AN ABSTRACT OF THE THESIS OF

Lei Zhang for the degree of Doctor of Philosophy in Zoology, presented on June 10, 1993.

Title: **Adaptive Significance of Polyploidy in Brine Shrimp (Artemia parthenogenetica)**

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Abstract approved:

To explore the adaptive significance of polyploidy in animals, I have investigated effects of polyploidy on physiological, life history and genetic characteristics, and geographic distributions in brine shrimp Artemia parthenogenetica. Based on laboratory studies of sympatric diploids and polyploids in three populations collected from China, Italy and Spain, I found that polyploid Artemia were more resistant to temperature changes than diploids. At optimal temperature and commonly used salinity (25°C, 90ppt), diploids had much higher higher fecundity and faster developmental rates than their sympatric polyploids. Polyploids had better reproductive performance than sympatric diploids at high temperature and low salinity (31°C, 30 ppt) which correspond to marginal environmental conditions. My electrophoretic results show that genetic divergence has occurred between

polyploids and diploid progenitors; some alleles occurred only in diploids, while others were restricted to polyploids.

My literature review reveals that in the Old World, below 25°N latitude, all Artemia populations are polyploids, while in temperate regions (between 35-45°N), diploids are the most common cytotype. Differences in relative fitness, latitudinal distributions and environmental conditions are considered for Chinese diploid and polyploid Artemia. I suggest that Artemia's habitats gradually become marginal southward along the China coast, and that the more southern distribution of polyploids along the China coast may be a result of combined effect of low salinity and high temperatures, rather than a result of temperature adaptation only. Thus, polyploidy in Artemia has led to divergence in physiological, life history, and genetic characteristics, and as a result, in geographic distributions. The occurrence of polyploidy in Artemia is also associated with the broader niches of asexual Artemia populations as compared with sexual populations, and consequently has extended the genus' distribution range.

Polyploidy is associated with an increased ability to withstand stressful temperatures in Artemia. An initial investigation of the biochemical basis of this association is reported.

Adaptive Significance of Polyploidy in Brine Shrimp (Artemia parthenogenetica)

By

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Prepared by: Lei Zhang

DEDICATION

To my wife,

Li, Hongyan

and to my parents,

Zhang, Xuezhi and Ouyang, Ying

ACKNOWLEDGEMENTS

First and Foremost I thank Dr. Charles E. King, who acted as my major professor, editor and friend during my M. S. and Ph. D. study in the Department of Zoology. Without Dr. King's careful guidance, encouragement and support, my achievement of a Ph.D. degree would not be possible. I would also like to thank Dr. George N. Somero who has acted as my co-adviser in the past two years. Without Dr. Somero's support and guidance, I would not be able to conduct biochemical analysis of polyploid and diploid *Artemia*. The other members of my committee (Drs. John E. Morris, Paul A. Roberts, Peter S. Dawson, Fred R. Rickson and Douglas E. Johnson) provided strong support during the development of my research.

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Table of Contents

Table of Contents (cont)

List of Figures

List of Figures (Cont)

List of Tables

List of Tables (Cont)

Adaptive Significance of Polyploidy in Brine Shrimp *Artemia parthenogenetica*

INTRODUCTION

Amplification of the genetic material (cellular DNA content) has been intimately connected with evolution of complex biochemical organization (Hinegardner 1976). One mechanism to increase cellular genetic material is polyploidisation. Polyploidy, although rare in animals, has been found in most major groups that possess an asexual mode of reproduction (reviewed by Suomalainen *et al* 1987; Beaton and Hebert 1988; Walsh and Zhang 1992). The general increase of cellular DNA content with organismal complexity, the existence of many duplicated gene loci and the evolution of salmonid and catostomid fishes by tetraploidisation have led Ohno (1970) and other authors (Allendorf and Thorgaard 1985, Ferris 1985) to suggest that polyploidy may have played a role in animal evolution. However, the scope and significance of this role remain unclear. To understand the adaptive significance of polyploidy in animals, we need more information on how polyploidy affects organismal physiological, life history and genetic characteristics. Each of these characteristics may in turn influence geographic distributions.

This dissertation is an investigation of the adaptive structure of sympatric diploid and polyploid brine shrimp Artemia parthenogenetica (Branchiopoda, Anostraca) collected from the coastal salterns of China, Italy and Spain. In Chapter 1, I examine effects of polyploidy on tolerance of temperature changes of Artemia and discuss the ecological implications of my results. In Chapter 2, I investigate effects of polyploidy on life history characteristics, and on norms of reactions to environmental changes of Artemia. In Chapter 3, I examine the genetic consequences of polyploidy in Artemia, and its effects on spatial and temporal distributions of allozyme variation in asexual populations. In Chapter 4, I relate my results from Chapters 1, 2 and 3 to the geographic distributions of diploid and polyploid Artemia and their environmental conditions in the Old World. I also consider potential explanations for these distributional patterns, and try to explore the adaptive significance of polyploidy in Artemia.

Polyploidy is associated with an increased ability to withstand stressful temperatures in Artemia. In Chapter 5, I report an initial investigation of the biochemical basis of this association.

Chapter 1. The effect of polyploidy on the tolerance of temperature changes of *Artemia* and its ecological implications

ABSTRACT

The responses to temperature changes of sympatric diploid and polyploid brine shrimp *Artemia parthenogenetica* from China, Italy and Spain were studied. In all three populations, polyploids have a higher tolerance of both cold stress (0°C) and heat stress (37.5°C). Sympatric diploids and pentaploids from China were selected for further study of their thermal behavior in a dark temperature gradient ranging from 12.5 to 35.1°C. Diploids generally selected temperatures above 19°C while pentaploids were more evenly distributed along the gradient. My study provides the first experimental evidence of differential temperature utilization by conspecific sympatric diploid and polyploid organisms. The ecological implications of the physiological and behavioral differences between diploids and pentaploids are discussed as they relate to the interactions of the two sympatric cytotypes in China coastal salterns.

INTRODUCTION

It has been recognized in many plant species and several animal species that polyploids tend to be found at high latitudes, high altitudes or at the boundary of a species' distribution when compared with diploid relatives (Berzychudek, 1985; Suomalainen et al., 1987; Beaton and Hebert, 1988). Most authors attribute this distribution pattern to the greater tolerance of stressful temperatures of polyploids than diploids (reviewed by Suomalainen et al., 1987). This hypothesis is based largely on the observations that polyploids tend to be found in "harsh temperature" environments. However, the evidence supporting this hypothesis is tenuous. First, the existing distribution patterns of diploids and polyploids do not necessarily indicates that the more northern distributions of polyploids are due to a direct temperature adaptation. Second, since most comparisons involve geographically isolated populations, the variability in tolerance may reflect unique environmental differences to which the populations have adapted. Few comparisons have been made of physiological differences between sympatric diploid and polyploid populations. The best studied case is that of the unisexual fish Poeciliopsis monacha-lucida

(Schultz, 1982). In this system, compared with sympatric diploids, triploids have a higher tolerance of cold stress, but a lower tolerance of high temperature stress. Furthermore, it is also unclear whether the more northern distributions of polyploids, in relation to diploids, result from different temperature requirements of the two cytotypes. In order to understand the role of polyploidy in animal evolution, it is essential to know both if, and how, polyploids are preadapted to stressful temperatures when compared to sympatric conspecific diploids.

Previous studies of temperature optima of aquatic invertebrates have concentrated on examining life history characteristics under a series of varying temperatures (King, 1972; Vanhaecke et al., 1984; Browne et al., 1988). However, the physiologically optimal temperatures of an ectotherm are not necessarily ecologically optimum temperatures (Huey and Slatkin, 1976; Huey and Bennett, 1987). That is, optimal levels for growth and reproduction may differ from temperatures chosen by the animal when confronted with a variety of possible choices. One approach to studying the selected temperatures of ectotherms is to permit them to choose their locations in an environment with a gradient of temperatures.

In this chapter, first I examine tolerance of stressful temperatures of sympatric diploid and polyploid brine shrimp Artemia parthenogenetica from China, Italy and Spain. Second, I investigate the behavior of Chinese diploids and pentaploids using an aquatic thermal gradient ranging from 12.5°C to 35.1°C to examine if sympatric diploid and polyploid Artemia have different temperature requirements. A starvation test is also conducted on Chinese diploids and pentaploids to determine if the two cytotypes are different in tolerance of food shortage. The results are discussed in relation to the potential interactions of sympatric diploid and pentaploid Artemia in China coastal salterns.

Brine shrimp of the genus Artemia (Branchiopoda, Anostraca) occur on every continent except Antarctica (Browne and Macdonald, 1982). Some species reproduce sexually, others by obligate parthenogenesis. Cyclic parthenogenesis has never been found. Members of the genus Artemia are usually the dominant animals in inland salt lakes and coastal solar salt works where high salinity excludes most predators (Browne, 1988). In the Old World, the genus Artemia consists of a number of sibling species that can be divided into sexual diploid groups (A. tunisiana and A. urmiana) and obligate parthenogenetic species (A. parthenogenetica)

composed of both diploid and polyploid individuals (Browne and Bowen, 1990; Lenz and Browne, 1990). Polyploidy has only been found in parthenogenetic Artemia. In the western hemisphere, Artemia reproduce solely by sexual reproduction, while in the Old World, A. parthenogenetica is the most common form and sexual forms are found in relatively fewer habitats (Browne and Macdonald, 1982). Parthenogenetic forms and sexual forms, as well as individuals of different ploidy levels, have been reported to co-occur in many Artemia populations. Based on an extensive allozyme survey of sexual and asexual Artemia from all over the world, Abreu-Grobois and Beardmore (1982) suggested that asexual Artemia of different ploidy levels have a monophyletic origin: asexual polyploid Artemia evolved from asexual diploid Artemia which themselves branched from ancestral sexual diploid A. tunisiana. This suggestion has been further supported by mtDNA analysis of Artemia from all over the world (Browne and Bowen 1991).

MATERIALS AND METHODS

Origin of the populations

Artemia are the predominant animals in coastal salterns where high salinity excludes most predators. A saltern is a series of interconnected artificial ponds having a sea water input. When the salinity in one pond increases to a certain level due to evaporation, the sea water is allowed to flow into the next pond with higher salinity. This transfer creates a salinity gradient between the input ponds and the terminal ponds where commercial sea salt is produced when the sea water becomes oversaturated. Artemia are usually found in ponds having salinities higher than 50 ppt (parts per thousand).

Artemia are capable of producing either active nauplii (ovoviviparous broods) or durable cysts (stress-resistant oviparous broods), depending upon genotypic features and environmental conditions (Gajardo and Beardmore 1989; Tackaert and Sorgeloos 1991). Individuals may produce a succession of broods throughout their life.

We collected Artemia cysts from a salt pond in the Dong Fang Hong Saltern of the Shandong Peninsula, China (King et al. 1988). In addition, Dr. Patrick Sorgeloos

of the Artemia Reference Center in Gent, Belgium, provided us with cysts that were collected from coastal salt ponds on the Adriatic Sea at Margherita di Savoia, Italy and from San Lucar on the Mediterranean Sea in southern Spain. The Spanish population is a mixture of sexual (equivalent to A. tunisiana, Bowen and Sterling 1978) and asexual individuals (A. parthenogenetica). Sexual and asexual forms were separated by noting morphological differences.

The ploidy level of a given adult female was determined by examining the chromosome numbers of its nauplii following the method of Appendix 1.

Morphological differences may also be used to separate diploid and polyploid Artemia (Amat 1980), however more precise characterization of the polyploids requires cytological analysis. Chromosome counting reveals that the Chinese population is composed of diploids and pentaploids; the Italian and the Spanish populations are each composed of diploids and tetraploids. Chromosome examinations of approximately 100 nauplii from each population indicated that different populations have different frequencies of polyploids. The proportions of polyploids in the cysts for Chinese and Italian populations are 10% and 62%, respectively. Polyploids make up about 66% of the asexual cysts in the Spanish population, sexual forms

make up about 44% of the cysts in this population. These results are included in table 3-5 in Chapter 3.

Tolerance of stressful temperatures (0°C and 37.5°C) of polyploids versus sympatric diploids in the three populations collected from China, Italy and Spain.

To examine survival rates of diploids and polyploids under stressful low (0°C) and high (37.5°C) temperatures, both newly hatched nauplii and 20-day old juveniles were tested. For each ploidy in each of the three populations, nauplii were produced and isolated in a mass culture of about 700 40-day old adults that had been cultured at 25°C and 35 ppt. These nauplii were divided into two groups. One group was directly used to examine survival rates of nauplii at stressful temperatures. The other group was cultured to 20-day old and then used to examine survival rates of juveniles at stressful temperatures. To determine survival rates at each temperature treatment, five replicates of 20 animals were used for each ploidy. For tests on nauplii, the experiment lasted for 48 hours for low temperature treatment and 12 hours for high temperature treatment. For tests on juveniles, the experiment lasted for 24 hours for low temperature treatment, and 12 hours for high temperature treatment.

Survival rate was calculated for each replicate and One-way ANOVA was used to compare difference between sympatric diploids and polyploids.

Thermal distributions of Chinese sympatric diploids and pentaploids

To determine if sympatric diploid and polyploid Artemia have different responses when provided with an environment with a gradient of temperatures, I examined the thermal distributions of Chinese diploids and pentaploids along a thermal gradient. This work was done in collaboration with Hugh Lefcort (Zhang and Lefcort 1991).

Three diploid clones and three pentaploid clones of different genotypes (as detected by celluose acetate gel electrophoresis) were isolated. These clones were maintained at a temperature of 25 $\pm$ 0.5°C in natural sea water with a salinity of approximately 35 ppt under 24-hr cool white fluorescent lighting at an intensity of 4,000-5,000 lux. The unicellular green alga Dunaliella tertiolecta was grown on 2X F medium at 25 $\pm$ 0.5°C and used as food. The Dunaliella were raised under a light intensity of 8,000-10,000 lux. Food was added daily at a concentration of approximately

17,090,000 cells/ml. This culture condition was used throughout my study unless specified.

To determine the thermal responses of diploids and pentaploids, equal numbers of 15-day old juveniles from each of three diploid clones were mixed to obtain diploid mixtures. Pentaploid mixtures were obtained in a similar manner. The animals were placed in a grey epoxy-painted wooden container. The container measured 273.0 cm x 27.0 cm x 46.0 cm and was filled with natural sea water to a depth of 1.5 cm. The warm end of the thermal gradient was created by placing a 0.5 l beaker of water containing two 200W immersion coils at one end. The cold end was created by immersing a cooling coil at the opposite end. Both the hot and cold elements were separated from the animals by closely spaced layers of 6 sq./cm plastic mesh placed 19 cm from the container ends. The length of the chamber available to the animals was therefore 234.0 cm. Temperatures, to the nearest tenth of a degree, were recorded at ten zones spaced 23.4 cm apart along the midline of the chamber. No vertical stratification of temperature was observed. Temperatures ranged from an average of 12.1 $\pm$ 1.1°C in zone 1 to an average of 35.1 $\pm$ 1.6°C in zone 10. The temperature of each zone varied by less than 2°C between trials. Once the gradient had been established, groups of five diploid

and five pentaploid animals from the mixtures were added to each of the ten zones. To avoid the confounding effect of light all tests were conducted in darkness and at a constant air temperature of 21.1 °C. Preliminary results indicated that the distribution of the animals after one hour in the presence of the gradient did not statistically vary from distributions after two and four hours. Therefore, at the end of one hour, barriers were placed between the zones and the number of animals of each cytotype present, as well as the temperature of each zone were recorded (diploids and pentaploids are morphologically distinguishable). All animals within a zone were assigned the same temperature. Temperatures were recorded using a K type thermocouple connected to an Omega HH 82 digital thermometer. Five trials of fifty diploids and fifty pentaploids each were run. Each trial involved different animals.

Determination of effects of semi-starvation on the survival of Chinese sympatric diploids and pentaploids

Food availability is often one of the limiting factors when animal population size approaches the environment's carrying capacity. This is especially the case during the summer, optimal growth season for

Artemia in the coastal salterns (reviewed by Tackaert and Sorgeloos 1991). The cytotype that has higher tolerance of low food resource conditions may have advantages during the middle of the population growth season. Such information is necessary in the understanding of potential interactions of sympatric diploid and polyploid Artemia.

To examine tolerance of Chinese diploids and pentaploids to low food resource condition , I examined their survival rates by culturing them under semi-starvation conditions in 135 um-filtered sea water at 25°C. No food was added to these cultures. Individuals at three developmental stages were tested: same age (neonatal nauplii), same size (5 mm juveniles) and same developmental stage (young adults with egg bags just visible). Three replicates of 20 individuals each were used for each clone. Survival was recorded at daily intervals until all of the individuals had died.

RESULTS

Tolerance of stressful temperatures

Fig 1-1 (a, b, c and d) show that in all three populations, for both nauplii and juveniles, polyploids

have significantly higher survival rates than sympatric diploids when exposed to stressful low and high temperatures (one-way ANOVA, $P < 0.01$). This also holds when comparisons were made between allopatric diploid and polyploids (one-way ANOVA, $P<0.01$).

