the two species are indistinguishable. The two species also appear to grow in similar habitats but they do differ to some extent, in geographical range (Landolt, 1975); Lemna turionifera occupying the more northerly parts of the Lemna minor distribution range in North America. The main trait which allows Lemna turionifera to occupy these more northerly ranges is the ability to form turions thus allowing this plant to survive severe winters without needing to resort to the sexual production of seeds in order to survive. Given the reproductive strategy used by Lemna minor or Lemna turionifera a high degree of reproductive isolation exists. Possession of turion forming abilities in Lemna turionifera, despite the similarities to Lemna minor, is adequate justification for defining Lemna turionifera as a closely related but distinct species from Lemna minor. This supports Landolt's (1975) definition of Lemna turionifera as a new species (sp. nov.).

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APPENDIX A

Hardy-Weinberg equilibrium holds for polyploidy.

Expected genotypic frequencies are calculated by squaring the gamete vector or raising the allele vector to the power of the ploidy level. For example, a two allele diploid population produces two gamete types p and q ; the expected genotypic array is calculated by expanding the binomial:

$$(p + q)^2 = p^2 + 2pq + q^2$$

For a three allele system, diploid populations produce three gamete types p , q and r . The expected genotypic array is calculated by expanding the trinomial:

$$(p + q + r)^2 = p^2 + 2pq + 2pr + q^2 + 2qr + r^2$$

If expected genotypic frequencies for tetraploids are required, the calculations are done similarly, but are somewhat more tedious. For example, the gametes produced by a two allele tetraploid population are: p^2 , $2pq$ and q^2 . To calculate the genotypic array we may either square this gametic array or expand the allele vector to the fourth power:

$$(p + q)^4 = (p^2 + 2pq + q^2)^2 = p^4 + 4p^3q + 6p^2q^2 + 4pq^3 + q^4$$

The three allele tetraploid is calculated as follows:

$$(p+q+r)^4 = (p^2+2pq+2pr+2qr+q^2+r^2)^2 = p^4+4p^3q+4p^3r+6p^2q^2+6p^2r^2+12p^2qr+4pq^3+12pq^2r+12pqr^2+q^4+4q^3r+6q^2r^2+4pr^3+4qr^3+r^4$$

Expected frequencies for higher order polyploids may be generated in a similar fashion.

One of the advantages of the electrophoretic technique is the codominant expression of alleles. If heterozygous

genotypes are not distinguishable by dosage effects, breeding tests must be run to unambiguously identify all genotypes. Genotypes may, then be assigned on the basis of progeny ratios (L: 1976). If breeding is not possible, other methods of genotypic assignment must be utilized. Mobility, heat stability, and other tests may be employed to classify zymogram bands into locus groups. Once this is done it is necessary to determine the nature, genetical or physiological, of any observed polymorphisms. At the population level, phenotypic frequencies may be employed to distinguish these two causes of polymorphism by comparing observed and expected Hardy-Weinberg frequencies.

Consider a population in which zymogram polymorphisms are observed. Assume that the single banded phenotypes are homozygotes; multiple-band phenotypes are then either heterozygotes or physiological modifications. In the case of genetic polymorphism, the number of observed homozygotes should equal the number of alleles. Not all homozygotes will, however, be observed in finite samples unless allele frequencies are all of intermediate value. This is especially true in polyploids. For example, the expected diploid homozygote frequency for an allele q , where q equals 0.2, is q^2 equal to 0.24. If 100 organisms are assayed, the chance of finding no q^2 homozygotes is 1.7 percent. If on the other hand the organism studied is a tetraploid, the expected homozygote frequency is 0.0016. In a sample of 100 organisms no homozygotes will be observed 85.2 percent of the time. Identification of homozygotes as a check on

a genetic model in polyploids is, thus, a large sample procedure.

The homozygote/heterozygote ratio does, nevertheless, provide an indication of the nature of an observed polymorphism. Genotypes, detectable genotypes and expected and observed homozygote/heterozygote ratios are given in Table A1 for diploids, tetraploids, and octoploids for three sets of allele frequencies in two and three allele populations. Note that the ratio decreases as ploidy increases, and that it decreases as allele number increases where alleles are equifrequent. If, however, the frequency of the most common allele is held constant, homozygote/heterozygote ratios remain roughly constant as allele number increases. This is because the homozygotes of relatively rare alleles contribute very little to the homozygosity sum.

Data for the A, B and C systems have been analyzed in this fashion (Table 12). The computer program and output follow.

Table A1. Ratio of Homozygotes to Heterozygotes. Assuming a population in Hardy-Weinberg equilibrium, the number of electrophoretically distinguishable genotypes, the number of genotypes and the homozygote/heterozygote ratio for M alleles at varying allele frequencies is given. Allele frequencies are recorded in parentheses in the allele column.

<u>Alleles = M</u>	<u>Number of</u>		<u>Number of Detectable Genotypes</u>	<u>Diploid</u>	<u>Expected Ratio</u>	
	<u>Genotypes</u>	<u>Genotypes</u>			<u>Homozygote/Tetraploid</u>	<u>Heterozygote/Octoploid</u>
	<u>D T O</u>	<u>D T O</u>	<u>D T O</u>			
2(.5, .5)	3 5 9	3 3 3	3 3 3	1.00	0.14	0.01
(.75, .25)	3 5 9	3 3 3	3 3 3	1.67	0.47	0.11
(.9, .1)	3 5 9	3 3 3	3 3 3	4.56	1.91	0.76
	<u>D T O</u>	<u>D T O</u>	<u>D T O</u>			
3(.33, .33, .33)	6 15 45	6 7 7	6 7 7	0.50	0.04	0.0+
(.75, .125, .125)	6 15 45	6 7 7	6 7 7	1.46	0.46	0.11
(.9, .05, .05)	6 15 45	6 7 7	6 7 7	4.40	1.91	0.76

THIS PROGRAM COMPUTES HOMOZYGOTE/HETEROZYGOTE RATIOS FOR
DIPLOIDS, TETRAPLOIDS AND OCTOPLLOIDS

```

5 INPUT A,B,C
6 N=A+B+C
9 PRINT "          SINGLE  DOUBLE          SINGLE          TOTAL  "
10 PRINT "A,B,C,N=";A,B,C,N
20 P1=((2*A)+B)/(2*N)
21 PRINT "FREQUENCY OF COMMON ALLELE BY H-W=";P1
30 P2=SQR(A/N)
31 PRINT "FREQUENCY OF COMMON ALLELE BY ROOT=";P2
40 Q1=((2*C)+B)/(2*N)
50 Q2=1-P2
60 H1=1-((P1!2)+(Q1!2))
70 H2=1-((P2!2)+(Q2!2))
71 PRINT "HET BY P1 AND P2=";H1,H2
80 R1=(P1!2+Q1!2)/H1
89 REM THE RATIO CALCULATED BY H-W
90 PRINT "HOMO/HETERONO=";R1
100 R2=(P2!2+Q2!2)/H2
109 REM THE RATIO CALCULATED BY SQUARE ROOT
110 PRINT "HOMO/HETEROFR=";R2
120 P4=SQR(P2)
140 PRINT "TETRAPLOID ALLELE FREQUENCY=";P4
150 Q4=1-P4
151 H4=1-(P4!4+Q4!4)
160 PRINT "TETRAHETERO=";H4
161 R4=((P4!4)+(Q4!4))/H4
170 PRINT "TETRA HOMO/HETERO=";R4
180 P8=SQR(P4)
211 PRINT "OCTOPLLOID ALLELE FREQUENCY=";P8
220 Q8=1-P8
230 H8=1-P8!8-Q8!8
231 PRINT "OCTOHETERO=";H8
240 R8=(P8!8+Q8!8)/H8
250 PRINT "OCTO HOMO/HETERO=";R8
260 R=(A+C)/B
270 PRINT "DATA HOMO/HETERO=";R
271 END

```


SAMPLE OUTPUT

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	72	68	0	140
FREQUENCY OF COMMON ALLELE BY H-W=	.7571428571429			
FREQUENCY OF COMMON ALLELE BY ROOT=	.7171371656			
HET BY P1 AND P2=	.367755102043		.405702902625	
HOMO/HETERONO=	1.719200887886			
HOMO/HETEROFR=	1.464857883691			
TETRAPLOID ALLELE FREQUENCY=	.84683951585			
TETRAHETERO=	.485164901654			
TETRA HOMO/HETERO=	1.061158693949			
OCTOPLOID ALLELE FREQUENCY=	.9202383631			
OCTOHETERO=	.485714284062			
OCTO HOMO/HETERO=	1.058823536415			
DATA HOMO/HETERO=	1.058823529412			

BRIDGENORTH

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	63	189	2	254
FREQUENCY OF COMMON ALLELE BY H-W=	.6200787401575			
FREQUENCY OF COMMON ALLELE BY ROOT=	.49802760572			
HET BY P1 AND P2=	.47116219232		.49999221932	
HOMO/HETERONO=	1.122411382535			
HOMO/HETEROFR=	1.000031123204			
TETRAPLOID ALLELE FREQUENCY=	.70571070965			
TETRAHETERO=	.744467872425			
TETRA HOMO/HETERO=	.3432413097196			
OCTOPLD ALLELE FREQUENCY=	.84006589602			
OCTOHETERO=	.7519680758564			
OCTO HOMO/HETERO=	.3298436889905			
DATA HOMO/HETERO=	.3439153439153			

B-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	29	7	0	36
FREQUENCY OF COMMON ALLELE BY H-W=	.9027777777778			
FREQUENCY OF COMMON ALLELE BY ROOT=	.89752746786			
HET BY P1 AND P2=	.175540123457		.183943824597	
HOMO/HETERONO=	4.696703296701			
HOMO/HETEROFR=	4.436442360546			
TETRAPLOID ALLELE FREQUENCY=	.94737926295			
TETRAHETERO=	.194436777381			
TETRA HOMO/HETERO=	4.143059936861			
OCTOPLD ALLELE FREQUENCY=	.97333409626			
OCTOHETERO=	.1944444444498			
OCTO HOMO/HETERO=	4.142857142715			
DATA HOMO/HETERO=	4.142857142857			

C-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	62	49	4	115
FREQUENCY OF COMMON ALLELE BY H-W=	.7521739130435			
FREQUENCY OF COMMON ALLELE BY ROOT=	.73425502026			
HET BY P1 AND P2=	.372816635164		.390249170963	
HOMO/HETERONO=	1.682283744018			
HOMO/HETEROFR=	1.562465405198			
TETRAPLOID ALLELE FREQUENCY=	.85688681882			
TETRAHETERO=	.460450078176			
TETRA HOMO/HETERO=	1.171788099074			
OCTOPLD ALLELE FREQUENCY=	.92568181295			
OCTOHETERO=	.4608695642695			
OCTO HOMO/HETERO=	1.169811325217			
DATA HOMO/HETERO=	1.34693877551			

IA-6

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	29	33	2	69
FREQUENCY OF COMMON ALLELE BY H-W=	.695652173913			
FREQUENCY OF COMMON ALLELE BY ROOT=	.64829765931			
HET BY P1 AND P2=	.423440453686 .45601560848			
HOMO/HETERONO=	1.361607142858			
HOMO/HETEROFR=	1.192907394844			
TETRAPLOID ALLELE FREQUENCY=	.8051693358			
TETRAHETERO=	.578269260182			
TETRA HOMO/HETERO=	.7292932159997			
OCTOPLOID ALLELE FREQUENCY=	.89731228444			
OCTOHETERO=	.5797101325564			
OCTO HOMO/HETERO=	.7250009368119			
DATA HOMO/HETERO=	.8157894736842			

B-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	20	16	0	36
FREQUENCY OF COMMON ALLELE BY H-W=	.7777777777778			
FREQUENCY OF COMMON ALLELE BY ROOT=	.7453559925			
HET BY P1 AND P2=	.34567901234 .379600873884			
HOMO/HETERONO=	1.892857142905			
HOMO/HETEROFR=	1.634345884845			
TETRAPLOID ALLELE FREQUENCY=	.86334002137			
TETRAHETERO=	.444095653351			
TETRA HOMO/HETERO=	1.251767141729			
OCTOPLOID ALLELE FREQUENCY=	.9291609233			
OCTOHETERO=	.4444444437659			
OCTO HOMO/HETERO=	1.250000003435			
DATA HOMO/HETERO=	1.25			