Distributions in the temperature gradient

Trials using control animals in the chamber without a gradient at a constant temperature of 21.1°C indicated a preference of both cytotypes for the ends of the chamber. No preference was noticed for one end over the other (diploid $\chi^2 = 0.802$, $P = 0.370$, pentaploid $\chi^2 = 0.247$, $P = 0.619$). χ^2 values for all five trials showed a significant difference in the distribution of diploids when compared to polyploids in the presence of a temperature gradient ($\chi^2 > 18.23$ with $P < 0.05$ in all replicates). Fig.1-2a indicates the average frequencies of diploids and pentaploids in each zone along the gradient. The variance of the mean temperature of the pentaploids (variance = 46.24) was broader than that of the diploids (variance = 36.19), suggesting that pentaploids were more evenly distributed along the thermal gradient than diploids. Since both cytotypes had been cultured for more than 30 generations at a constant temperature of 25 $\pm$ 0.5°C,

acclimation is an unlikely cause of the different temperature distributions.

Fig.1-2b indicates the percentage of total animals after one hour in zones with temperature below 17°C (non-optimal growth temperatures) compared to controls (animals tested at a water temperature of 21.1°C in all zones). The majority of the diploids left low temperature zones (i.e., zone 1-3) and moved to warmer zones, while a greater percentage of the pentaploids remained in low temperature zones (paired t-test, $P = 0.003$; Bonferroni adjusted $P = 0.009$).

Effects of semi-starvation on the survival of Chinese diploids and pentaploids

At 25°C, under semi-starvation conditions, diploid clones show significantly higher survival rates than pentaploid clones (OW-ANOVA, $p < 0.01$). This is true for same age (Fig. 1-3a), same size (Fig. 1-3b) and same developmental stage comparisons (Fig. 1-3c). Except for two diploid individuals from the same developmental stage comparison, all of the experimental animals died before reaching reproductive maturity. The two diploid individuals surviving to maturity produced 15 and 45 cysts respectively. These results

indicate that diploids were less stressed than pentaploids under semi-starvation conditions.

DISCUSSION

Thermoregulatory behavior may influence both an organism's fitness (Orcutt and Porter, 1983) and the distribution of a species (Vinogradov, 1970). The diurnal vertical migration of zooplankton may confer protection from "visual" predators, preservation and widening of the distribution range, and gene flow between groups of individuals at different water layers (Vinogradov, 1970). Thermoclines have been found to play an important role in limiting the dimensions of an animal's vertical migration and dispersal range (reviewed by Vinogradov, 1970). Animals that have a wider temperature range may have greater dispersal abilities and be able to exploit a greater variety of resources than animals with a narrow temperature optimum.

My results show that polyploid Artemia are more tolerant of both low and high temperature stress than conspecific sympatric diploids. When diploids and pentaploids were placed in the thermal gradient, after one hour, the majority of diploids left low temperature zones (below 17°C) and moved to warmer zones, while

pentaploids were relatively more evenly distributed along the thermal gradient. These results suggest that pentaploids are more robusted to temperature changes than sympatric diploids. This would afford pentaploid Artemia a high potential to exploit a wider range of temperature microhabitats. This difference of diploids and pentaploids may have ecological implications.

In the salterns of northeast China (between latitudes of 35-41°N), diploids are the most common form, making up an average of 85% in the cyst population (Wang, 1986). Pentaploids may be disadvantaged during the middle of the Artemia population growth season when intraspecific competition is important, because they have a lower intrinsic rate of increase (table 2-1 in Chapter 2) and lower tolerance of low food resource conditions (fig 1-3) when compared to sympatric diploids. Contrarily, the robustness of pentaploids to temperature changes may afford them an ability to exploit a wider range of temperature microhabitats than diploids, spatially or temporally, thereby reducing intraspecific competition.

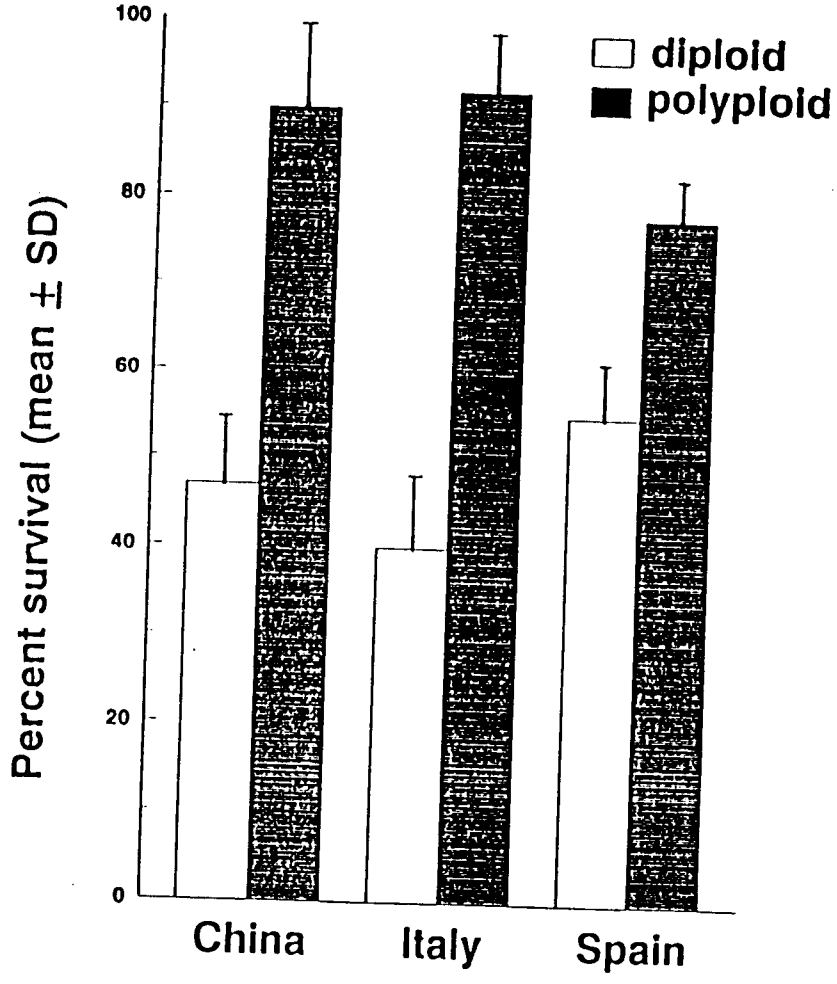

Fig 1-1a. Percent survival of nauplii after cold shock (0°C) for 48 hours.

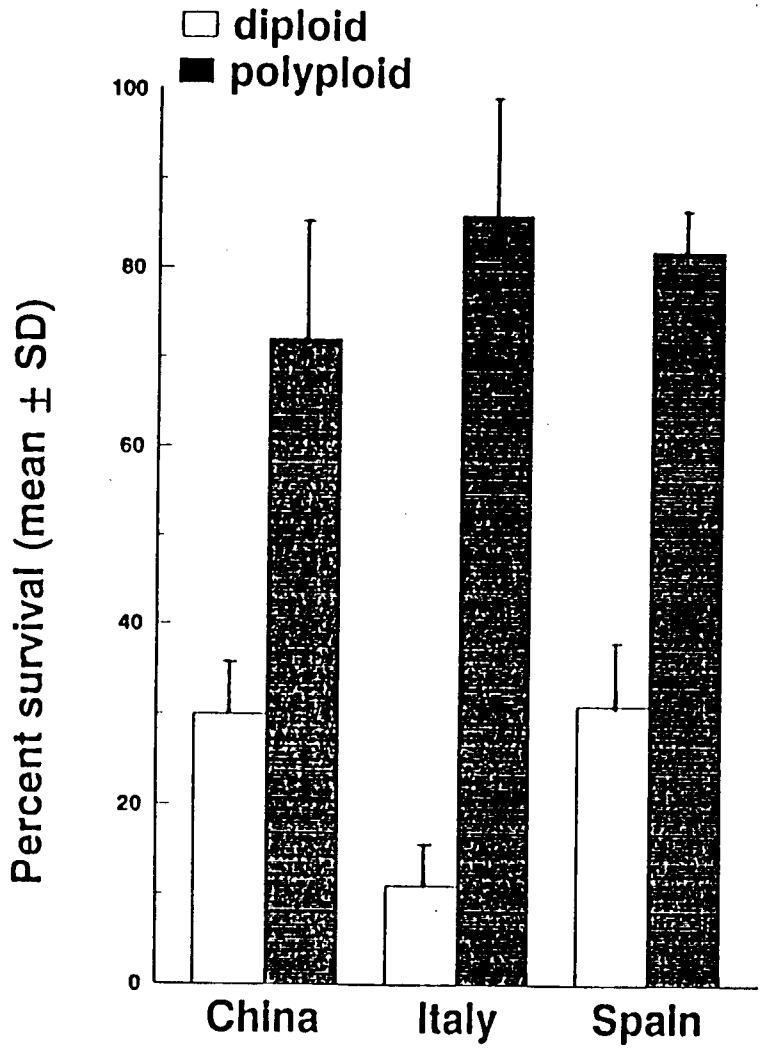

Fig 1-1b. Percent survival of nauplii after heat shock (37°C) for 12 hours.

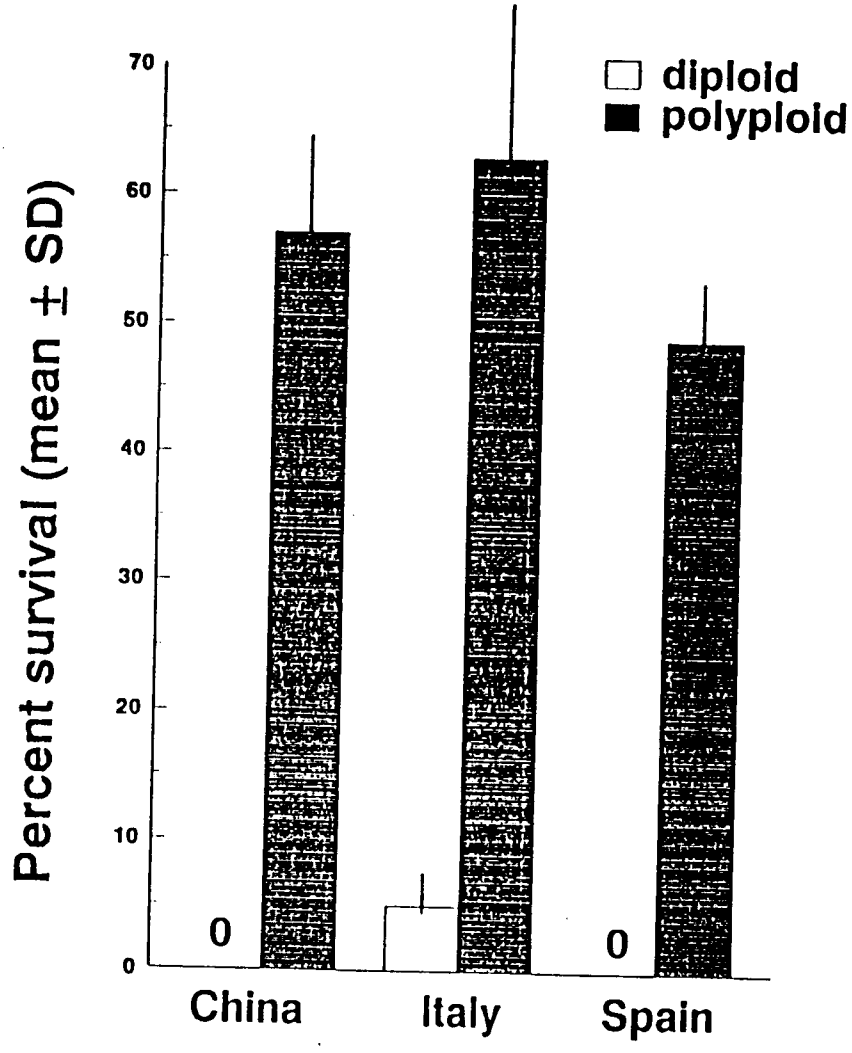

Fig 1-1c. Percent survival of 20-day old juveniles after cold shock (0°C) for 24 hours.

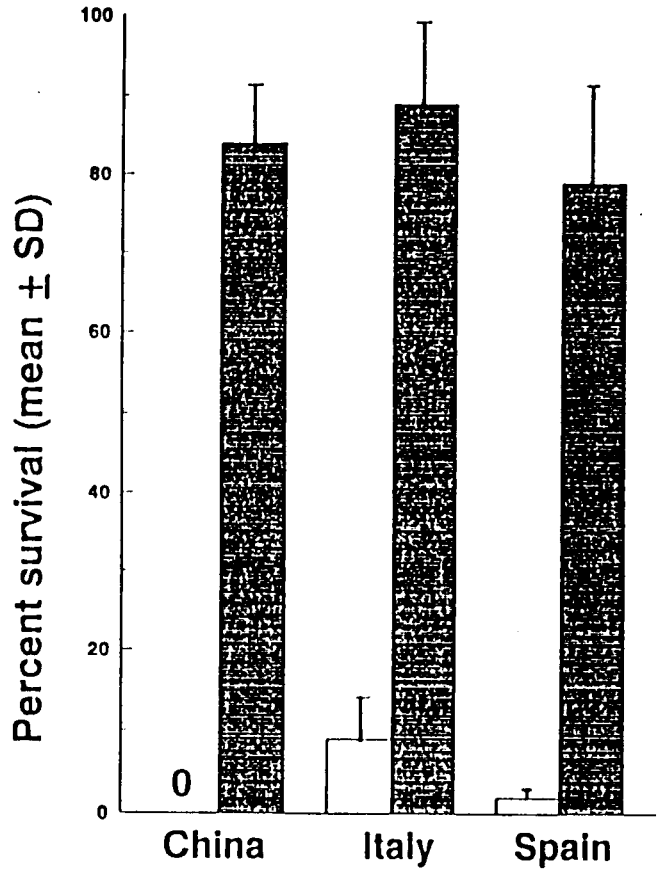

Fig 1-1d. Percent survival of 20-day old juveniles after heat shock (37°C) for 12 hours.

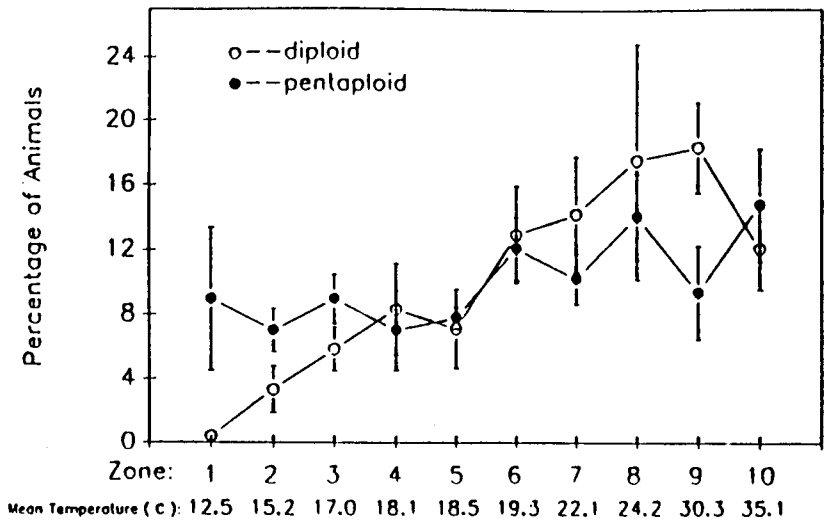

Fig 1-2a. Percentage (± SE) of diploids and pentaploids in each zone along a thermal gradient.

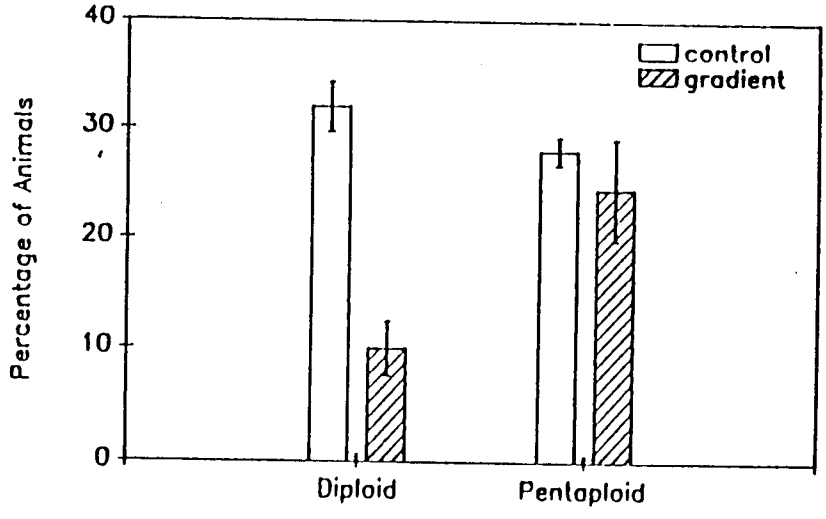

Fig 1-2b. Percentage (± SE) of total animals in zones with temperatures below 17°C, compared to controls in these zones.

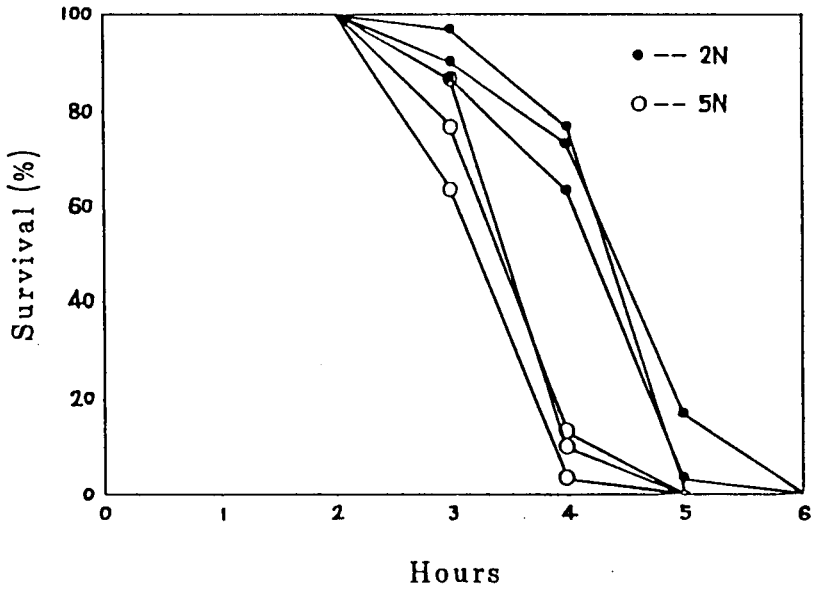

Fig 1-3a. Survival (%) of the individuals in each clone under semi-starvation conditions. Animals are at the same age (neonatal nauplii).

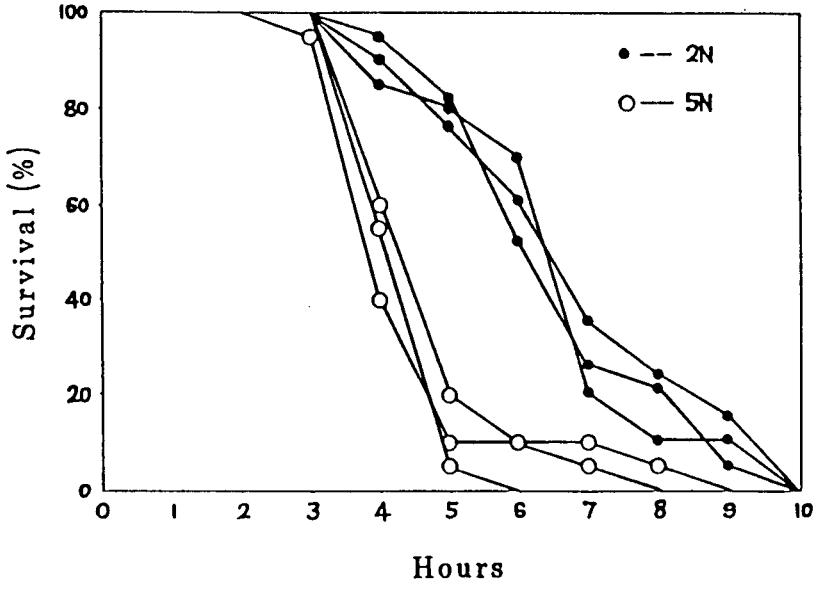

Fig 1-3b. Survival (%) of the individuals in each clone under semi-starvation conditions. Animals are at the same size (5 mm juveniles).

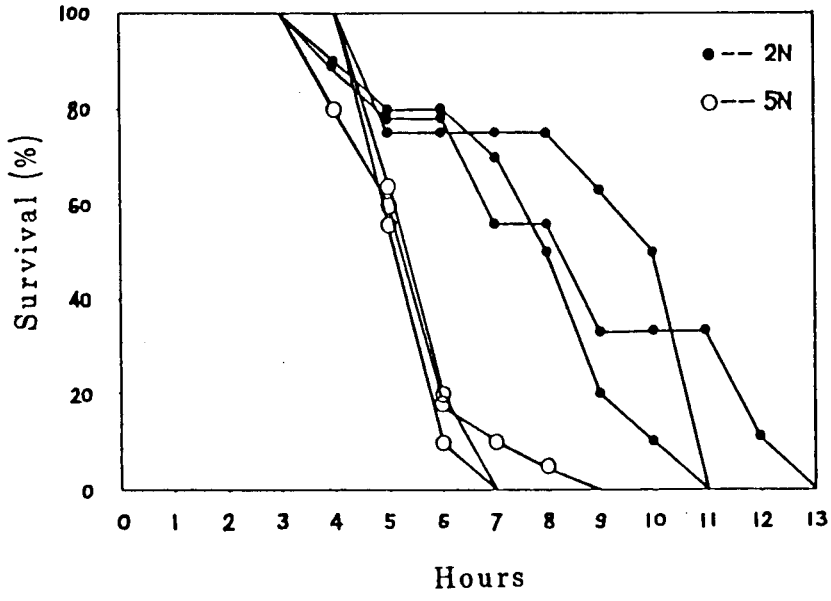

Fig 1-3c. Survival (%) of the individuals in each clone under semi-starvation conditions. Animals are at the same developmental stage (young adults whose egg bags are just visible).

Chapter 2. Effects of polyploidy on life history characteristics

ABSTRACT

In order to study how polyploidy affects life history patterns in animals, I have examined sympatric diploid and polyploid brine shrimp (Artemia parthenogenetica) from China, Italy and Spain under laboratory conditions. At optimal temperature and commonly used salinity (25°C and 90 ppt), diploids from the three populations had much higher intrinsic rates of increase, higher fecundity, faster developmental rates, and larger brood sizes than their sympatric polyploids.

The Chinese and Italian populations were selected for further analysis to determine the life history responses of diploids and polyploids to temperature and salinity changes. Under intermediate and high salinities (90 ppt and 180 ppt), Chinese and Italian polyploids produced most of their offspring as dormant cysts while their sympatric diploids produced most of their offspring as nauplii. This relationship is reversed in the Spanish diploid-polyploid complex.

For the Chinese population at 25°C, pentaploid clones had higher developmental rates than diploid clones at 35 ppt; at 90 ppt, diploid clones had higher

developmental rates than the pentaploids. Italian diploids and tetraploids had different responses to variation in both temperature (25°C and 31°C) and salinity (30 ppt and 180 ppt): diploids have better reproductive performance than polyploids at high salinity even at a high temperature of 31°C; tetraploids have better reproductive performance than sympatric diploids at low salinity and high temperature.

My results demonstrate that relative fitness of the two cytotypes is a function of environmental conditions and that sympatric diploids and polyploids respond differently to environmental changes. Chinese and Italian polyploids are expected to have lower fitness than their sympatric diploids when the physical environment is not stressful and when intraspecific competition is important. However, polyploids may have advantages over sympatric diploids in stressful habitats or when they encounter short-term lethal temperatures. These results suggest that polyploid Artemia have evolved a suite of life-history characteristics adapting them to environments that contrast to those of their sympatric diploids.

INTRODUCTION

Our current knowledge about the effects of polyploidy on life history characteristics is primarily obtained from studies of higher plants in which polyploidy has been viewed as a major evolutionary force (reviewed by Levin 1983). Demographically, autopolyploid plants have lower developmental rates, delayed reproduction, larger sizes at maturity, longer life spans and reduced fecundities when compared with closely related diploids (Lewis 1980, Van Dijk and Van Delden 1990). Exceptions have also been reported. For example, Lumaret *et al.* (1987) found that habitat differentiation occurred between diploids and polyploids in the plant *Dactylis glomerata*; an *in situ* comparison of plant performance showed that where plants of each cytotype were more common, they also had higher fecundity.

Relatively few studies have been conducted with animals and contradictory findings have been reported. For example, tetraploid *Daphnia pulex* have lower developmental rates and smaller broods than their sympatric diploids when measured at 25°C (Weider 1987). These diploids and tetraploids use different microhabitats in nature (Hebert and Emery 1990). In contrast to what is generally found in plants, Browne *et al* (1984) reported that three polyploid *Artemia*

parthenogenetica populations from India (Madras and Kutch) and Turkey have larger brood sizes and earlier reproduction than two diploid populations from Spain and France under optimal temperature and salinity (25°C, 90 ppt). At a stressful low temperature (15°C), Madras triploids retain a larger brood size; however, at 30°C, the brood size of triploids is larger than Spanish diploids, but smaller than French diploids (Browne et al 1988). These results suggest that comparisons of the life history patterns of diploids and polyploids are strongly influenced by the environmental conditions used in the study.

In this chapter, I compare life history patterns of sympatric diploid and polyploid A. parthenogenetica in three populations collected from China, Italy and Spain. To examine how diploids and polyploids respond to environmental changes, the Chinese and Italian populations are selected for further analysis at different salinities and temperatures.

MATERIALS AND METHODS

Life history determinations of diploids and polyploids under optimal condition of 25°C and 90 ppt salinity.

To determine how polyploidy affects life history traits under optimal temperature and salinity, I examined life history patterns of sympatric diploids and polyploids from the three populations at 25 ± 0.5°C and 90 ppt salinity (sea water at 35 ppt + 55 g NaCl/l). Reproductive output of *Artemia* has been found to be optimal in this medium (Browne *et al.* 1984). Animals were maintained under 24-hr cool-white fluorescent lighting at an intensity of 4,000-5,000 lux. A unicellular green alga, *Dunaliella tertiolecta*, was grown on 2X F medium at 25 ± 0.5°C and used as food.

Nauplii hatched from cysts on the same day were mass-cultured at low density and then separated to start clonal lines as soon as females showed signs of ovarian development. The ploidy level of each clone was determined by examining the chromosome numbers of its offspring. Approximately 20 females were isolated and maintained for each cytotype in each of the three populations (Table 2-1). Second generation individuals were used for the experiments to reduce maternal effects. Individuals were maintained in 150 ml jars

containing 100 ml 90 ppt brine. The medium of each culture was changed daily. After the experimental animals reached reproductive maturity, their offspring were counted and removed from the jars at daily intervals. Numbers of nauplii and diapause cysts produced by each clone were separately recorded. In my study, a "diapause cyst" is defined as an oviparous offspring having a clear, coffee-colored shell which does not hatch in 90 ppt brine for at least one week. Each individual culture was maintained until the parental animal had died. One-way analysis of variance (ANOVA) was used to analyze life history variation within and between cytotypes in each population.

The relative fitness of diploids and polyploids under each treatment was measured by calculating their intrinsic rates of increase (r) following the procedures of Birch (1948). Only nauplii were used for calculating $\underline{r}$ because cysts require a diapause and do not immediately contribute to growth of the population. The reproductive contribution to $\underline{r}$ of individuals that produced only cysts was therefore regarded as zero.

Life history determinations of Chinese diploids and pentaploids at a suboptimal salinity of 35 ppt

To determine whether life history patterns measured at 90 ppt (which is common in Artemia's habitats) are similar to those at the suboptimal low salinity of 35 ppt (which is generally regarded as rare in Artemia's habitats), I examined life history patterns of three diploid and three pentaploid multilocus genotypes from China. The multilocus genotypes were identified using cellulose acetate gel electrophoresis and will hereafter be referred to as "clones." Three replicates of 5 newly-hatched nauplii were used for each of the six clones. To examine life history patterns after shifting the salinity from 35 ppt to 90 ppt, I transferred newly-hatched nauplii produced in 35 ppt sea water to 90 ppt brine. These nauplii were cultured to maturity and, to eliminate or reduce maternal effects, their first generation offspring were used for the life history determinations. One-way ANOVA was used to compare the life history variation between cytotypes.

Reproductive performance of Italian diploids and tetraploids under various salinities (30 ppt and 180 ppt) and temperatures (25°C and 31°C).

To compare the responses of diploids and polyploids to suboptimal salinities and temperatures, I focused on the survivorship, total fecundity and percentage of cyst production of Italian diploids and polyploids. Nauplii that were hatched from cysts on the same day were mass-cultured at 90 ppt. At an age of 20 days, which is about two-thirds of the prereproductive period (Table 2-1), diploid and polyploid juveniles (which are morphologically distinguishable) were removed from mass culture and subjected to four treatments (25°C, 30 ppt; 25°C, 180 ppt; 31°C, 30 ppt; 31°C, 180 ppt). About 50 juveniles of each cytotype were used for each treatment. The culture medium was refreshed daily and more than 80% of these juveniles were able to develop to maturity under each treatment. After the animals reached reproductive maturity, ten individuals of each cytotype from each of the four treatments were chosen haphazardly and used to measure fecundity. Numbers of nauplii and cysts produced by each individual were recorded at three-day intervals.

To compare the survivorships of diploids and polyploids under the four treatments, newly-hatched

nauplii were used. Since most Italian tetraploids produced cysts in their first three broods at 90 ppt, I had to induce nauplii production by transferring tetraploids to low salinity medium of 30 ppt. About 700 40-day old adults of each cytotype that had been cultured at 90 ppt and 25°C were isolated and transferred to 30 ppt. At this salinity, Italian tetraploids produce most of their offspring as nauplii (Table 2-5). Five replicates of 100 nauplii from the 30ppt-adult mixture were used for each treatment. Since most non-senescent mortality occurs in the first nine days of adult life (Vanhaecke et al. 1984), survivorship was measured as the percentage of animals alive at day 20. Responses of the two cytotypes were analyzed using both two-way and three-way ANOVA.

RESULTS

Life history patterns of sympatric diploids and polyploids under optimal temperature and salinity.

In this section, I compare the life history patterns of sympatric diploids and polyploids from the three populations under optimal conditions of 25°C and 90 ppt. In all three populations, diploids had significantly higher intrinsic rates of increase,

higher net fecundities (total number of offspring per female), larger brood sizes and faster developmental rates than sympatric polyploids (Table 2-1, $P < 0.01$). Cytotypes with longer mean life spans also produced more broods. For example, Chinese diploids had significantly longer life spans and produced more broods than sympatric pentaploids. An opposite pattern was observed in the Spanish population where tetraploids had longer life spans and more broods than diploids ($P < 0.01$, Table 2-1). Italian diploids and tetraploids had similar life spans and number of broods per female. The frequency of encysted offspring was measured by calculating the ratio of the number of cysts per female to the total number of offspring (including cysts) per female. Chinese and Italian polyploids produced significantly higher percentages of encysted offspring than their sympatric diploids, while an opposite pattern was observed in the Spanish population where tetraploids never produced any cysts ($P<0.01$, Table 2-1).

To determine if the observed life history patterns at 90 ppt would be altered by a suboptimal salinity of 35 ppt, I looked at life history patterns of three diploid and three pentaploid clones of Chinese population at both salinities. I found that Chinese diploid clones still had significantly higher

fecundities, larger brood sizes, longer life spans, and lower frequencies of cyst production at 35 ppt than the pentaploid clones ($P < 0.01$, Table 2-2). At 35 ppt, although both cytotypes had similar intrinsic rates of increase, the pentaploids had significantly faster developmental rates than the diploids (Table 2-2). Moreover, the three pentaploids clones that produced all of their offspring as cysts at 90 ppt produced most of their offspring as nauplii at low salinity (Table 2-2).

Responses of Italian diploids and tetraploids to suboptimal salinity and temperature

To evaluate the effects of environmental stress, I focused on the Italian diploids and tetraploids and examined their responses to temperature (25°C and 31°C) and salinity (30 ppt and 180 ppt) variation. All three factors (ploidy level, temperature and salinity) had significant main effects and interactions on survivorship and reproduction (Table 2-3).

At a low salinity of 30 ppt, tetraploids had significantly higher survivorship than diploids under both optimal (25°C) and high (31°C) temperatures (one-way ANOVA, $P< 0.01$, Fig. 2-1a). At a high salinity of 180 ppt, diploids had significantly higher survivorship

than tetraploids at both 25°C and 31°C ($P < 0.01$, one-way ANOVA, Fig. 2-1a). The interaction of salinity and temperature also had a significant influence on survivorship (Table 2-4).

At 30 ppt, although tetraploids had significantly lower fecundity than diploids at 25°C, they had significantly higher fecundity than diploids at 31°C ($P < 0.01$ in both cases by one-way ANOVA, Fig. 2-1b). At 180 ppt, diploids had higher fecundities than tetraploids at both 25°C and 31°C ($P<0.01$, one-way ANOVA, Fig. 2-1b). The interactive effects of salinity and temperature on fecundity were significant for tetraploids, but not for diploids (Table 4). Salinity level had significant effects on the fecundity of tetraploids, but not on diploids (Table 2-4). For example, at 31°C, with a change in salinity from 30 ppt to 180 ppt the fecundity of tetraploids was reduced by 84.4% while that of diploids was only reduced by 5.6% (Fig. 2-1b).

Tetraploids had higher frequencies of cyst production than diploids at both temperatures and salinities tested ($P <0.01$, one-way ANOVA, Fig. 2-1c). They produced most of their offspring as cysts at high salinity, and most as nauplii at low salinity. Diploids had low frequencies of cyst production under a wide range of salinities (Table 2-5). Only at high

salinity and high temperature did the frequency of cyst production increase (Fig. 2-1c). The frequency of cyst production in tetraploids was significantly affected by salinity changes, but not by temperature changes. Diploids showed an opposite pattern (Table 2-4).