C-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	28	19	0	47
FREQUENCY OF COMMON ALLELE BY H-W=	.7978723404255			
FREQUENCY OF COMMON ALLELE BY ROOT=	.77184498499			
HET BY P1 AND P2=	.322544137615 .352200608276			
HOMO/HETERONO=	2.10035037723			
HOMO/HETEROFR=	1.839290950958			
TETRAPLOID ALLELE FREQUENCY=	.87854708752			
TETRAHETERO=	.404037732756			
TETRA HOMO/HETERO=	1.475016363395			
OCTOPLOID ALLELE FREQUENCY=	.9373084271			
OCTOHETERO=	.4042553189314			
OCTO HOMO/HETERO=	1.473684211857			
DATA HOMO/HETERO=	1.473684210526			

LA-7

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	33	26	2	61
FREQUENCY OF COMMON ALLELE BY H-W=	.7540983606557			
FREQUENCY OF COMMON ALLELE BY ROOT=	.73551587784			
HET BY P1 AND P2=	.370868046228 .389064542575			
HOMO/HETERONO=	1.696376311566			
HOMO/HETEROFR=	1.570267630614			
TETRAPLOID ALLELE FREQUENCY=	.85762222327			
TETRAHETERO=	.458605462513			
TETRA HOMO/HETERO=	1.180523525643			
OCTOPLD ALLELE FREQUENCY=	.92607895088			
OCTOHETERO=	.4590163925585			
OCTO HOMO/HETERO=	1.178571432763			
DATA HOMO/HETERO=	1.346153846154			

B-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	4	7	1	12
FREQUENCY OF COMMON ALLELE BY H-W=	.625			
FREQUENCY OF COMMON ALLELE BY ROOT=	.57735026919			
HET BY P1 AND P2=	.46875 .48803387172			
HOMO/HETERONO=	1.133333333333			
HOMO/HETEROFR=	1.049038105646			
TETRAPLOID ALLELE FREQUENCY=	.75933568565			
TETRAHETERO=	.663339811409			
TETRA HOMO/HETERO=	.5075229660593			
OCTOPLD ALLELE FREQUENCY=	.87168554237			
OCTOHETERO=	.666666593184			
OCTO HOMO/HETERO=	.500000165336			
DATA HOMO/HETERO=	.7142857142857			

C-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	26	43	0	69
FREQUENCY OF COMMON ALLELE BY H-W=	.6884057971014			
FREQUENCY OF COMMON ALLELE BY ROOT=	.61384981404			
HET BY P1 AND P2=	.42900651124 .47407643968			
HOMO/HETERONO=	1.330966952249			
HOMO/HETEROFR=	1.109364474377			
TETRAPLOID ALLELE FREQUENCY=	.7834856821			
TETRAHETERO=	.62099081672			
TETRA HOMO/HETERO=	.6103297715133			
OCTOPLD ALLELE FREQUENCY=	.88514726577			
OCTOHETERO=	.6231883755018			
OCTO HOMO/HETERO=	.6046512407982			
DATA HOMO/HETERO=	.6046511627907			

TH

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	38	45	1	84
FREQUENCY OF COMMON ALLELE BY H-W=	.7202380952331			
FREQUENCY OF COMMON ALLELE BY ROOT=	.67259270913			
HET BY P1 AND P2=	.402990362814		.44042351352	
HOMO/HETERONO=	1.481448918573			
HOMO/HETEROFR=	1.270541806471			
TETRAPLOID ALLELE FREQUENCY=	.82011749715			
TETRAHETERO=	.546572025911			
TETRA HOMO/HETERO=	.8295850365436			
OCTOPLOID ALLELE FREQUENCY=	.90560338844			
OCTOHETERO=	.5476190413055			
OCTO HOMO/HETERO=	.8260869775749			
DATA HOMO/HETERO=	.8666666666667			

B-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	18	8	0	26
FREQUENCY OF COMMON ALLELE BY H-W=	.8461538461538			
FREQUENCY OF COMMON ALLELE BY ROOT=	.83205029434			
HET BY P1 AND P2=	.260355029587		.279485204059	
HOMO/HETERONO=	2.840909090891			
HOMO/HETEROFR=	2.578006940893			
TETRAPLOID ALLELE FREQUENCY=	.91216790907			
TETRAHETERO=	.307632794548			
TETRA HOMO/HETERO=	2.250628729197			
OCTOPLOID ALLELE FREQUENCY=	.95507481857			
OCTOHETERO=	.3076923076835			
OCTO HOMO/HETERO=	2.250000000093			
DATA HOMO/HETERO=	2.25			

C-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	41	31	0	72
FREQUENCY OF COMMON ALLELE BY H-W=	.7847222222222			
FREQUENCY OF COMMON ALLELE BY ROOT=	.75461542818			
HET BY P1 AND P2=	.337866512345		.370341967463	
HOMO/HETERONO=	1.95974878676			
HOMO/HETEROFR=	1.700207073075			
TETRAPLOID ALLELE FREQUENCY=	.86868603545			
TETRAHETERO=	.43025822217			
TETRA HOMO/HETERO=	1.324185683093			
OCTOPLOID ALLELE FREQUENCY=	.93203328023			
OCTOHETERO=	.430555550747			
OCTO HOMO/HETERO=	1.322580647755			
DATA HOMO/HETERO=	1.322580645161			

SB

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	16	20	1	37
FREQUENCY OF COMMON ALLELE BY H-W=	.7027027027027			
FREQUENCY OF COMMON ALLELE BY ROOT=	.65759594922			
HET BY P1 AND P2=	.41782322863		.45032703357	
HOMO/HETERONO=	1.39335664338			
HOMO/HETEROFR=	1.22060841445			
TETRAPLOID ALLELE FREQUENCY=	.81092289968			
TETRAHETERO=	.566289494345			
TETRA HOMO/HETERO=	.7658812497609			
OCTOPLOID ALLELE FREQUENCY=	.90051257608			
OCTONETERO=	.5675675579728			
OCTO HOMO/HETERO=	.7619047916899			
DATA HOMO/HETERO=	.85			

GU

A-SYSTEM

	SINGLE	DOUBLE	SINGLE	TOTAL
A,B,C,N=	4	16	0	20
FREQUENCY OF COMMON ALLELE BY H-W=	.6			
FREQUENCY OF COMMON ALLELE BY ROOT=	.4472135955			
HET BY P1 AND P2=	.48		.494427191	
HOMO/HETERONO=	1.083333333333			
HOMO/HETEROFR=	1.022542485937			
TETRAPLOID ALLELE FREQUENCY=	.66874030498			
TETRAHETERO=	.787958671883			
TETRA HOMO/HETERO=	.2691020934008			
OCTOPLOID ALLELE FREQUENCY=	.81776543396			
OCTONETERO=	.7999987836851			
OCTO HOMO/HETERO=	.2500019004949			
DATA HOMO/HETERO=	.25			

THIS PROGRAM COMPUTES DIPLOID, TETRAPLOID AND OCTOPLOID
HARDY-WEINBERG EXPECTATIONS FOR THREE PHENOTYPES; IT ALSO
COMPUTES CHI-SQUARE GOODNESS-OF-FIT

```

8 DIM A(5)
9 REM K= NUMBER OF PHENOTYPES
10 K=3
15 FOR I=1 TO K: INPUT A(I):PRINT "PHENOTYPE FREQUENCY=";A(I):NE
XT I
19 N=0
20 FOR I=1 TO K:N=N+A(I):NEXT I
23 PRINT "PHENOTYPE TOTAL =" ;N
25 P=A(1)/N:PRINT "COMMON HOMOZYGOTE FREQUENCY=" ;P
30 D=SQR(P):PRINT "DIPLOID ALLELE FREQUENCY=" ;D
35 T=SQR(D):PRINT "TETRAPLOID ALLELE FREQUENCY=" ;T
40 O=SQR(T):PRINT "OCTOPLOID ALLELE FREQUENCY=" ;O
50 S=((D*D)+(1-D)*(1-D))*N:PRINT "EXP DIPLOID HOMOZYGOTE SUM=" ;S

55 H=N-S:PRINT "EXP DIPLOID HETEROZYGOTES=" ;H
60 REM C AND L ARE CHI-SQUARE NUMERATORS
61 C=((A(1)+A(3))-S)
70 IF C]0 THEN 80
71 C=-C
80 Y=(C-0.5)!2/S
81 PRINT "HOMOZYGOTE CHI (YATES)=" ;Y
90 L=A(2)-H
92 IF L]0 THEN 100
95 L=-L
100 M=(L-0.5)!2/H
101 PRINT "HETEROZYGOTE CHI (YATES)=" ;M
110 B=Y+M:PRINT "DIPLOID CHI-SQUARE VALUE=" ;B
120 E=(T!4+(1-T)!4)*N:PRINT "EXP TETRAPLOID HOMO SUM =" ;E
130 F=N-E:PRINT "EXP TETRAPLOID HETEROZYGOTES =" ;F
140 G=(O!8+(1-O)!8)*N:PRINT "EXP OCTOPLOID HOMO SUM =" ;G
150 Q=N-G:PRINT "EXP OCTOPLOID HETEROZYGOTES=" ;Q
160 C=((A(1)+A(3))-E)
170 IF C]0 THEN 180
171 C=-C
180 Y=(C-0.5)!2/E
190 PRINT "TETRAPLOID HOMO CHI (YATES)=" ;Y
200 L=A(2)-F
202 IF L]0 THEN 210
205 L=-L
210 M=(L-0.5)!2/F
211 PRINT "TETRAPLOID HETERO CHI (YATES)=" ;M
220 B=Y+M:PRINT "TETRAPLOID CHI-SQUARE VALUE=" ;B
230 C=((A(1)+A(3))-G)
240 IF C]0 THEN 250
242 C=-C

```



```
250 Y=(C-0.5)!2/G
251 PRINT "OCTOPLD HOMO CHI (YATES)=";Y
260 L=A(2)-Q
262 IF L>0 THEN 270
263 L=-L
270 M=(L-0.5)!2/Q
271 PRINT "OCTOPLD HETERO CHI (YATES)=";M
280 B=Y+M:PRINT "OCTOPLD CHI-SQUARE VALUE=";B
281 END
```


SAMPLE OUTPUT

PHENOTYPE FREQUENCY= 72
PHENOTYPE FREQUENCY= 68
PHENOTYPE FREQUENCY= 0
PHENOTYPE TOTAL = 140
COMMON HOMOZYGOTE FREQUENCY= .5142857142857
DIPLOID ALLELE FREQUENCY= .7171371656
TETRAPLOID ALLELE FREQUENCY= .34683951585
OCTOPLOID ALLELE FREQUENCY= .92023883631
EXP DIPLOID HOMOZYGOTE SUM= 83.20159363174
EXP DIPLOID HETEROZYGOTES= 56.7984063683
HOMOZYGOTE CHI (YATES)= 1.376465296649
HETEROZYGOTE CHI (YATES)= 2.016326048259
DIPLOID CHI-SQUARE VALUE= 3.392791344908
EXP TETRAPLOID HOMO SUM = 72.07703976848
EXP TETRAPLOID HETEROZYGOTES = 67.9229692316
EXP OCTOPLOID HOMO SUM = 72.00000023132
EXP OCTOPLOID HETEROZYGOTES= 67.9999997687
TETRAPLOID HOMO CHI (YATES)= 2.48200200E-03
TETRAPLOID HETERO CHI (YATES)= 2.63379801E-03
TETRAPLOID CHI-SQUARE VALUE= 5.11580001E-03
OCTOPLOID HOMO CHI (YATES)= 3.47221899E-03
OCTOPLOID HETERO CHI (YATES)= 3.67646719E-03
OCTOPLOID CHI-SQUARE VALUE= 7.14868619E-03