Effects of salinity level on the frequency of cyst production of diploids and polyploids in the three populations

Since Artemia cysts are very stress-resistant, frequency of cyst production may be an important component of relative fitness. Table 2-5 presents a summary of the frequencies of cyst production in the three populations at optimal temperature (25°C) and different salinities (30 ppt, 90 ppt and 180 ppt). Chinese and Italian populations had very similar responses to salinity changes. For polyploids, the percentage of cyst production increased with salinity while diploids in the two populations produced most of their offspring as nauplii irrespective of salinity. Spanish diploids and tetraploids showed an opposite pattern; diploids produced most of their offspring as cysts at intermediate and high salinities while tetraploids produced all of their offspring as nauplii at all salinities tested.

DISCUSSION

Effects of polyploidy on life history patterns

It is known that polyploidization within an asexual lineage is associated with an increase in heterozygosity (reviewed by Suomalainen et al 1987). This is also the case in Artemia: polyploids have higher levels of heterozygosity than diploids (Abreu-Grobois and Beardmore 1982; Chapter 3 in this thesis). In addition, genetic divergence has also occurred following the polyploidisation in Artemia; at some loci sympatric diploids and polyploids in my study have different alleles (Chapter 3). Life history differences between diploids and polyploids may therefore have their origin in allelic divergence and increased heterozygosity as well as in the increased DNA content.

It has been suggested that there is a strong inverse correlation between DNA content and developmental rate in eucaryotes (reviewed by Levin, 1983, Cavaller-Smith 1985). In animals, most of the evidence for this inverse correlation is from observations made under laboratory conditions that usually favor diploids (Goin et al. 1968; Weider 1987). My results from 25°C, 90 ppt salinity (which

are common levels in Artemia's habitats) support this generalization (table 2-1). However, when cultured in 35 ppt sea water (which is rare in Artemia's habitats) at 25°C, the three pentaploid clones from China have faster developmental rates than their three sympatric diploid clones (table 2-2). Browne et al. (1984) also reported higher developmental rates for Indian polyploid Artemia than Spanish and French diploid Artemia at 25°C and 90 ppt salinity. Thus while ploidy level may have a strong effect on developmental rates, the expression of this effect is greatly influenced by environmental conditions.

A second generalization, based on studies of many plant species and a smaller number of animals (Dewey 1980, Schultz 1980, Weider 1987), is that polyploids have larger body size at maturity and reduced fecundities. My measures at 25°C, 90 ppt support these studies (table 2-1); diploids had smaller brood sizes and higher fecundity than their sympatric polyploids. Polyploids have larger cyst sizes and body size at maturity than diploids (Zhang and King, unpub. data; Amat 1980; Wang et al. 1990). However, at low salinity of 30 ppt and high temperature of 31°C , Italian tetraploids have significantly higher fecundity than sympatric diploids (fig 2-1b). Thus the relationship between fecundity rates and cytotype may

differ with both population and environment. Similar conclusions may be drawn from the relationship I observed between life span and cytotype. For example, Chinese and Italian polyploids had shorter life spans while Spanish tetraploids had a longer life span than their sympatric diploids (table 2-1). In agreement with the work on allopatric diploid and polyploid populations (Browne et al. 1988), my results further demonstrate that the comparisons of life history traits between diploids and polyploids can be altered by the environmental conditions tested.

Nevertheless, my results show that at salinities that are common in Artemia's habitats, polyploids have lower developmental rates, smaller brood sizes and lower fecundities than sympatric diploids (table 2-1). Under the same conditions diploids consistently have higher intrinsic rates of increase than sympatric polyploids.

Therefore it seems reasonable to suggest that under optimal conditions diploids generally have higher fitness. It is primarily in suboptimal environments that polyploids sometimes appear to have an advantage over diploids.

Responses of diploids and polyploids to environmental stress

A. Cyst production:

Under some circumstances cyst production in Artemia is an important component of fitness because encysted embryos are protected from many environmental stresses. Cysts may act as a "seed pool" permitting population survival in periodically stressful or unpredictable environments (Versichele and Sorgeloos 1980; Lenz and Browne 1991). However, when intraspecific competition is important, high frequencies of cyst production may be disadvantageous because Artemia cysts require a diapause (from several days to several months) and do not immediately contribute to the growth and competitive ability of the parental clone.

Different genotypes or populations may have different rates of cyst production (Browne et al. 1984; Gajardo and Beardmore 1989) and it has been reported that cyst production is usually induced by adverse conditions such as stressful salinities, temperatures, or low oxygen concentrations (Tackaert and Sorgeloos 1991). In my study, both cytotype and population had significant effects on the frequencies of cyst production. Under salinities common in coastal

salterns (>60 ppt), Chinese and Italian polyploids produced most offspring as cysts while their sympatric diploids produced most of their offspring as nauplii (Table 2-5). This relationship is reversed in Spanish tetraploids and diploids. Furthermore, cytotypes that have high frequencies of cyst production at intermediate and high salinities may shift to produce nauplii at low salinities (Table 2-5).

The different patterns of cyst production by Spanish and Italian tetraploids are particularly interesting. Measures of the genetic similarity of the two groups show that they are closely related (Nei's standard genetic identity I = 0.964, Chapter 3) even though their collection sites are rather widely separated (41° 25'N - 16° 05'E for the former, vs. 36° 43'N - 6° 23'W for the latter). As indicated in Table 2-1, tetraploids from the two populations differ in most of the life history traits examined. Moreover, Italian tetraploids produced most of their offspring as cysts while Spanish tetraploids produced all of their offspring as active nauplii (Table 2-5). Thus the life history characteristics of this cytotype appear to have undergone extensive geographical divergence.

B. Polyploidy as a buffer against environmental stress

A number of investigators have proposed that polyploids are genetically better buffered against environmental stress than closely related diploids (see, for example, Levin 1983; Suomalainen et al. 1987). This perception is based largely on the observation that polyploids tend to be found in extreme environments such as high latitudes, high elevations, or arid areas when compared with diploid relatives (Bierzychudek 1985; Suomalainen et al. 1987; Beaton and Hebert 1988; Zhang and Lefcort 1991). However, the physiological responses of diploids and polyploids to stressful conditions do not show a consistent pattern of polyploid superiority as might be expected from the geographical distributions. For example, when examined under laboratory conditions, diploid and triploid salamanders (Ambystoma laterale-texanum) have similar thermal tolerance limits, while triploid fish (Poeciliopsis monacha-lucida) have lower resistance to heat stress but higher resistance to cold stress than diploids (Schultz 1982; Licht and Bogart 1989). Tetraploid Daphnia pulex from the low arctic of Canada have lower tolerance of high temperatures than the temperate diploid populations (Macisaac et al. 1985). Moreover, at a stressful high temperature of 30°C and 90 ppt, Madras (India) triploid Artemia do not

have substantially higher fitness than Spanish and French diploids (Browne et al. 1988).

My data support the conclusion that there is a positive relationship between polyploidy and resistance to environmental stress. As mentioned earlier, Chinese pentaploids had higher developmental rates and similar intrinsic rates of increase than diploids at low salinity and optimal temperatures (table 2-2). The polyploid Artemia in my study had higher survival rates than sympatric diploids after a short exposure to cold and heat shocks irrespective of the culture salinities (Chapter 1; Zhang Lei, per. obs.). Polyploids can survive a 30-minute heat shock of 40°C, develop to maturity and reproduce while diploids can not (Zhang and King, unpub. data). Obviously, this difference may give polyploids at least short-term advantages over sympatric diploids, because most coastal salt ponds are shallower than one meter and their temperature approaches lethal levels on hot summer afternoons. However, this short-term advantage may not apply to sublethal temperatures unless the thermal stress is also combined with low salinity stress. At low salinities and high temperatures, Italian tetraploids had both higher survivorship and higher fecundity than diploids (Fig. 2-1a and 2-1b). As will be discussed in

Chapter 4, low salinities and high temperatures correspond to marginal environmental conditions.

These results indicate that polyploids have advantages over their sympatric diploids in utilizing spatially or temporally marginal environments. Note, however, that identifying stressful environments in physiological terms is a tautological process and different measures of stress response may lead to different categorizations. Furthermore, if my results are typical it may be the interaction between different environmental elements, rather than their separate effects, that produces physiological stress and decrease in fitness.

Table 2-1. Life history characteristics (mean ± SE) of diploid and polyploid *A. parthenogenetica* at 25°C, 90 ppt. V: significance level of an ANOVA comparing the two cytotypes in each population (-: P>0.01; * : P < 0.01).

Characteristic	China			Italy			Spain		
	2N	5N	V	2N	4N	V	2N	4N	V
Cytotype frequency (%)	90	10		38	62		34	66	
No. of animals tested for life history traits	19	20		20	20		20	23	
Intrinsic rate of increase	0.202 ±0.019	0.115 ±0.011	*	0.205 ±0.009	0.142 ±0.012	*	0.100 ±0.007	0.007 ±0.005	*
Total offspring per female	1744 ±166	317 ±66	*	776 ±104	418 ±63	*	1034 ±156	643 ±91	*
Offspring per brood	177 ±6	57 ±5	*	151 ±12	88 ±7	*	129 ±10	63 ±7	*
% offspring encysted	5.3 ±2.8	89.5 ±6.9	*	4.2 ±3.6	55.3 ±11.7	*	55.4 ±7.0	0 0	*
Number of broods	9.8 ±0.9	4.7 ±0.8	*	4.7 ±0.5	4.4 ±0.4	-	7.3 ±0.9	9.6 ±1.1	*
Age at first reproduction (days)	26.6 ±0.9	33.7 ±1	*	29.5 ±0.7	33.5 ±1.1	*	35.3 ±0.9	44.6 ±1.1	*
Life span (days)	102.5 ±7.1	68.4 ±5.3	*	54.9 ±2.4	60.9 ±2.9	-	89.5 ±6.0	110.8 ±8.9	*

Table 2-2. Life history characteristics (mean ± SE) of the Chinese diploids and pentaploids at 25°C under different salinities (S: ppt). Data are pooled from the three clones of each cytotype. ANOVA significance levels: -: P>0.01, * : P < 0.01.

Characteristic	S	Diploids	Pentaploids	ANOVA
Intrinsic rate of increase	35	0.205 ± 0.007	0.225 ± 0.008	-
	90	0.190 ± 0.005	0	*
Total offspring per female	35	909 ± 97	50 ± 7	*
	90	1241 ± 125	197 ± 18	*
Offspring per brood	35	157 ± 18	46 ± 18	*
	90	161 ± 29	55 ± 6	*
% offspring encysted	35	5.8 ± 1.4	38.9 ± 7.4	*
	90	5.3 ± 1.4	100	*
Age at first reproduction (days)	35	23.0 ± 0.0	17.3 ± 0.5	*
	90	29.3 ± 1.3	34.1 ± 1.6	*
Life span (days)	35	57.7 ± 9.7	23.7 ± 5.1	*
	90	85.0 ± 6.7	66.0 ± 14.1	*

Table 2-3. Results of a three-way ANOVA on the life history traits of Italian diploids and tetraploids. Factors are: ploidy level (P), salinity (S) and temperature (T). Significance levels: -: P>0.05, *: P<0.05, **: P<0.01.

Factor	Survivorship	Fecundity	% cyst
P	**	*	**
S	**	-	**
T	**	**	**
P X S	**	-	**
P X T	**	**	**
S X T	**	**	-
P X S X T	**	**	-

Table 2-4. Results of a two-way ANOVA on the life history traits of Italian diploids and tetraploids. Significance levels: -: P>0.05, *: P<0.05, **: P<0.01.

Factor	Survivorship		Fecundity		% cyst	
	2N	4N	2N	4N	2N	4N
Temperature	**	**	**	**	**	-
Salinity	**	**	-	*	-	**
Temperature X salinity	*	**	-	**	-	-

Table 2-5. Approximate frequency (%) of cyst production of diploids and polyploids in the three populations at optimal temperature (25°C) and different salinities.

Salinity (ppt)	China		Italy		Spain	
	2N	5N	2N	4N	2N	4N
30	5.8	38.9*	1.1	15.2	7.3	0**
90	5.3	89.5	4.2	55.3	55.4	0
180	0	100**	0	93.3	100	0**

*: measured at 35 ppt (Table 2).
**: measured from single mass culture of about 120 individuals.

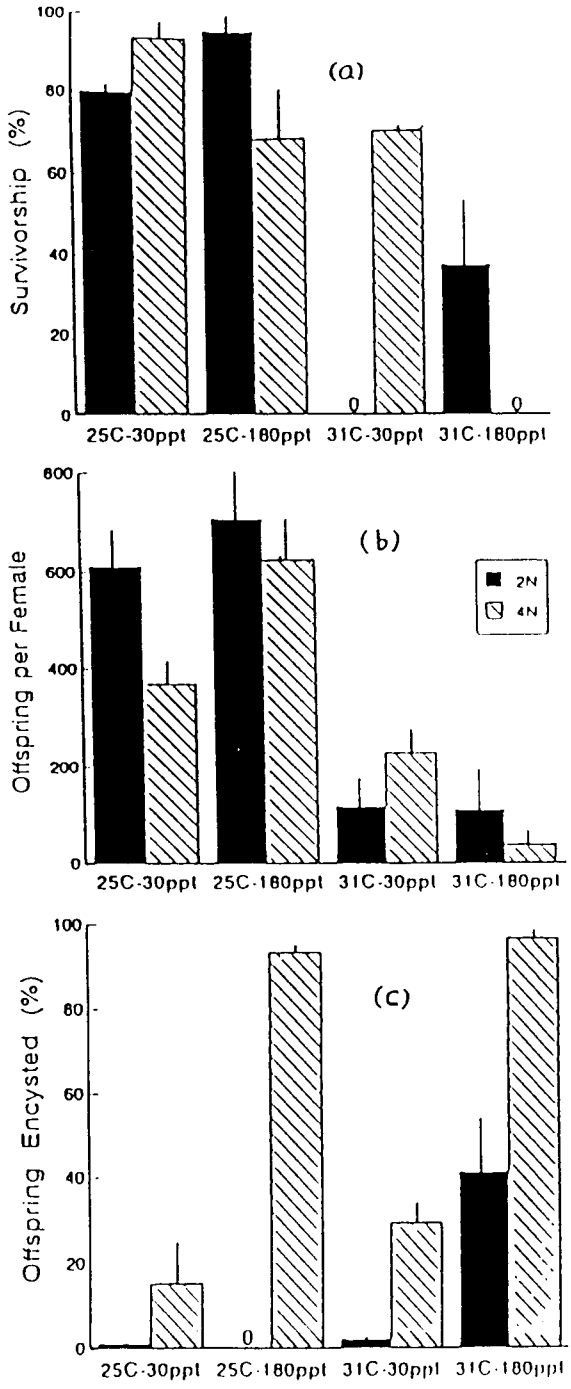

Fig 2-1. Effects of different temperature (25°C, 31°C) and salinity (30 ppt, 180 ppt) combinations on the life history traits of Italian diploids and tetraploids.

a. Survivorship
b. Fecundity (offspring/female)
c. Percent offspring encysted

Chapter 3. Effects of polyploidy on genetic variation in Artemia

ABSTRACT

I examined genetic variation in sympatric diploid and polyploid brine shrimp A. parthenogenetica from each of three populations (China, Italy and Spain). Italian and Spanish tetraploids are closely related (genetic identity index, I = 0.964). Diploids and tetraploids within each of the two European populations are also closely related (mean I = 0.905). Most alleles found in diploids also exist in sympatric polyploids. In contrast, the asexual Artemia (2N, 4N and 5N) in my study share few alleles with their close sexual relative, A. tunisiana (mean I = 0.002). These results, as well as the work of other authors, strongly suggest that at least the tetraploid Artemia in my study have an autopolyploid origin.

Clonal diversity of polyploid Artemia can be very high, at least in some population. Both diploids and polyploids had low clonal diversities in the populations dominated by polyploids and high clonal diversities in the population dominated by diploids.

The most common genotypes of sympatric diploid and polyploid Artemia frequently differed. Some alleles

occurred only in diploids, while others were restricted to polyploids. These results suggest that polyploidy in Artemia has led to genetic divergence from diploid progenitors, and that ploidy-level variation must also be considered in developing an understanding of spatial and temporal allozyme polymorphism in asexual populations.

INTRODUCTION

The success of polyploids is often correlated with extreme environments (Bierzychudek 1985, Suomalainen et al. 1987; Chapter 3). Most researchers attribute this distribution pattern to polyploids having higher tolerance of environmental stress than diploids (reviewed in previous chapters). However, higher tolerance per se may not necessarily enable polyploids to colonize and become adapted to new environments; also required is a pool of sufficient genetic variation to permit a genetic response to the changing environment.

In the context of parthenogenetic populations, two components of genetic variation need to be looked at separately. The first component is gene diversity, calculated as mean heterozygosity per individual. The second component is within-population clonal diversity (number of genotypically unique clones and their relative frequencies in a sample).

One hypothesized consequence after the acquisition of polyploidy in a parthenogenetic lineage is an increase in functional heterozygosity through evolutionary time (Suomalainen et al 1987). More heterozygous individuals are expected to have greater biochemical versatility and be better able to cope with

changing environments (reviewed by Barber 1970). However, the supporting evidence for this hypothesis is less than ideal. Most of the diploid-polyploid systems examined so far have involved comparisons between allopolyploids (which are of hybrid origin) and their sexual diploid parents (Suomalainen et al. 1987), or comparisons between amphimictic diploids and obligate apomictic polyploids (Becak and Pueyo 1970, Parker and Selander 1976), or comparisons among populations from different geographic origins (Abreu-Grobois and Beardmore 1982). Such systems are potentially flawed because they can not separate joint effects of breeding system, environmental parameters and ploidy levels on the levels of heterozygosity.

Relatively few studies have explored the genetic diversity of conspecific diploid and polyploid clones. Since asexual organisms reproduce by clonal propagation and natural selection directly acts on clones with different relative fitness, clonal diversity is an important component to use in comparing genetic variation of diploids and polyploids. A comparison of clonal diversities in asexual diploid and polyploid populations does not reveal a consistent pattern. Results of Lokki et al. (1975) indicate that the numbers of multilocus genotypes of tetraploid parthenogens of Solenobia triquetrella from central

Europe and Finland were comparable to those of the asexual diploids in Switzerland. In Artemia parthenogenetica, clone numbers of polyploid populations have been reported to be lower than those of allopatric diploid populations (Abreu-Grobois and Beardmore 1982, Browne and Hoope 1990); polyploid populations had a tendency to be monoclonal. In Daphnia pulex, clonal diversities of high-arctic polyploid populations have been reported to be greater than those of low-arctic and temperate asexual diploid populations (Weider et al. 1987). In this system, levels of variation in polyploids may be related to the polyphyletic origin of their ancestors (Weider et al. 1987, Crease et al. 1989). A similar result has also been reported among the populations of the Austrian plant Galium austriacum where the seven diploid populations have lower genotypic variation than the tetraploid population; in this study, only one tetraploid population was examined and all populations reproduced by outbreeding (Samuel 1990). Results from these studies suggest that clonal diversities of allopatric diploid and polyploid populations vary from species to species, and may also be influenced by the origin of the polyploids. Again, there is the likelihood that these studies confounded the independent effects of ploidy levels, environmental

parameters and breeding system on clonal diversity. To avoid this potentially important problem, the genetic consequences of polyploidisation should be evaluated using a sympatric diploid and autopolyploid complex that reproduces asexually. This design reduces the confounding effects of ecological and environmental isolation and of variation derived from differences in breeding systems. The brine shrimp Artemia parthenogenetica may provide such a system because diploids and polyploids co-occur in many populations and the two cytotypes have a monophyletic origin (Abreu-Grobois and Beardmore 1982; Browne and Bowen 1991).

In this chapter, I first compare levels of genetic variation of sympatric diploids and polyploids in three pond populations collected from China, Italy and Spain. Then I discuss genetic consequence after polyploid-isation in Artemia. Finally, I discuss the role of polyploidy in the geographic distributions of allozyme polymorphism in asexual populations.

MATERIALS AND METHODS

Allozyme analysis

Allozyme variation was assayed using cellulose acetate gel electrophoresis following the methods of Hebert and Payne (1985) as modified by Zhang and King (1991. appendix 2). To determine whether the asexual A. parthenogenetica from the Spanish population share allozyme variation with their sympatric sexual, A. tunisiana - which is generally regarded as their close relative, I also examined genetic variation in 50 Spanish A. tunisiana female adults. For each cytotype, 50 to 60 adult females from each of the three populations were examined for their allozyme variation. Each of these females was examined for allozyme variation at eleven loci: malate dehydrogenase (fast system, Mdh-1 and slow system, Mdh-2), isocitrate dehydrogenase (Idh-1 and Idh-2), glutamate oxaloacetate transaminase (Got-1 and Got-2), glucose-6-phosphate dehydrogenase (G6pd-2), 6-phosphogluconate dehydrogenase (6Pgd), phosphoglucose isomerase (Pgi), lactate dehydrogenase (Ldh), and malic enzyme (Me). Alleles at each locus were numbered according to the proportional electrophoretic mobilities of their products relative to that of the most common allele

(e.g., 6Pgd^{100}). Electromorphs were compared using an asexual clone from the Spanish population as an internal control on each gel plate. These methods permitted us to identify multilocus genotypes (referred to as "clones" hereafter) for each individual.

Clonal diversity, C, was calculated using Simpson's diversity index: $C = 1 - \Sigma^{n}_{i=1}(P_i^2)$, where P_i is the frequency of the ith clone and n is the total number of clones in the population. Gene diversity, H, was calculated in the same way as expected heterozygosity (Nei 1975): $H = (M - \Sigma^{m}_{i=1}X_i^2)/M$, where X_i is the frequency of the ith allele, m is the total number of alleles over all loci, and M is the number of loci scored (M = 11). Genetic identity (I) and distance (D) between two populations (X and Y) were calculated according to Nei (1975):

$$I = \frac{\Sigma^{m}_{i=1}(X_i * Y_i)}{(\Sigma^{m}_{i=1}X_i^2 * \Sigma^{m}_{i=1}Y_i^2)^{1/2}} \quad \text{and} \quad D = -\ln(I).$$

where X_i and Y_i are the frequencies of the ith allele at a given locus in populations X and Y.

RESULTS

Genetic variation in diploids and polyploids

The relative mobilities of the electromorphs and the allele frequencies found in my samples at each locus are presented in table 3-1. The amount of allozyme variation not only differs among the 11 loci scored, but also differs from population to population. Intra- or interpopulational variation occurs in 8 of the 11 enzyme loci; Ldh, Me and Got-2 were monomorphically single-banded in the asexual populations of my sample. The Ldh, Me and Got-2 loci of Spanish sexual A. tunisiana were also monomorphically single banded, but contained different alleles than those of the asexual Artemia (table 3-1).

Some diploid individuals from the Chinese and Italian populations showed "null" activity in the Idh loci (tables 3-2 and 3-3). Whenever this was observed, samples from these individuals were heavily loaded and re-examined to make sure that the absence of activity was not due to inadequate sample quantities. Such individuals are designated electromorph "N".
The banding patterns of the heterozygous tetraploids appeared to be balanced as detected by cellulose acetate gel electrophoresis under my laboratory

conditions. In pentaploids, all heterozygous genotypes are unbalanced, and there are four ways to be a heterozygote for two alleles. I have seen obvious unbalanced enzyme activity in Pgi, Mdh-1, Idh-1 and Idh-2 loci. However, my electrophoretic results did not permit me to confidently assign allele dosages on the basis of these differences in banding intensity. I have therefore, arbitrarily, designated heterozygotes as having two doses of one allele and three of the second.

Table 3-1 shows that some alleles are ploidy specific and the apparent polymorphism at these loci is due to the simultaneous occurrence in the geographic population of diploids and polyploids. For example, the Idh-2 locus was monomorphic in Italian diploids. However, viewing the Italian population as a whole, this locus appears to be polymorphic because the sympatric tetraploids had different alleles. Similar examples can also be found for the Idh-1 and Idh-2 loci in the Spanish population, and the Got-1 and Idh-1 loci in the Chinese population.

There were no common patterns of electrophoretic variation (multilocus genotypes) shared by the sympatric diploids and polyploids (tables 3-2 to 3-4). This diversification reflected not only loci that were more heterozygous in polyploids than in diploids, but

also the presence of different heterozygotes in both diploids and polyploids. For example, all Italian diploids had the cf genotype at the Mdh-1 locus while all the sympatric tetraploids had the ffgg genotype (table 3-3). Similar patterns can also be found in the 6Pgd locus in the Chinese and Spanish populations.

Among the three populations studied, Spanish tetraploids had the lowest levels of genetic variation; only two (6Pgd and G6pd-2) of the 11 loci were variable. Chinese pentaploids were the most variable in my samples. Both diploids and polyploids from the Chinese population were more variable than their counterparts from the other two populations with respect to number of clones, clonal diversity, mean number of alleles per locus, proportion of polymorphic loci and gene diversity (table 3-5).

Genetic clonal diversity of sympatric diploids and polyploids varied with the population (tables 3-2 to 3-5). In each population, there were usually one or two predominant clones that made up over 50% of the total number of individuals, and a larger number of rare clones. The Chinese population had the highest clonal diversity index; clone diversities were similar in diploids (clone number n = 18, C = 0.813) and in pentaploids (n = 17, C = 0.806). Italian tetraploids had a higher clonal diversity index than their

sympatric diploids (n = 12, C = 0.776 vs. n = 6, C = 0.349). Spanish tetraploids were very homogeneous (C = 0.183); only three clones were identified from 50 individuals. Spanish diploids had a higher clonal diversity (n = 6, C = 0.530) than sympatric tetraploids even though the latter had a higher gene diversity.

A summary of the genetic variation in tables 3-1 to 3-4 for all loci scored is presented in table 3-5. Polyploids have a greater gene diversity than diploids when compared with both sympatric and allopatric diploids. Although within each population polyploids had a greater number of alleles per locus than diploids, the difference is not statistically significant (paired-t test, $P > 0.01$ for all three populations). In addition, polyploids had a greater proportion of polymorphic loci than sympatric diploids (table 3-5).

Genetic similarities among the populations.

Nei's measure of genetic identity (table 3-6) calculated for all loci between populations indicates that tetraploids from Italy and Spain are closely related (I = 0.964) even though their collection sites are widely separated (41° 25'N - 16° 05'E for the former, vs. 36° 43'N - 6° 23'W for the latter, Vanhaecke

et al. 1987). Sympatric diploids and tetraploids from the Italian and Spanish populations are more closely related (I = 0.865 and 0.945 respectively) than sympatric diploids and pentaploids from the Chinese population (I = 0.747).

San Lucar sexual A. tunisiana shared almost no alleles with sympatric asexuals or the Italian asexual population (I = 0.001 ± 0.001 and I = 0.000 ± 0.000 respectively). Chinese asexual Artemia also had low genetic identity with the San Lucar sexual Artemia (I = 0.006 ± 0.005). However, the I value for Spanish sexual and Chinese asexual diploids (I = 0.011) is an order of magnitude larger than the I values between Spanish sexual and either Spanish asexuals or Italian asexuals.

The average genetic distance between Chinese pentaploids and Italian and Spanish tetraploids found in my study (D = 0.464 ± 0.028) is far larger than that between European pentaploid populations from Bulgaria and Turkey and tetraploid populations (D = 0.045 ± 0.027, Abreu-Grobois and Beardmore 1982). This difference may reflect the geographic isolation of China's coastal Artemia populations from European populations as well as the relative ages of the two groups of pentaploids. The average genetic distance (D> 6.71 ± 0.61) between asexual populations and the

San Lucar sexual population in my results is far larger than the reported distance between A. tunisiana and asexual Artemia (D = 1.023 ± 0.295, Abreu-Grobois and Beardmore 1991). The discrepancy between the two studies may be due to differences in electrophoretic techniques and, in addition, to the smaller numbers of populations and enzyme loci examined in my study.

DISCUSSION

My data are subject to three constraints. First, a saltern is a series of interconnected ponds where salinity gradually increases due to evaporation as sea water flows from one pond to the next. My samples were collected from one pond in each of the three salterns (China, Italy and Spain). Therefore, the findings of gene diversity, clone diversity and ploidy ratio in a pond population may not reflect the dynamic situation in the saltern as a whole. Second, homogeneity of any two individuals at the eleven loci does not demonstrate homogeneity at other, non-assayed, loci. Third, my sample sizes of 50-60 individuals for each cytotype in each of the three populations are not large, and it is likely that additional rare clonal types occur. The number of multilocus genotypes found in each population must therefore be regarded as a minimum estimate.

Clonal diversities of sympatric diploids and polyploids

My study reveals that clonal diversity of polyploids can be very high, at least in some Artemia populations. Pentaploids from the Chinese sample had high clonal diversity, whereas tetraploids from Italian and Spanish samples had low clonal diversities. For diploids, in addition to the high levels I observed in the Chinese population, Browne and Hoopes (1990) identified 63 clones from a sample of 545 asexual diploids in a French population. By contrast, clonal diversities of diploids in the polyploid-dominated populations (Italy and Spain) are low. Founder effects, as well as the relative age of each population, might well explain why clonal diversities of diploids are high in some populations and low in others. It is also not clear if the habitats dominated by polyploid Artemia have certain environmental conditions leading to truncation of clonal diversity.

Genetic consequences after polyploidization in Artemia

There is strong evidence indicating that asexual diploid and polyploid Artemia have a monophyletic origin (Abreu-Grobois and Beardmore 1982, Browne and Bowen 1991). Moreover, asexual diploids and

tetraploids have a very close genetic identity (table 3-6 in this paper; Abreu-Grobois and Beardmore 1982); most alleles found in diploids also occur in sympatric polyploids (table 3-1). I also found that asexual Artemia from Italy and Spain share almost no allozyme variation with their sexual ancestor A. tunisiana. Individuals from the Italian population even have a single mtDNA RFLP genotype (Browne and Bowen 1991). These observations strongly suggest that tetraploid Artemia have an autopolyploid origin. The present data do not allow me to discriminate if the Chinese pentaploids are of allo- or autopolyploid origin.

My data may contribute to the eventual understanding of the evolutionary consequences of autopolyploidy in A. parthenogenetica. In all three populations, sympatric diploids and polyploids are usually electrophoretically different. Tetraploid populations are also known to have unique genotypes that are different from allopatric diploid populations at some loci (Abreu-Grobois and Beardmore 1982, 1991). These results indicate that genetic divergence has occurred between diploids and polyploids following their separation by the autopolyploid event. An increased level of heterozygosity in polyploids is one source of genetic divergence.

A second source of variation is apparent in polyploid individuals having unique alleles that are rare or absent in sympatric diploids (table 3-1). In diploid Artemia, genetic variation can be created by either mutation or endomeiotic recombination, while in polyploids, endomeiosis appears to be absent and mutation is the only source of variation (Barigozzi 1974). Once generated by these mechanisms, the expression and maintenance of the variation are likely to reflect, at least in part, the differential selective pressures (sympatric diploids and polyploids in my study are significantly different in relative fitness, Chapters 1 and 2) acting on diploid and polyploid Artemia. That is, ploidy-dependent selection may reinforce genetic divergence between the two cytotypes. Thirdly, the genetic variation present in asexual diploids at the time of polyploidization, as well as the founder effects, may also influence genetic divergence.

The genetic divergence of sympatric diploids and polyploids may have played an important role in the adaptation of the two sympatric cytotypes. Diploids have significantly higher relative fitness than sympatric polyploids in the three populations of my study when measured under optimal culture conditions; polyploids have higher thermotolerance than diploids

(Chapters 1 and 2 in this thesis). Competition under optimal conditions led to the exclusion of polyploids by sympatric diploids in three generations in Chinese and Italian populations (Podrabsky and Zhang, unpubl.). However, the two cytotypes must still be regarded as "ecologically similar", and they are expected to compete when they co-occur in a limited environment. The exploitation of different microhabitat resources, either spatially or temporally, would reduce this intraspecific competition. The genetic variation provided by autopolyploidisation may be used for population subdivision.

Polyploidy and geographic distributions of allozyme polymorphism

Although distributions of allozyme polymorphism have often been shown to be non-random and have received extensive study (reviewed by Lumaret 1984), there are only a few cases in which selection has been shown to act directly on specific alleles or gene complexes (Allison 1956; reviewed by Lumaret 1984; Hochachka and Somero 1984). The investigation of genetic variation between sympatric diploids and polyploids is potentially relevant to the geographic distribution of allozyme polymorphism in asexual populations.

As mentioned earlier, many polyploids tend to be found in extreme environments, such as high latitudes, when compared to conspecific diploids (Bierzychudek 1985, Suomalainen et al. 1987; Beaton and Hebert 1988). As will be discussed in Chapter 4, non-random distributions of diploids and polyploids also exist in Artemia. Polyploid Artemia populations tend to increase in frequency with decreasing latitudes in the Old World (Chapter 4 in this thesis). In addition, polyploid Artemia also tend to be found in inland salt lakes. Results of Amat Domenech (1980) show that almost all inland salt lake Artemia populations in Spain are composed of polyploids, although diploid populations have also been found in inland salt lakes in many other areas of the Old World (e.g., Abreu-Grobois and Beardmore 1982; L. Zhang, personal observations). This biogeographic pattern may lead to a non-random distribution of ploidy-specific allozyme variation (irrespective of their individual selective values), arising from selection for elevated ploidy levels in certain environments. For example, Lumaret (1984) reported that there is a positive correlation between allele diversity at the GOT-1 locus and environmental unpredictability in the plant Dactylis glomerata; most populations with high allele diversity were polyploids. My results suggest that one of the

effects of polyploidisation may be a functional genetic divergence from the diploid progenitors. The effects of ploidy-level variation must, therefore, also be considered in developing an understanding of spatial and temporal distributions of allozyme polymorphism in asexual populations.

Table 3-1. Allozyme frequency in diploid and polyploid *Artemia* (frequency of null allele is not included). Two columns appear under each enzyme locus. On the left is a lower case letter used to designate a particular allele in tables 2-4; on the right is a number that indicates mobility of the allele relative to that of the most common alleles, designated as "100".