BRIDGENORTH

A-SYSTEM

PHENOTYPE FREQUENCY= 63
 PHENOTYPE FREQUENCY= 189
 PHENOTYPE FREQUENCY= 2
 PHENOTYPE TOTAL = 254
 COMMON HOMOZYGOTE FREQUENCY= .248031496063
 DIPLOID ALLELE FREQUENCY= .49802760572
 TETRAPLOID ALLELE FREQUENCY= .70571070965
 OCTOPLOID ALLELE FREQUENCY= .84006589602
 EXP DIPLOID HOMOZYGOTE SUM= 127.0019762923
 EXP DIPLOID HETEROZYGOTES= 126.9980237077
 HOMOZYGOTE CHI (YATES)= 29.78294667789
 HETEROZYGOTE CHI (YATES)= 29.78387361843
 DIPLOID CHI-SQUARE VALUE= 59.56682029632
 EXP TETRAPLOID HOMO SUM = 64.90516040413
 EXP TETRAPLOID HETEROZYGOTES = 189.0948395959
 EXP OCTOPLOID HOMO SUM = 63.00010873247
 EXP OCTOPLOID HETEROZYGOTES= 190.9998912676
 TETRAPLOID HOMO CHI (YATES)= 2.52915102E-03
 TETRAPLOID HETERO CHI (YATES)= 8.68109110E-04
 TETRAPLOID CHI-SQUARE VALUE= 3.39726013E-03
 OCTOPLOID HOMO CHI (YATES)= 3.57090465E-02
 OCTOPLOID HETERO CHI (YATES)= 1.17784036E-02
 OCTOPLOID CHI-SQUARE VALUE= 4.74874501E-02

B-SYSTEM

PHENOTYPE FREQUENCY= 29
 PHENOTYPE FREQUENCY= 7
 PHENOTYPE FREQUENCY= 0
 PHENOTYPE TOTAL = 36
 COMMON HOMOZYGOTE FREQUENCY= .8055555555556
 DIPLOID ALLELE FREQUENCY= .89752746786
 TETRAPLOID ALLELE FREQUENCY= .94737926295
 OCTOPLOID ALLELE FREQUENCY= .97333409626
 EXP DIPLOID HOMOZYGOTE SUM= 29.37802231463
 EXP DIPLOID HETEROZYGOTES= 6.62197768537
 HOMOZYGOTE CHI (YATES)= 5.06451917E-04
 HETEROZYGOTE CHI (YATES)= 2.24684473E-03
 DIPLOID CHI-SQUARE VALUE= 2.75329665E-03
 EXP TETRAPLOID HOMO SUM = 29.00027601431
 EXP TETRAPLOID HETEROZYGOTES = 6.99972398569
 EXP OCTOPLOID HOMO SUM = 28.9999999981
 EXP OCTOPLOID HETEROZYGOTES= 7.00000000019
 TETRAPLOID HOMO CHI (YATES)= 3.61109258E-03
 TETRAPLOID HETERO CHI (YATES)= 3.56762727E-02
 TETRAPLOID CHI-SQUARE VALUE= 4.42873653E-02
 OCTOPLOID HOMO CHI (YATES)= 3.62068964E-03
 OCTOPLOID HETERO CHI (YATES)= 3.57142856E-02
 OCTOPLOID CHI-SQUARE VALUE= 4.43349753E-02

BRIDGENORTH

C-SYSTEM

PHENOTYPE FREQUENCY= 62
PHENOTYPE FREQUENCY= 49
PHENOTYPE FREQUENCY= 4
PHENOTYPE TOTAL = 115
COMMON HOMOZYGOTE FREQUENCY= .5391304347826
DIPLOID ALLELE FREQUENCY= .73425502026
TETRAPLOID ALLELE FREQUENCY= .85688681882
OCTOPLD ALLELE FREQUENCY= .92568181295
EXP DIPLOID HOMOZYGOTE SUM= 70.12134533891
EXP DIPLOID HETEROZYGOTES= 44.8786546611
HOMOZYGOTE CHI (YATES)= .187020685365
HETEROZYGOTE CHI (YATES)= .2922133509356
DIPLOID CHI-SQUARE VALUE= .4792340363006
EXP TETRAPLOID HOMO SUM = 62.04824100979
EXP TETRAPLOID HETEROZYGOTES = 52.9517589903
EXP OCTOPLD HOMO SUM = 62.00000010901
EXP OCTOPLD HETEROZYGOTES= 52.999999891
TETRAPLOID HOMO CHI (YATES)= .1920222061431
TETRAPLOID HETERO CHI (YATES)= .225009335935
TETRAPLOID CHI-SQUARE VALUE= .4170315420781
OCTOPLD HOMO CHI (YATES)= .1975806325074
OCTOPLD HETERO CHI (YATES)= .2311320615508
OCTOPLD CHI-SQUARE VALUE= .4287126940582

LA-6

A-SYSTEM

PHENOTYPE FREQUENCY= 29
 PHENOTYPE FREQUENCY= 33
 PHENOTYPE FREQUENCY= 2
 PHENOTYPE TOTAL = 69
 COMMON HOMOZYGOTE FREQUENCY= .4202898550725
 DIPLOID ALLELE FREQUENCY= .64829765931
 TETRAPLOID ALLELE FREQUENCY= .8051693358
 OCTOPOLOID ALLELE FREQUENCY= .89731228444
 EXP DIPLOID HOMOZYGOTE SUM= 37.53492301444
 EXP DIPLOID HETEROZYGOTES= 31.46507698556
 HOMOZYGOTE CHI (YATES)= .9703042624062
 HETEROZYGOTE CHI (YATES)= 1.157483129843
 DIPLOID CHI-SQUARE VALUE= 2.127787392249
 EXP TETRAPLOID HOMO SUM = 29.09942104746
 EXP TETRAPLOID HETEROZYGOTES = 39.99057895254
 EXP OCTOPOLOID HOMO SUM = 29.00000085361
 EXP OCTOPOLOID HETEROZYGOTES= 39.99999914639
 TETRAPLOID HOMO CHI (YATES)= 6.74110113E-02
 TETRAPLOID HETERO CHI (YATES)= 4.91627303E-02
 TETRAPLOID CHI-SQUARE VALUE= .1165737416539
 OCTOPOLOID HOMO CHI (YATES)= 7.75861163E-02
 OCTOPOLOID HETERO CHI (YATES)= 5.62499371E-02
 OCTOPOLOID CHI-SQUARE VALUE= .1333360534897

B-SYSTEM

PHENOTYPE FREQUENCY= 20
 PHENOTYPE FREQUENCY= 16
 PHENOTYPE FREQUENCY= 0
 PHENOTYPE TOTAL = 36
 COMMON HOMOZYGOTE FREQUENCY= .5555555555556
 DIPLOID ALLELE FREQUENCY= .7453559925
 TETRAPLOID ALLELE FREQUENCY= .86334002137
 OCTOPOLOID ALLELE FREQUENCY= .9291609233
 EXP DIPLOID HOMOZYGOTE SUM= 22.33436854001
 EXP DIPLOID HETEROZYGOTES= 13.66563145999
 HOMOZYGOTE CHI (YATES)= .1506605362302
 HETEROZYGOTE CHI (YATES)= .2462314273915
 DIPLOID CHI-SQUARE VALUE= .3968919636217
 EXP TETRAPLOID HOMO SUM = 20.01255647938
 EXP TETRAPLOID HETEROZYGOTES = 15.98744352062
 EXP OCTOPOLOID HOMO SUM = 20.00000002443
 EXP OCTOPOLOID HETEROZYGOTES= 15.99999997557
 TETRAPLOID HOMO CHI (YATES)= 1.18726053E-02
 TETRAPLOID HETERO CHI (YATES)= 1.48617373E-02
 TETRAPLOID CHI-SQUARE VALUE= 2.67343426E-02
 OCTOPOLOID HOMO CHI (YATES)= 1.24999987E-02
 OCTOPOLOID HETERO CHI (YATES)= 1.56249984E-02
 OCTOPOLOID CHI-SQUARE VALUE= 2.81249972E-02

LA-6

C-SYSTEM

PHENOTYPE FREQUENCY= 23
PHENOTYPE FREQUENCY= 19
PHENOTYPE FREQUENCY= 0
PHENOTYPE TOTAL = 47
COMMON HOMOZYGOTE FREQUENCY= .5957446808511
DIPLOID ALLELE FREQUENCY= .77134498499
TETRAPLOID ALLELE FREQUENCY= .87854708752
OCTOPLD ALLELE FREQUENCY= .9373084271
EXP DIPLOID HOMOZYGOTE SUM= 30.44657141123
EXP DIPLOID HETEROZYGOTES= 16.55342858877
HOMOZYGOTE CHI (YATES)= .1244521167202
HETEROZYGOTE CHI (YATES)= .2289036521153
DIPLOID CHI-SQUARE VALUE= .3533557683355
EXP TETRAPLOID HOMO SUM = 28.01022656047
EXP TETRAPLOID HETEROZYGOTES = 18.98977343953
EXP OCTOPLD HOMO SUM = 28.00000001022
EXP OCTOPLD HETEROZYGOTES= 18.99999998978
TETRAPLOID HOMO CHI (YATES)= 8.56394437E-03
TETRAPLOID HETERO CHI (YATES)= 1.26319580E-02
TETRAPLOID CHI-SQUARE VALUE= 2.11959024E-02
OCTOPLD HOMO CHI (YATES)= 8.92857106E-03
OCTOPLD HETERO CHI (YATES)= 1.31578942E-02
OCTOPLD CHI-SQUARE VALUE= 2.20864652E-02

LA-7

A-SYSTEM

PHENOTYPE FREQUENCY= 33
 PHENOTYPE FREQUENCY= 26
 PHENOTYPE FREQUENCY= 2
 PHENOTYPE TOTAL = 61
 COMMON HOMOZYGOTE FREQUENCY= .5409836065574
 DIPLOID ALLELE FREQUENCY= .73551587784
 TETRAPLOID ALLELE FREQUENCY= .85762222327
 OCTOPLD ALLELE FREQUENCY= .92607895038
 EXP DIPLOID HOMOZYGOTE SUM= 37.26706290319
 EXP DIPLOID HETEROZYGOTES= 23.73293709681
 HOMOZYGOTE CHI (YATES)= 8.37874267E-02
 HETEROZYGOTE CHI (YATES)= .1315686841061
 DIPLOID CHI-SQUARE VALUE= .2153561106725
 EXP TETRAPLOID HOMO SUM = 33.02506678672
 EXP TETRAPLOID HETEROZYGOTES = 27.97493321328
 EXP OCTOPLD HOMO SUM = 33.00000005393
 EXP OCTOPLD HETEROZYGOTES= 27.9999994607
 TETRAPLOID HOMO CHI (YATES)= 6.58720237E-02
 TETRAPLOID HETERO CHI (YATES)= 7.77634737E-02
 TETRAPLOID CHI-SQUARE VALUE= .143635497461
 OCTOPLD HOMO CHI (YATES)= 6.81818131E-02
 OCTOPLD HETERO CHI (YATES)= 8.03571372E-02
 OCTOPLD CHI-SQUARE VALUE= .1485389504007

B-SYSTEM

PHENOTYPE FREQUENCY= 4
 PHENOTYPE FREQUENCY= 7
 PHENOTYPE FREQUENCY= 1
 PHENOTYPE TOTAL = 12
 COMMON HOMOZYGOTE FREQUENCY= .3333333333333
 DIPLOID ALLELE FREQUENCY= .57735026919
 TETRAPLOID ALLELE FREQUENCY= .75983568565
 OCTOPLD ALLELE FREQUENCY= .87168554287
 EXP DIPLOID HOMOZYGOTE SUM= 6.143593539451
 EXP DIPLOID HETEROZYGOTES= 5.85640646055
 HOMOZYGOTE CHI (YATES)= 6.74218828E-02
 HETEROZYGOTE CHI (YATES)= 7.07281242E-02
 DIPLOID CHI-SQUARE VALUE= .1381500071578
 EXP TETRAPLOID HOMO SUM = 4.039922263098
 EXP TETRAPLOID HETEROZYGOTES = 7.96007773691
 EXP OCTOPLD HOMO SUM = 4.000000881792
 EXP OCTOPLD HETEROZYGOTES= 7.99999911821
 TETRAPLOID HOMO CHI (YATES)= 5.23949497E-02
 TETRAPLOID HETERO CHI (YATES)= 2.65916403E-02
 TETRAPLOID CHI-SQUARE VALUE= 7.89365900E-02
 OCTOPLD HOMO CHI (YATES)= 6.24997657E-02
 OCTOPLD HETERO CHI (YATES)= 3.12493932E-02
 OCTOPLD CHI-SQUARE VALUE= 9.37496589E-02