Locus	China		Italy		Spain		
	2N	5N	2N	4N	2N	4N	sexual
Mdh-1							
a 91							0.86
b 93							0.13
c 95			0.50				
d 96	0.43	0.12					
e 98	0.57	0.88					
f 100			0.50	0.50	0.50	0.50	
g 105				0.50	0.50	0.50	
Mdh-2							
a 82							0.25
b 84							0.75
c 87			0.50				
d 93			0.50	0.48		0.50	
e 98	0.02	0.04					
f 100	0.98	0.96		0.52	1.00	0.50	
Pgi							
a 93			0.02				0.37
b 96							0.63
c 100	0.34	0.40	0.98	0.52	0.90	0.50	
d 136	0.12						
e 150	0.12						
f 171	0.43	0.60		0.48	0.10	0.50	
6Pgd							
a 92				0.48	0.45		
b 100			0.93	0.48	0.45	0.49	
c 104	0.10	0.40		0.02	0.05	0.50	
d 107	0.38	0.02			0.05	0.01	0.25
e 111	0.14	0.56	0.07	0.02			
f 115	0.38	0.02					
g 152							0.63
h 170							0.13

Table 3-1 cont.

Locus	China		Italy		Spain		
	2N	5N	2N	4N	2N	4N	sexual
G6pd-2							
a 60							0.50
b 70							0.50
c 85	0.36	0.47					
d 90			0.02		0.02		
e 95	0.02	0.45			0.12		
f 100			0.85	0.92	0.71	0.92	
g 105	0.62	0.08	0.13	0.08	0.16	0.08	
Idh-1							
a 96		0.31					
b 98		0.14		0.05			
c 100	0.96		0.98	0.58	1.00	0.50	
d 104	0.04	0.55		0.37		0.50	
e 108							0.33
f 125							0.65
g 131							0.02
Idh-2							
a 96	0.01	0.40					
b 100	0.91			0.33	1.00	0.50	
c 103							1.00
d 105		0.14					
e 108	0.01		1.00	0.67		0.50	
f 111		0.46					
Got-1							
a 100		0.36					
b 112							0.16
c 118	1.00	0.64	1.00	1.00	1.00	1.00	
d 129							0.84
Got-2							
a 64							1.00
b 100	1.00	1.00	1.00	1.00	1.00	1.00	
Ldh							
a 96							1.00
b 100	1.00	1.00	1.00	1.00	1.00	1.00	
Me							
a 94							1.00
b 100	1.00	1.00	1.00	1.00	1.00	1.00	

Table 3-2: Clonal genotypes of the Chinese 2N and 5N *Artemia*. *Ldh*, *Me* and *Got-2* are found to be monomorphically single-banded. See page 61 for the designation of pentaploid heterozygous genotype.

Numbers of clones	Clone freq.	Mdh-1	Mdh-2	Pgi	6Pgd	G6p-2	Idh-1	Idh-2	Got-1
2N (total = 55)									
2	0.04	de	ef	cf	df	gg	cc	N	cc
1	0.02	de	ff	cf	df	gg	cc	N	cc
1	0.02	de	ff	ff	df	gg	cc	N	cc
2	0.04	de	ff	ff	df	gg	cc	bb	cc
1	0.02	de	ff	ff	ce	gg	cc	bb	cc
1	0.02	de	ff	ff	ee	cc	cc	ae	cc
2	0.04	de	ff	cf	df	gg	dd	bb	cc
23	0.42	de	ff	cf	df	gg	cc	bb	cc
1	0.02	de	ff	cf	ce	ee	cc	bb	cc
2	0.04	de	ff	cf	ce	gg	cc	bb	cc
3	0.05	de	ff	cf	ce	cc	cc	bb	cc
1	0.02	ee	ff	ff	ee	cc	cc	bb	cc
1	0.02	ee	ff	ed	ce	cc	cc	bb	cc
10	0.18	ee	ff	ed	df	cc	cc	bb	cc
1	0.02	dd	ff	cf	ce	cc	cc	bb	cc
1	0.02	dd	ff	ed	ce	cc	cc	bb	cc
1	0.02	dd	ff	ed	df	cc	cc	bb	cc
1	0.02	dd	ff	cc	ce	cc	cc	bb	cc
5N (Total = 51)									
1	0.02	eeeee	fffff	ccfff	eeeee	eeggg	bbbbb	ddddd	aaccc
13	0.26	eeeee	fffff	ccfff	cceee	eeeee	aaddd	aafff	aaccc
1	0.02	eeeee	fffff	ccfff	cceee	eeggg	bbbbb	ddddd	aaccc
1	0.02	eeeee	fffff	ccfff	cceee	eeggg	aaddd	aafff	aaccc
1	0.02	eeeee	fffff	ccfff	cceee	ggggg	aaddd	aafff	aaccc
1	0.02	eeeee	fffff	ccfff	cceee	ccccc	bbbbb	ddddd	ccccc
17	0.33	eeeee	fffff	ccfff	cceee	ccccc	aaddd	aafff	aaccc
1	0.02	ddddd	fffff	ccfff	ccccc	ccccc	bbbbb	ddddd	ccccc
1	0.02	ddddd	fffff	ccfff	ddfff	eeeee	aaddd	aafff	aaccc
1	0.02	ddddd	fffff	ccfff	cceee	eeeee	bbbbb	ddddd	ccccc
1	0.02	ddddd	fffff	fffff	cceee	eeeee	bbbbb	ddddd	ccccc
1	0.02	eeeee	fffff	fffff	ccccc	ccccc	bbbbb	ddddd	ccccc
1	0.02	eeeee	fffff	ccccc	cceee	ccccc	aaddd	aafff	aaccc
1	0.02	eeeee	eefff	ccfff	ddfff	eeeee	ddddd	aaaaa	aaccc
1	0.02	eeeee	eefff	ccfff	cceee	ggggg	ddddd	aaaaa	aaccc
3	0.06	eeeee	eefff	ccfff	cceee	ccccc	ddddd	aaaaa	aaccc
5	0.10	ddeee	fffff	ccfff	cceee	eeeee	aaddd	aafff	aaccc

Table 3-3. Clonal genotypes of the Italian 2N and 4N *Artemia*. *Ldh*, *Me*, *Got-1* and *Got-2* are monomorphically single-banded.

Numbers of clones	Clone freq.	Mdh-1	Mdh-2	Pgi	6Pgd	G6pd-2	Idh-1	Idh-2
2N (Total = 60)								
48	0.80	cf	cd	cc	bb	ff	cc	ee
1	0.02	cf	cd	cc	bb	ff	N	ee
4	0.07	cf	cd	cc	bb	gg	cc	ee
2	0.03	cf	cd	cc	bb	df	cc	ee
4	0.07	cf	cd	cc	ee	gg	cc	ee
1	0.02	cf	cd	aa	bb	ff	cc	ee
4N (Total = 60)								
25	0.42	ffgg	ddff	ccff	aabb	ffff	ccdd	bbee
8	0.13	ffgg	ddff	ccff	aabb	ffff	cccc	bbee
3	0.05	ffgg	ddff	ccff	aabb	ffff	dddd	bbee
3	0.05	ffgg	ddff	ccff	aabb	ffff	dddd	eeee
8	0.13	ffgg	ddff	ccff	aabb	ffff	cccc	eeee
5	0.08	ffgg	ddff	ccff	aabb	ffff	ccdd	eeee
2	0.03	ffgg	ddff	ccff	aabb	ffff	bbbb	eeee
2	0.03	ffgg	ddff	ccff	aabb	gggg	cccc	eeee
1	0.02	ffgg	ddff	ccff	aabb	gggg	bbbb	bbee
1	0.02	ffgg	ddff	ccff	aabb	gggg	ccdd	bbee
1	0.02	ffgg	ffff	cccc	ccee	ffff	ccdd	bbee
1	0.02	ffgg	ffff	cccc	ccee	gggg	cccc	eeee

Table 3-4. Clonal genotypes of Spanish 2N and 4N *Artemia*. *Ldh*, *Me*, *Got-1* and *Got-2* are monomorphically single-bended, and *Mdh-1* are monomorphically double-banded.

Numbers of clones	Clone freq.	*Mdh-2*	*Pgi*	*6Pgd*	*G6pd-2*	*Idh-1*	*Idh-2*
2N (Total = 51)							
34	0.67	ff	cc	ab	ff	cc	bb
5	0.10	ff	cc	ab	ee	cc	bb
5	0.10	ff	cc	ab	gg	cc	bb
2	0.04	ff	cc	ab	de	cc	bb
2	0.04	ff	ff	cd	ff	cc	bb
3	0.06	ff	ff	cd	gg	cc	bb
4N (Total = 50)							
45	0.90	ddff	ccff	bbcc	ffff	ccdd	bbee
4	0.08	ddff	ccff	bbcc	gggg	ccdd	bbee
1	0.02	ddff	ccff	ccdd	ffff	ccdd	bbee

Table 3-5. Summary of genetic variation in each population.

	China		Italy		Spain		
	2N	5N	2N	4N	2N	4N	Sexual
Numbers of individuals examined	55	51	60	60	51	50	50
Estimated cytotype frequency (%)	90	10	38	62	19	37	44
Number of loci scored for each individual	11	11	11	11	11	11	11
Number of multilocus genotypes	18	17	6	12	6	3	44
Diversity of multilocus genotypes	0.813	0.806	0.349	0.776	0.530	0.183	0.975
Mean number of alleles per locus (s.e. of mean)	2.18 0.35	2.18 0.30	1.55 0.21	1.91 0.28	1.73 0.36	1.73 0.19	1.82 0.23
Proportion of polymorphic loci	0.45	0.73	0.36	0.64	0.36	0.64	0.64
Gene diversity	0.238	0.320	0.134	0.286	0.157	0.287	0.259

A locus was considered polymorphic if the frequency of its most common allele < 0.95.

Table 3-6. Nei's standard genetic identity (I, above the diagonal) and genetic distance (D, below diagonal) among populations and ploidy levels.

	China		Italy		Spain		
	2N	5N	2N	4N	2N	4N	Sexual
China2N	--	0.747	0.603	0.718	0.831	0.733	0.011
China5N	0.292	--	0.483	0.629	0.613	0.656	0.001
Italy2N	0.507	0.728	--	0.865	0.761	0.836	0.001
Italy4N	0.331	0.464	0.145	--	0.884	0.964	0
Spain2N	0.186	0.489	0.274	0.123	--	0.945	0.001
Spain4N	0.311	0.422	0.179	0.036	0.058	--	0.000
Spain sexual	4.471	7.356	7.082	+ ∞	6.571	8.047	--

Chapter 4. Geographic distributions and adaptive significance of polyploidy in Artemia

ABSTRACT

I have reviewed geographic distributions of diploid and polyploid Artemia in the Old World using information of Artemia populations in which both mode of reproduction and chromosome number of the dominant cytotypes have been either reported, or determined by myself. Below 25°N latitudes, all Artemia populations are polyploids, while in temperate regions (between 35-45°N), diploids are the most common cytotype.

Differences in relative fitness, latitudinal distributions and environmental conditions are considered for Chinese diploids and polyploids Artemia. I suggest that Artemia's habitats gradually become marginal southward along the China coast, and that the more southern distribution of polyploids along China coast may be a result of combined effect of low salinity and high temperatures, rather than a result of temperature adaptation only.

The distribution of Chinese polyploids in the low latitudes and the underlying mechanism strongly suggest that polyploids having advantages over diploid Artemia in marginal habitats. Thus, polyploidy in Artemia has

led to divergence in physiological, demographic and genetic characteristics, and as a result, in geographic distributions. The occurrence of polyploidy in Artemia is also associated with the broader niches of asexual Artemia populations, and consequently has extended the genus' distribution range.

INTRODUCTION

Levin (1983) hypothesized that polyploidy in plants may "... greatly alter the cytological, biochemical, physiological, and developmental character of organisms... which could suit them to conditions which are beyond the limits of their diploid progenitors". As mentioned earlier, it has been recognized in many plant species and several animal species that polyploids do tend to be found at high latitudes, high altitudes or in arid areas (Berzychudek, 1985; Suomalainen et al., 1987; Beaton and Hebert, 1988). For example, all Daphnia pulex populations in the high arctic areas of Canada are tetraploids while most temperate populations are diploids (Beaton and Hebert 1988). Triploid lizards of the Heteronotia binoei have a wider distribution on Australian dry desert than their bisexual diploids (Moritz 1984). The adaptive mechanisms underlying the geographic distributions of polyploids remain unclear. That is, we still do not know if the high frequency of polyploids in "harsh-temperature" habitats is due to a direct or indirect adaptation to "harsh temperatures". Among possible indirect factors are a number of effects that are either temperature-correlated, or latitudinally (or altitudinally) correlated. If the latter is the case,

how do these factors interact and determine the distribution patterns of diploids and polyploids? Such knowledge is essential in understanding adaptive significance of polyploidy in plants and animals.

Hebert and Emery (1990) report that the polyploid Daphnia pulex in the high arctic areas of Canada are melanic forms having higher tolerance of UV radiation than non-melanic diploids. Thus latitude-correlated factors such as the intensity of UV light may also be a potential selective force in certain species.

In this chapter, I first review the geographic distributions of diploid and polyploid Artemia in the Old World. Then, I focus on Chinese Artemia and explore potential factors determining the distribution patterns of diploid and polyploid Artemia along the China coast. Finally, I try to explore the adaptive significance of polyploidy in Artemia, as it is related to a universal phenomenon -- parthenogenetic forms having different geographic distributions from sexual relatives.

INVESTIGATION APPROACH AND RESULTS

Literature review of Old World asexual diploid and polyploid, and sexual Artemia

Artemia occur in both inland salt lakes and coastal salterns. Since the water chemistry of inland salt lakes shows considerable variation, it is very difficult to separate the joint effects of temperatures, salinities and specific water chemical compositions. Therefore, my literature review (Table 4-1) only deals with Artemia populations found in coastal salterns where the chemical composition of sea water is relatively constant.

Table 4-1 is based on populations of Artemia in which both mode of reproduction and chromosome number of the dominant cytotypes (i.e., the most common cytotype in a population) have been either reported, or determined by myself. The result is summarized in table 4-2, which clearly shows that asexual diploid and polyploid Artemia in the Old World differ in their distribution patterns. Table 4-2 shows that polyploids are found with increasing frequency at low latitudes. Below the latitudes of 25°N, all Artemia populations are polyploids. Almost all diploids occur in temperate regions (between 35-45°N), and there they are the most

common cytotype. Polyploids occur throughout the genus' latitudinal range. At higher and colder latitudes (above 45°N), the small sample size (only two populations are known for ploidy composition above 45°N) precludes the speculation on distribution pattern.

Yearly average temperatures and precipitations along the China coast

Polyploid and diploid Artemia clearly differ in their distributions along the China coast (table 4-1): all populations below the latitudes of 25°N are polyploids while all populations in the north (above 35°N) are diploids. Temperature and salinity are the predominant factors that determine the geographic distributions of Artemia (reviewed by Vanhaecke et al 1987). The salinity in coastal salterns is controlled by the intensity of evaporation and yearly precipitation.

I obtained the information about the yearly average temperature and precipitation in the different latitudes along the China coast from the National Meteorological Bureau of China. Table 4-3 shows that with decreasing latitudes along the China coast, both yearly average temperature (as well as average summer temperature) and precipitation increase. Correlated

with these changes is the reduction of Artemia biomass in the low latitudes (Li et al 1990. table 4-3). Most China's commercial Artemia cysts are supplied by the northern provinces (Liao Ning, He Bei, Shandong and north Jiang Su). In these areas, Artemia adults occur only in warmer seasons (late spring, summer and early autumn. (Chen 1975)) while in cold seasons Artemia exist in the form of dormant cysts. In the areas that receive a precipitation of above 1000mm/year (south Jiang Su, Fu Jian, Kan Tang and Hai Nan provinces), Artemia adults are found sparsely, but throughout the year (Chen 1975). Their cyst production never reaches any commercial scale (table 4-3).

Responses to temperature and salinity changes of Chinese diploid and polyploid Artemia that were collected from the northern and the southern coast of China

Information about responses to temperature and salinity changes of Chinese diploids and polyploids is necessary in order to explore the selective forces influencing distribution patterns along the China coast. Although I did not examine norm of reactions to temperature and salinity changes of Chinese diploids and polyploids, I did for Italian diploids and polyploids. Chinese

diploid-polyploid complexes are very similar to Italian diploid-polyploid complex in response to salinity changes: polyploids collected from Dong Fang Hong Saltern (China) produce most offspring as cysts at high salinities, and most offspring as nauplii at low salinity (table 2-5). It is also quite likely that the two systems responds similarly to temperature changes. To check if this is the case, I examined survival rates of Chinese diploids and polyploids at different temperatures (25°C and 31°C) and salinities (30 ppt and 90 ppt).

Two Chinese populations were used. One of those was collected from Dong Fang Hong Saltern (about 37°N in China: North-China hereafter) and has been repeatedly used in Chapters 1,2 and 3. This population has both diploids and pentaploids. The other population is a southern population (about 19°N: South-China hereafter) which I collected from San Yia Saltern of Hinan Province in the Summer of 1992. This population is a mixture of tetraploids and pentaploids.