IA-7

C-SYSTEM

PHENOTYPE FREQUENCY= 26
PHENOTYPE FREQUENCY= 43
PHENOTYPE FREQUENCY= 0
PHENOTYPE TOTAL = 69
COMMON HOMOZYGOTE FREQUENCY= .3768115942029
DIPLOID ALLELE FREQUENCY= .61384981404
TETRAPLOID ALLELE FREQUENCY= .7634856821
OCTOPLD ALLELE FREQUENCY= .88514726577
EXP DIPLOID HOMOZYGOTE SUM= 36.28872566165
EXP DIPLOID HETEROZYGOTES= 32.71127433835
HOMOZYGOTE CHI (YATES)= 2.64046610436
HETEROZYGOTE CHI (YATES)= 2.9292392919
DIPLOID CHI-SQUARE VALUE= 5.56970539626
EXP TETRAPLOID HOMO SUM = 26.15163364638
EXP TETRAPLOID HETEROZYGOTES = 42.84836635362
EXP OCTOPLD HOMO SUM = 26.00000209038
EXP OCTOPLD HETEROZYGOTES= 42.99999790962
TETRAPLOID HOMO CHI (YATES)= 4.64059408E-03
TETRAPLOID HETERO CHI (YATES)= 2.83229272E-03
TETRAPLOID CHI-SQUARE VALUE= 7.47288630E-03
OCTOPLD HOMO CHI (YATES)= 9.61530344E-03
OCTOPLD HETERO CHI (YATES)= 5.81390515E-03
OCTOPLD CHI-SQUARE VALUE= 1.54292086E-02

TII

A-SYSTEM

PHENOTYPE FREQUENCY= 38
 PHENOTYPE FREQUENCY= 45
 PHENOTYPE FREQUENCY= 1
 PHENOTYPE TOTAL = 84
 COMMON HOMOZYGOTE FREQUENCY= .452380952381
 DIPLOID ALLELE FREQUENCY= .67259270913
 TETRAPLOID ALLELE FREQUENCY= .82011749715
 OCTOPLD ALLELE FREQUENCY= .90560338844
 EXP DIPLOID HOMOZYGOTE SUM= 47.00442486513
 EXP DIPLOID HETEROZYGOTES= 36.99557513487
 HOMOZYGOTE CHI (YATES)= 1.198108321027
 HETEROZYGOTE CHI (YATES)= 1.522246710605
 DIPLOID CHI-SQUARE VALUE= 2.720355031632
 EXP TETRAPLOID HOMO SUM = 38.08794982348
 EXP TETRAPLOID HETEROZYGOTES = 45.91205017652
 EXP OCTOPLD HOMO SUM = 38.00000053034
 EXP OCTOPLD HETEROZYGOTES= 45.99999946966
 TETRAPLOID HOMO CHI (YATES)= 4.45771822E-03
 TETRAPLOID HETERO CHI (YATES)= 3.69805633E-03
 TETRAPLOID CHI-SQUARE VALUE= 8.15577455E-03
 OCTOPLD HOMO CHI (YATES)= 6.57893332E-03
 OCTOPLD HETERO CHI (YATES)= 5.43477114E-03
 OCTOPLD CHI-SQUARE VALUE= 1.20137044E-02

B-SYSTEM

PHENOTYPE FREQUENCY= 18
 PHENOTYPE FREQUENCY= 8
 PHENOTYPE FREQUENCY= 0
 PHENOTYPE TOTAL = 26
 COMMON HOMOZYGOTE FREQUENCY= .6923076923077
 DIPLOID ALLELE FREQUENCY= .83205029434
 TETRAPLOID ALLELE FREQUENCY= .91216790907
 OCTOPLD ALLELE FREQUENCY= .95507481857
 EXP DIPLOID HOMOZYGOTE SUM= 18.73338469451
 EXP DIPLOID HETEROZYGOTES= 7.26661530549
 HOMOZYGOTE CHI (YATES)= 2.90755869E-03
 HETEROZYGOTE CHI (YATES)= 7.49570650E-03
 DIPLOID CHI-SQUARE VALUE= 1.04032652E-02
 EXP TETRAPLOID HOMO SUM = 18.00154734178
 EXP TETRAPLOID HETEROZYGOTES = 7.99845265822
 EXP OCTOPLD HOMO SUM = 18.00000000023
 EXP OCTOPLD HETEROZYGOTES= 7.99999999977
 TETRAPLOID HOMO CHI (YATES)= 1.38018720E-02
 TETRAPLOID HETERO CHI (YATES)= 3.10628896E-02
 TETRAPLOID CHI-SQUARE VALUE= 4.48647616E-02
 OCTOPLD HOMO CHI (YATES)= 1.38888888E-02
 OCTOPLD HETERO CHI (YATES)= 3.12499999E-02
 OCTOPLD CHI-SQUARE VALUE= 4.51388888E-02

TH

C-SYSTEM

PHENOTYPE FREQUENCY= 41
PHENOTYPE FREQUENCY= 31
PHENOTYPE FREQUENCY= 0
PHENOTYPE TOTAL = 72
COMMON HOMOZYGOTE FREQUENCY= .5694444444444
DIPLOID ALLELE FREQUENCY= .75461542818
TETRAPLOID ALLELE FREQUENCY= .86868603545
OCTOPLD ALLELE FREQUENCY= .93203328923
EXP DIPLOID HOMOZYGOTE SUM= 45.33537834248
EXP DIPLOID HETEROZYGOTES= 26.66462165752
HOMOZYGOTE CHI (YATES)= .3244734599914
HETEROZYGOTE CHI (YATES)= .5516729701661
DIPLOID CHI-SQUARE VALUE= .8761455292575
EXP TETRAPLOID HOMO SUM = 41.02140809379
EXP TETRAPLOID HETEROZYGOTES = 30.97859199621
EXP OCTOPLD HOMO SUM = 41.00000003462
EXP OCTOPLD HETEROZYGOTES= 30.99999996538
TETRAPLOID HOMO CHI (YATES)= 5.58367715E-03
TETRAPLOID HETERO CHI (YATES)= 7.39382535E-03
TETRAPLOID CHI-SQUARE VALUE= 1.29775025E-02
OCTOPLD HOMO CHI (YATES)= 6.09756012E-03
OCTOPLD HETERO CHI (YATES)= 8.06451502E-03
OCTOPLD CHI-SQUARE VALUE= 1.41620751E-02

SB

A-SYSTEM

PHENOTYPE FREQUENCY= 16
 PHENOTYPE FREQUENCY= 20
 PHENOTYPE FREQUENCY= 1
 PHENOTYPE TOTAL = 37
 COMMON HOMOZYGOTE FREQUENCY= .4324324324324
 DIPLOID ALLELE FREQUENCY= .65759594922
 TETRAPLOID ALLELE FREQUENCY= .81092289968
 OCTOPLOID ALLELE FREQUENCY= .90051257608
 EXP DIPLOID HOMOZYGOTE SUM= 20.33789975758
 EXP DIPLOID HETEROZYGOTES= 16.66210024242
 HOMOZYGOTE CHI (YATES)= .3959934472142
 HETEROZYGOTE CHI (YATES)= .4833529337194
 DIPLOID CHI-SQUARE VALUE= .8793463809336
 EXP TETRAPLOID HOMO SUM = 16.04728870925
 EXP TETRAPLOID HETEROZYGOTES = 20.95271129075
 EXP OCTOPLOID HOMO SUM = 16.00000035501
 EXP OCTOPLOID HETEROZYGOTES= 20.99999964499
 TETRAPLOID HOMO CHI (YATES)= 1.27714728E-02
 TETRAPLOID HETERO CHI (YATES)= 9.78143162E-03
 TETRAPLOID CHI-SQUARE VALUE= 2.25529045E-02
 OCTOPLOID HOMO CHI (YATES)= 1.56249774E-02
 OCTOPLOID HETERO CHI (YATES)= 1.19047452E-02
 OCTOPLOID CHI-SQUARE VALUE= 2.75297226E-02

GU

A-SYSTEM

PHENOTYPE FREQUENCY= 4
 PHENOTYPE FREQUENCY= 16
 PHENOTYPE FREQUENCY= 0
 PHENOTYPE TOTAL = 20
 COMMON HOMOZYGOTE FREQUENCY= .2
 DIPLOID ALLELE FREQUENCY= .4472135955
 TETRAPLOID ALLELE FREQUENCY= .66374030498
 OCTOPLOID ALLELE FREQUENCY= .81776543396
 EXP DIPLOID HOMOZYGOTE SUM= 10.11145618
 EXP DIPLOID HETEROZYGOTES= 9.88854382
 HOMOZYGOTE CHI (YATES)= 3.114135005932
 HETEROZYGOTE CHI (YATES)= 3.184335432312
 DIPLOID CHI-SQUARE VALUE= 6.298470518244
 EXP TETRAPLOID HOMO SUM = 4.24082656234
 EXP TETRAPLOID HETEROZYGOTES = 15.75917343766
 EXP OCTOPLOID HOMO SUM = 4.000024326298
 EXP OCTOPLOID HETEROZYGOTES= 15.99997567371
 TETRAPLOID HOMO CHI (YATES)= 1.58390987E-02
 TETRAPLOID HETERO CHI (YATES)= 4.26233463E-03
 TETRAPLOID CHI-SQUARE VALUE= 2.01014334E-02
 OCTOPLOID HOMO CHI (YATES)= 6.24935385E-02
 OCTOPLOID HETERO CHI (YATES)= 1.56235033E-02
 OCTOPLOID CHI-SQUARE VALUE= 7.81170419E-02

APPENDIX B

A seasonal check was carried out on August 18, 1977. Colonies from SB, LA-6, LA-7, and BN-4 were run. It was found that although the smear between A and C was beginning to clear, it had not entirely disappeared and as yet, the A and C systems had not become as distinct as was found after August 15, 1976 and from May 19, 1977 to June 20, 1977.

APPENDIX C

As mentioned in the Results and Discussion Sections, MDH pattern changes suggestive of a seasonal variability in the enzyme were observed (Figure 23). Several technique cross checks were initiated in order to varify these results. All buffer systems with each starch type (Materials and Methods) were rerun and band pattern form was not changed on any of these systems.

Also, in 1976, the mass-grind controls were used. These, as stated, were prepared by grinding a mixture of several individuals from each available population. This homogenate was frozen so that the same control system could be used throughou the survey season. The control was prepared during the July 20 to August 15, 1976 phase when the A and C systems appeared to be connected by a dark smear. During the August 15 to December 9, 1976 phase all live individuals, independent of culture condition (field, indoor and outdoor holding facilities) yielded patterns which contained distinct A, B and C systems. These particular forms of A and C were not yielded by control individuals which were frozen during an earlier phase. This phenomenon suggests that the changes in the A and C systems are more likely due to changes within the plants than to changes in technique or other external rhythmic changes.

APPENDIX DAssumptions of the Hardy-Weinberg Model for Polyploids

- 1.) Model assumes a two allele case.
- 2.) Double bands represent heterozygotes
Single bands represent homozygotes
- 3.) Hardy-Weinberg normally requires randomly mating populations and Lemna minor populations obviously do not bear this characteristic. However, for the application of this model to Lemna minor, it is assumed that the apomictic mitotic reproduction utilized by this plant neither reduces nor increases the genetic variability remaining from the last round of random outcrossing.
- 4.) It is also assumed that regardless of the ploidy level, all genotypic heterozygotes produce indistinguishable phenotypes.



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