To examine survivorship of diploids and polyploids under different temperatures (25°C and 31°C) and salinities (30 ppt and 90 ppt), newly produced nauplii were used. Nauplii were produced and isolated in a mass culture of about 700 40-day old adults that had been cultured at 25°C and 35 ppt. Five replicates of

20 animals were used for each ploidy at each treatment. One-way ANOVA was used to compare difference between diploids and polyploids.

Fig 4-1 shows that at high salinity and high temperatures (90ppt, 31C), diploids have significantly higher survival rates than both sympatric and allopatric polyploids (one-way ANOVA, $P<0.01$). At low salinity and high temperature (30 ppt, 30C), polyploids have significantly higher survival rates than diploids (one-way ANOVA, $P<0.01$). These results are in agreement with what are found in Italian diploids polyploids.

DISCUSSION

Distribution patterns of diploid and polyploid Artemia in the Old World and the potential selectives forces

My literature review reveals that diploid and polyploid Artemia have different distributions in the Old World: polyploids tend to increase in frequency toward the equator while diploids predominate in the temperate regions (table 4-1 and 4-2). This is particularly true along the China coast. The environmental conditions along the China coast, together with my experimental results, may help explore the selective forces

underlying the latitudinal trend of polyploids in China.

In China, the coastal regions that receive a yearly average precipitation of less than 1000mm are the center of the Artemia cyst industry. In regions that receive a precipitation of above 1000mm/year, Artemia are only found sparsely and their cyst production never reaches a commercial scale (table 4-3). Moreover, southeast Asia countries (e.g. Thailand, Philippines and Malaysia which all have relatively highly frequent rains) do not have natural Artemia distributions (reviewed by Vanhaecke et al 1987). The low Artemia biomass in low latitudes of China can be attributed to frequent rains that decrease saltern salinity and disturb Artemia's growth and reproduction (reviewed by Vanhaecke et al 1987). Also, in this area the dry season is too short for Artemia to grow and maintain a large population size. High yearly average temperature and summer temperature are clearly not the limiting factor because Artemia can grow and reproduce in these areas as long as culture salinities are kept high (reviewed by Vanhaecke et al 1987). This evidence indicates that Artemia habitats gradually become marginal southward along the China coast. Thus the predominance of polyploids below the latitudes of 25°N

in China indicates that polyploid Artemia have advantages over diploids in marginal habitats.

Is the more southern distribution of polyploid Artemia due to temperature adaptation? If this is the case, one would expect to find that polyploid Artemia have better reproductive performance than diploids when cultured at high temperature and a variety of salinities. My laboratory results do not support this hypothesis. Although polyploid Artemia have higher survival rates when exposed to short term heat shock, this short-term advantage does not have long term ecological consequence because polyploids have even worse reproductive performance than sympatric diploids when cultured at high temperature and high salinities that commonly occur in nature; it is only at low salinities and high temperatures that Italian tetraploids have better reproductive performance than sympatric diploids (fig 2-1a, 2-1b and 2-1c). This is also true for Chinese polyploids that were collected from both north and south China (fig 4-1). These results strongly suggest that Chinese polyploids have significant advantages over diploids at low salinity and high temperatures. This is a typical environmental conditions in the low latitudes along the China coast. Therefore, the more southern distribution of polyploids

along the China coast could be a result of combined effects of low salinity and high temperatures.

Adaptive significance of polyploidy and geographic parthenogenesis in Artemia

It has been recognized for more than 50 years in both plants and animals that parthenogenetic forms have different geographic distributions than their sexual relatives. Parthenogens generally tend to be found at higher latitudes and altitudes (Vandel, 1928; reviewed by Suomalainen et al., 1987). This distribution pattern has been termed "geographic parthenogenesis" (Vandel, 1928; reviewed by Suomalainen et al., 1987). The cause of geographic parthenogenesis has been debated (reviewed by Bierzychidet 1985; Beaton and Hebert 1988). Most recent authors (Glesener and Tilman, 1978; Bell, 1982; Browne and MacDonald, 1982) attribute the geographic parthenogenic trend to direct selection for breeding systems. That is, an asexual reproduction mode is selected for in situations when physical, rather than biotic, factors are important. These situations usually correspond to habitats in high latitudes, high elevations or arid regions. Sexual forms, with their potential for rapid genetic change,

are dominant in situations when biological interactions are important.

Suomalainen (1962; 1969) argued that geographical parthenogenesis is an indirect consequence of selection for elevated ploidy level in some habitats, because parthenogenesis is strongly correlated with the occurrence of polyploidy. As mentioned earlier, polyploidy is found in most major animal groups that posses an asexual mode of reproduction. This hypothesis has gained some support for plants (Bierzychudek, 1985) and the cladoceran Daphnia pulex (Beaton and Hebert, 1988). The information I have gathered permits me to evaluate the applicability of these ideas to Artemia.

It is generally accepted that the genus Artemia originated in the Mediterrnaean Sea (Badaracco et al., 1987; reviewed by Browne and Bowen 1990). As mentioned earlier, there is strong evidence to suggest that asexual polyploid Artemia evolved from asexual diploid Artemia which themselves branched from ancestral sexual Artemia tunisiana (Abreu-Grobois and Beardmore, 1982; Browne and Bowen, 1990). Although A. tunisiana (sexual form) has ecological advantages at low temperature (Browne et al., 1988; Browne and Halanych, 1989; Lenz and Browne, 1990), and is the dominant form in winter when they are sympatric with A. parthenogenetica in

Spanish salinas (Perez, 1987), they have never been found north of 40°N (Browne and Macdonald, 1982; Browne, 1988; Lenz and Browne, 1990). In the Old World, sexual populations are only found in the center of Artemia's distribution (between latitudes of 25-40°N, table 4-2) while their parthenogenetic relatives are distributed far beyond this range. Sexual forms are clearly at a selective disadvantage when compared to parthenogenetic forms at low and high latitudes in the Old World. My literature review reveals that polyploid Artemia have a wider latitudinal distribution than asexual diploid Artemia (table 4-2). The distribution of Chinese polyploids in the low latitudes and the underlying mechanism strongly suggest that polyploids having advantages over diploid Artemia in marginal habitats. Thus, polyploidy in Artemia leads to divergence in physiological, demographic and genetic characteristics, and as a result, in geographic distributions. The occurrence of polyploidy in Artemia is clearly associated with niche breadth in asexual Artemia populations, and consequently is also associated with the broad distribution range of this genus. This evidence supports Suomalainen's hypothesis; that is, geographic parthenogenesis arises indirectly as a result of selection for elevated ploidy level in marginal habitats.

Table 4-1. Locations, ploidy number (where reported) and the approximate latitudes of sexual and parthenogenetic Artemia in the Old World. Dominant cytotype is defined as the most common cytotype in a population.

Locality	dominant cytotype	Latitude (degree)	Reference
Sri Lanka	pp	5-10	16
India			
Tuticorin	pp	5-10	12
Madras	pp	10-15	10
Kutch	pp	20-25	6
Israel			
Athlit	pp	25-30	7, 11
Kalia	pp	30-35	7, 11
Turkey			
Izmir	pp	35-40	2
Tunisia			
Megrine	sd	30-35	8, 9
Chott Ariana	sd	35-40	7
Eygpt			
Wadi Natrun	sd	30-35	8, 9
Libya			
unknown location	sd	25-30	8
China			
Dongfang	pp	15-20	14
Ying Ge Hai	pp	15-20	14
San Ya	pp	15-20	14
Xu Wen	pp	15-20	14
Zhanjiang	pp	15-20	14
Dong Fang Hong	pd	35-40	14
Nanwan	pd	35-40	13
Jime	pd	35-40	13
Yangkou	pd	35-40	13
Huanghuai	pd	35-40	13
Hangu	pd	35-40	13
Daqing He	pd	35-40	13
Gao Dao	pd	35-40	14
Xiao Tan	pd	35-40	14
Yengkou	pd	40-45	13
Spain			
Janubio	pd	25-30	3
Ayamonte	pd	35-40	3
Isla Cristina	pd	35-40	3
San Fernando	pd	35-40	3
San Fernando	sd	35-40	3
Cabo de Gata	pd	35-40	3
San Pedro del Pinatar	pd	35-40	3

Table 4-1 continued.

Locality	Dominant cytotype	latitude (degree)	Reference
Bras de Port	pd	35-40	3
Bonmati	pd	35-40	3
Calpe	pd	35-40	3
San Lucar	PP	35-40	14
San Lucar	sd	35-40	14
San Felix	sd	35-40	3
San Pedro del Pinatar	sd	35-40	3
Bras de Port	sd	35-40	3
Salinera Espanola	sd	35-40	3
Ibiza	sd	35-40	3
San Antonio	pp	40-45	3
Playa Tierzo	pp	40-45	3
Delta del Ebro	pp	40-45	2
Portugal			
Alcochete	pp	35-40	3
Italy			
Comachio	pp	40-45	7
Margherita di Savoia	pp	40-45	14
Santa Gilla	pd	35-40	2
San Bartollomeo	bd	35-40	7
France			
Sete	pd	40-45	5
Salin de Giraud	pd	40-45	2
La Palme	pd	40-45	15
Greece			
Citros, Pieria	pp	40-45	1
M. Embolon	pp	40-45	1
Yugoslavia			
Istria	pp	> 45	5
Soviet Union			
Odessa	pp	> 45	5

Note: pd: parthenogenetic diploid. pp: parthenogenetic polyploid. sd: sexual diploid. Populations which are considered to be transplanted are not included. Reference code: 1, Abatzopoulos et al. (1986); 2, Abreu-Grobois and Beardmore (1982); 3, Amat Domenech (1980); 4, Badaracco et al. (1987); 5, Barigozzi (1980); 6, Browne (1980); 7, Browne and Macdonald (1982); 8, Browne (1988); 9, Browne and Bowen (1990); 10, R.A. Browne, personal communication; 11, Goldschmidt (1952); 12, Vanhaecke et al. (1984); 13, Wang (1986); 14, Zhang (unpub data); 15, Gilchrist (1960); 6, Badaracco et al. (1991).

Table 4-2. Summary of Table 2. Distribution patterns of sexual diploid, asexual diploid and polyploid *Artemia* populations in the Old World.

Latitude (degree)	Number of sexual populations	Number of asexual populations		
		di-ploid	poly-ploid	percent polyploid populations
5-10	0	0	2	100
10-15	0	0	1	100
15-20	0	0	5	100
20-25	0	0	1	100
25-30	1	1	1	50
30-35	2	0	1	100
35-40	9	17	3	15
40-45	0	4	7	64
> 45	0	0	2	100
Sum	12	22	23	

Note: Populations with unknown ploidy compositions are not included.

Table 4-3. Approximate yearly average temperature, summer temperature, precipitation, and level of Artemia cyst supply for commercial market in each latitude during 1987-1990 along the China coast.

Name of coastal provinces	Average latitude (°N)	Yearly average temperature (°C)	Yearly average summer temperature (°C)	Yearly average precipitation (mm/year)	level of Artemia cyst supply for commercial market
Hai Nan	19	25.6	28	1425	no supply
Kan Tang	22	22.5	28	1500	no supply
Fu Jian	25.5	18.5	27.5	1350	no supply
Zhe Jiang	29	17	28.5	1275	no supply
Jiang Su	33	14.5	27.5	1000	little
Shandong	36	13	26	865	great
He Bei	38.5	8.5	24	600	great
Liao Ning	41	7	23	800	great

The meteorological information is obtained from National Meteorological Bureau of China.

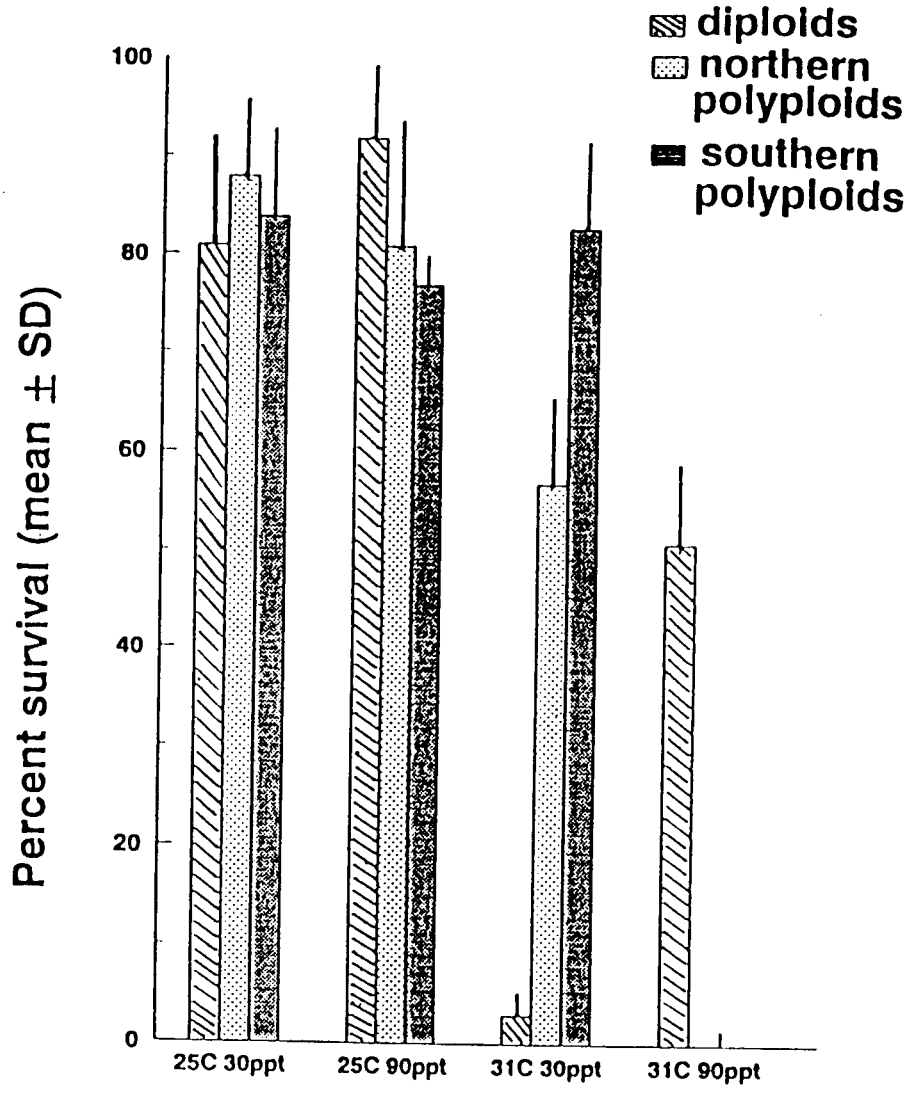

Fig 4-1. Percent survival of Chinese diploids and polyploids at different temperatures and salinities after 4 days' culture.

Chapter 5. Biochemical basis for the higher tolerance of stressful temperatures of polyploid than diploid *Artemia*

ABSTRACT

Polyploid individuals may have higher dosages of genes coding for enzymes or other proteins contributing to their higher thermal tolerance. To examine if this is the case, I first focused on Italian sympatric diploids and polyploids and examined their activities of lactic dehydrogenase (LDH), malate dehydrogenase (MDH) and citrate synthase (CS). At all three culture temperatures (15°C, 22°C and 30°C), diploids and polyploids had similar LDH activities. For MDH and CS, diploids had significantly higher enzyme activities than those of polyploids. So, polyploid individuals do not necessarily have higher enzyme activities than diploids.

Second, I asked whether polyploid individuals have higher levels of heat shock proteins than diploids. These proteins are generally viewed as being relevant to organismal thermotolerance. When endogenous levels of heat shock protein 70 (**HSP70**) were assayed using Italian *Artemia* cultured at 22°C, polyploids were found to have significantly higher **HSP70** levels than

diploids. This is also true when allopatric diploid and polyploid Artemia cysts were compared.

At 30°C, polyploids have a higher thermotolerance than diploids. However, at that temperature, Italian diploids and polyploids have similar **HSP70** levels. These results suggest that caution should be used in assigning adaptive significance to **HSP70** levels in Artemia.

INTRODUCTION

Increased thermal tolerance is probably one of the most important physiological consequences of polyploidisation in Artemia. This characteristic may have played an important role in extending the distribution range of the genus. However, the biochemical basis for the higher thermotolerance of polyploids than diploids remains unclear.

Two distinctly different genetic mechanisms may contribute to the differential thermotolerance of diploids and polyploids. First, it is known that polyploid individuals have higher levels of average heterozygosity than diploid individuals. This enables polyploids to be more biochemically diverse and be better able to cope with environment changes than diploids. I have reported in Chapter 3 that this appears to be the case in polyploid Artemia.

Second, polyploid individuals may have higher dosages of genes coding for enzymes. It is of interest to know if polyploids have higher rates of enzyme production which may possibly contribute to their higher thermal tolerance. Extensive studies in this area have been done in plants (reviewed by Levin 1983), yet the pattern is far from clear. These studies indicate that the ranking of enzyme activity between

diploids and autopolyploids depends not only on species, but also on the enzymes being considered. For example, when compared with its diploid progenitor, the tetraploid plant Lycopersicon esculentum had higher activity of malate dehydrogenase, but lower activity of peroxidase (Albuzio et al. 1978); in a ploidal series of n, 2n, 3n, 4n and 6n of plant Datura innoxia, Cullis and Davis (1974) found an absence of differential amplification of RNA cistrons with different ploidy levels. In animals, most research has been conducted with fishes. Loss of duplicated gene expression is a common phenomenon in salmonid and catostomid fishes where tetraploidisation occurred about 25-100 and 50 million years ago, respectively; approximately 50% of the additional loci created by tetraploidy are no longer detectable by their protein products (Allendorf and Thorgaard 1985; Ferris 1985). This phenomenon has also been reported in other tetraploid organisms such as frogs and cyprinids where overall levels of isozyme activity are comparable to those of diploid relatives (Beck and Pueyo 1970; reviewed by Levin 1983). These studies demonstrate the complexity of the effect of polyploidy on gene activity. However, most of the above studies were conducted at optimal temperatures, and few studies have examined enzyme activities of polyploids versus

diploids when animals are cultured at suboptimal high and low temperatures. Moreover, the activity of the enzymes studied may not be directly related to organismal thermotolerance.

The proteins which have been generally viewed as the most likely to be directly relevant to organismal thermotolerance are heat shock proteins (**HSPs**). **HSPs** are groups of highly-conserved proteins (with various molecular weights) that increase rapidly in concentration when organisms are exposed to heat shock (Lindquist 1986). It has been shown in many -- but not all -- species that **HSPs** are positively correlated with organismal thermotolerance, and the enhancement of their synthesis can also be elicited by many other kinds of stress (reviewed by Lindquist 1986). Although many studies have reported a positive correlation between **HSP** concentrations and organismal thermotolerance, there is only one direct study of this relationship; Sanchez and Lindquist (1990) demonstrated that deleting and re-inserting the **HSP104** (which has a molecular weight of 104 KD) gene influences yeast thermo-tolerance. Nevertheless, the striking positive correlation between **HSP** concentration and organismal thermotolerance in many species, the rapid increase of their synthesis when organisms are exposed to thermal stress, and the close relationship between their

induction temperature and the organism's environment have led many authors to hypothesize that **HSPs** may have played a crucial role in the acquisition of organismal thermotolerance (reviewed by Lindquist 1986). The ecological significance of heat shock proteins has recently been demonstrated in the field. Dietz and Somero (1992) provided the first example, using goby fish, that poikilothermic organisms vary the concentration of HSP90 on a seasonal basis, increasing the level in summer and decreasing the level in winter. Ulmasov et al. (1992) also reported that lizard species inhabiting the southern desert had a higher constitutive level of **HSP70** than their relatives in the middle and northern area in the former Soviet Union. These results strongly suggest that **HSPs** plays a crucial role in organismal adaptation to high temperature habitats, spatially or temporally. The production and the potential adaptive value of these proteins have not been investigated in any diploid-polyploid complex.

HSPs can be divided into several classes according to their molecular weight. The relative abundance of each **HSP** class varies from species to species (reviewed by Lindquist 1986). Miller and Mclennan (1988) examined **HSP** production in sexual Artemia franciscana

and found that the predominant **HSPs** were **HSP68** (belongs to **HSP70** family) and **HSP89** (belongs to **HSP90** family).

In this Chapter, I first examine activities of three enzymes (lactic dehydrogenase, malate dehydrogenase and citrate synthase) in Italian sympatric diploids and tetraploids that are cultured at different temperatures (low temperature of 15°C, optimal temperature of 22°C and high temperature of 30°C). Then I focus on **HSP70** and examine its level in Italian sympatric diploids and polyploids that were cultured at 22°C and 30°C. Allopatric diploid and tetraploid Artemia cysts were also examined to check if the findings of **HSP70** between sympatric populations also hold for allopatric populations (collected from China, India and France).

MATERIALS AND METHODS

Determination of enzyme activities of polyploids versus diploids at low (15°C), optimal (22°C) and high (30°C) temperatures.

To examine activities of LDH, MDH and CS of Italian polyploids versus sympatric diploids cultured at 22°C, ten 30-day old individuals of each ploidy were randomly selected and each individual was examined for

activities of the three enzymes following the methods presented in Appendix 3. One way-ANOVA was used to examine differences of diploids and polyploids at each temperature.

To examine enzyme activities of diploids and polyploids acclimated at 15°C and 30°C, 22°C-cultured 20-day old animals were transferred to 15°C and 30°C for a 14-day acclimation. At the end of acclimation at each temperature, ten individuals of each ploidy were randomly selected and each individual was examined for activities of the three enzymes following the methods in Appendix 3.

Determination of HSP70 levels of polyploids versus diploids cultured at optimal (22°C) and high (30°C) temperatures.

It is possible that polyploids have a larger quantity of **HSP70** even under optimal temperatures; this would enable polyploids to have higher survival rates than diploids when exposed to lethal heat shock, given that **HSP70** plays an important role in the acquisition of thermotolerance. To determine their constitutive level of **HSP70**, diploids and polyploids were raised under optimal conditions of 90 ppt salinity and 22°C. Ten individuals of each ploidy were randomly selected and

the **HSP70** level of each individual was examined following the methods of Appendix 4. Protein concentration of each protein sample was determined using the Bradford method (Pierce Company's product). 20 ug protein were loaded into each lane for each Artemia sample.

To examine if higher level of **HSP70** has long term ecological consequences in Artemia, I performed two experiments. First, I examined **HSP70** levels of Italian diploids and polyploids that had been transferred from 22°C to 30°C for two weeks of acclimation. Ten individuals of each ploidy were randomly selected and the **HSP70** level of each individual was examined following the methods of Appendix 4. 20 ug protein were loaded into each lane for each Artemia sample. Diploids and polyploids from this culture were also examined for tolerance of lethal high temperature by exposing them to 39°C for 5 minutes. Five replicates of ten individuals each were used for this experiment.

Second, I examined **HSP70** levels in two diploid cyst populations and two polyploid cyst populations. The diploid populations were collected from Yiangko Saltern of Shandong Peninsula, China (39°10'N), and from Salin de Giraud Saltern, France (43°24'N). The polyploid populations were collected from southern China (San Yia Saltern, Hainan Province. approximately 19°N), and from

southern India (Tuticorin, 8°50'N). The Chinese polyploid cysts were collected by Mr. Zhongwen Chen of Hianan Fishery Research Institute in 1992. The Chinese diploid cysts were collected by myself in the summer of 1987. The Indian polyploid cysts and French diploid cysts were provided by Dr. Patrick Sorgeloos of the Artemia Reference Center in Belgium. About 100 mg of Artemia cysts were decapsulated and cleaned following the method of Miller and McLennan (1988). After homogenization and centrifugation, supernatant was obtained. 30 ug of protein from each sample were loaded onto each lane of the SDS-gel. The **HSP70** level of each sample was determined following the protocols described in Appendix 4.

RESULTS

Enzyme activities of polyploids versus diploids at low (15°C), optimal (22°C) and high (30°C) temperatures.

Results of the comparison of diploids and polyploids for enzyme activities varied among enzymes. When cultured at all three temperatures, diploids and polyploids have similar LDH activities (fig 5-1a. one-way ANOVA, $P>0.05$); for MDH and CS, diploids have

significantly higher enzyme activities than those of polyploids (fig 5-1b and 5-1c, one-way ANOVA, $P<0.01$).

Since body weight variation also has effects on tissues enzyme activities (Somero and Childress 1980), body weight of each individual used for enzyme assay is also included (fig 5-1d), and its effects on my measurement will be discussed later. Fig 5-1d shows that at 15°C and 22°C, polyploid individuals have significantly greater body weights than those of diploid individuals (one-way ANOVA, $P<0.01$), although the difference is only about 1.5mg. At 30°C, differences in body weight are not significant (one-way ANOVA, $P>0.01$).

Constitutive levels of HSP70 of polyploids versus diploids at optimal (22°C) and high (30°C) temperatures

Three protein bands (72.5 KD, 68.7 KD and 50.9 KD) were detected using immunoblotting techniques (fig 5-2a). 68.7KD-protein and 50.9KD-protein bands can only be detected in the presence of primary antibody (fig 5-2a). Therefore, 68.7KD-protein belongs to **HSP70** family.

50.9KD-protein also reacts with primary antibody. This protein may have a similar epitope as **HSP70** and was therefore identified by primary antibody. Another

possibility is that 50.9KD-protein is derived from 68.7KD-proteins that were broken into smaller molecular weights during the process of sample treatment.

Results of immunoblotting analysis (fig 5-2b) clearly demonstrate that under optimal conditions of 25°C, Italian polyploids have significantly higher endogenous level of **HSP70** than the sympatric diploids; the endogenous levels of **HSP70** in diploid adult are too low to be detected under my experimental conditions. When diploid cysts from northern China and France are compared with polyploid cysts from southern China and India, polyploids have significantly higher endogenous levels of **HSP70** than diploids (Fig 5-2c).

When cultured at a high temperature of 30°C, the **HSP70** levels of diploids and polyploids are very similar (fiq 5-2d), yet polyploids have higher survival rates than diploids after exposure to a lethally high temperature of 39°C for 5 minutes (fig 5-2e).

DISCUSSION

Enzyme activities

My comparisons of enzyme activities between diploids and polyploids vary with enzymes. For activity of enzyme LDH, polyploids and diploids are not

significantly different, although the mean of the former is slightly higher than the latter. For citric acid cycle enzymes MDH and CS, diploids have higher activities than polyploids. These results hold even at suboptimal low and high temperatures. Therefore, polyploid Artemia individuals, although they presumably have higher gene dosage, may not have higher enzyme activities than diploid individuals.

One possible explanation for the above results is that differences in enzyme activities result from their differences in body size. Somero and Childress (1980) found in teleost fishes that the activity of LDH increases with increasing body size. For CS, the activity decreases with increasing body size. MDH show an intermediate pattern, varying among species, which may reflect the mixed aerobic and anaerobic functions of this enzyme. In my study, polyploid individuals are larger and heavier than diploids at 15°C and 22°C (fig 5-1d), therefore, I can not rule out the possibility that the difference of diploids and polyploids in CS activities at 15°C and 22°C are results of their differences in body size, and may have nothing to do with ploidy effect. However, this possibility is ruled out at high temperature (30°C) where polyploids and diploids have similar body weight, and yet diploids

have significantly higher activities of MDH and CS than polyploids.

An alternative explanation for the higher MDH and CS activities of diploids than polyploids is related to their culturing salinity. Animals used for enzyme assays had been cultured at 90 ppt which is commonly used to culture Artemia by most Artemia scientists. As mentioned in Chapter 2, at this salinity, polyploid Artemia develop much slower and are less fecund than diploid Artemia at all temperatures tested. In addition, at this salinity Italian polyploids produced most of their offspring as dormant cysts which are usually induced by unfavorable environmental conditions. So it is quite likely that a salinity of 90 ppt favors diploids and stresses polyploids. As a result, diploids develop faster and have higher enzyme activities.

Endogenous HSP70 synthesis of diploids versus polyploids

The endogenous level of HSP70 of Italian polyploids is significantly higher than that of sympatric diploids under optimal temperature. This is also true when polyploids from southern China and India are compared with diploids from northern China and France. Thus, a

higher level of **HSP70** is positively correlated with the higher thermotolerance of polyploids, as well as with their habitat temperatures. It is possible, but unproven, that the high level of **HSP70** of polyploid Artemia plays an important role in their higher thermotolerance.

After acclimation to 30°C for two weeks, although polyploids retain a higher thermotolerance (fig 5-2e), they do not significantly differ in **HSP70** levels (fig 5-2d). This suggests that **HSP70** is not the only factor that determines thermotolerance in Artemia. Other non-investigated heat shock proteins, such as **HSP90**, may also be involved in determining thermotolerance.

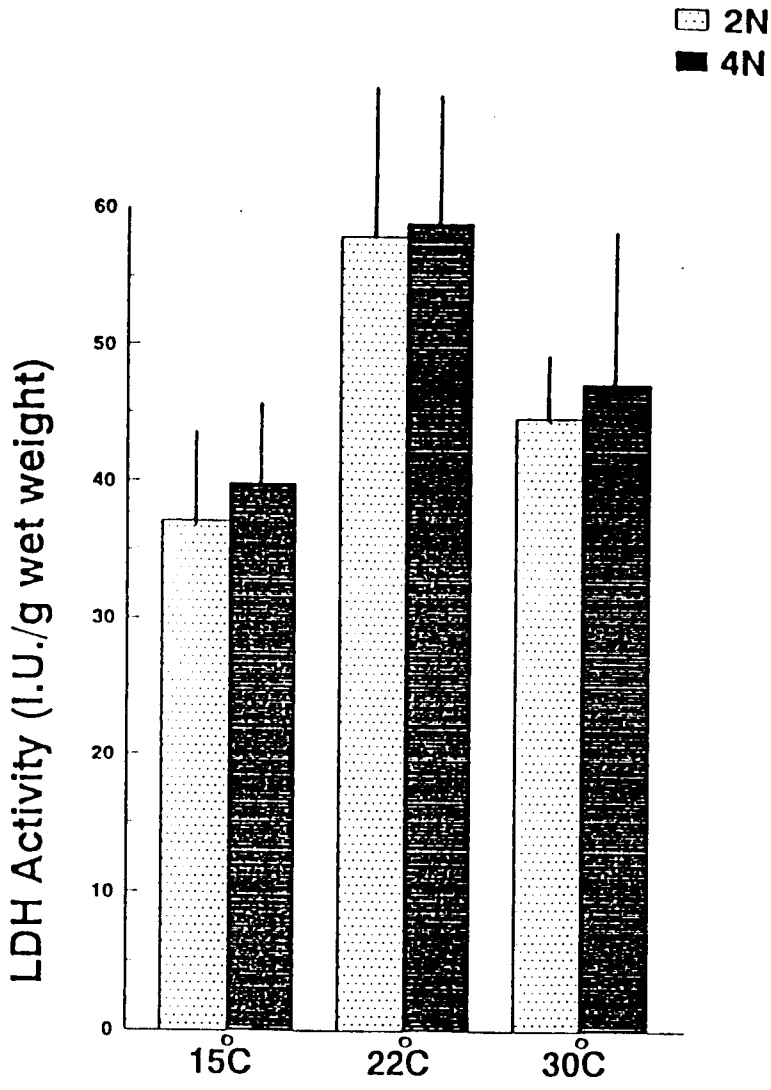

Fig 5-1a. Lactic dehydrogenase (LDH) activities (mean ± SD) of animals cultured at different temperatures.

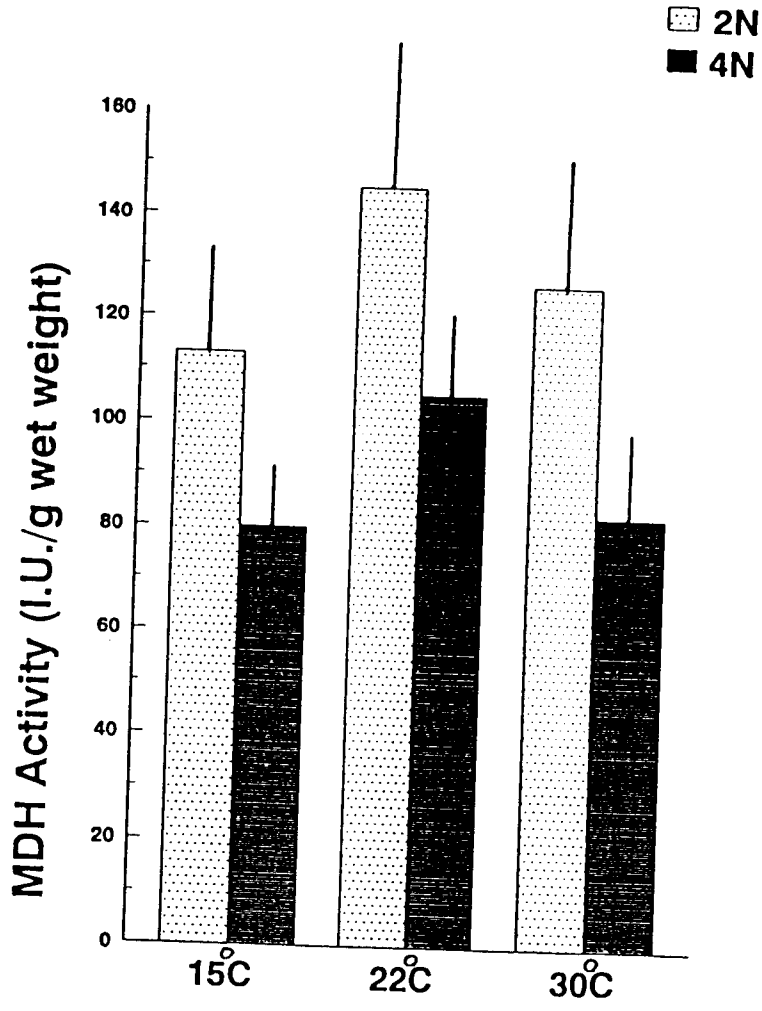

Fig 5-1b. Malate dehydrogenase (MDH) activities (mean ± SD) of animals cultured at different temperatures.

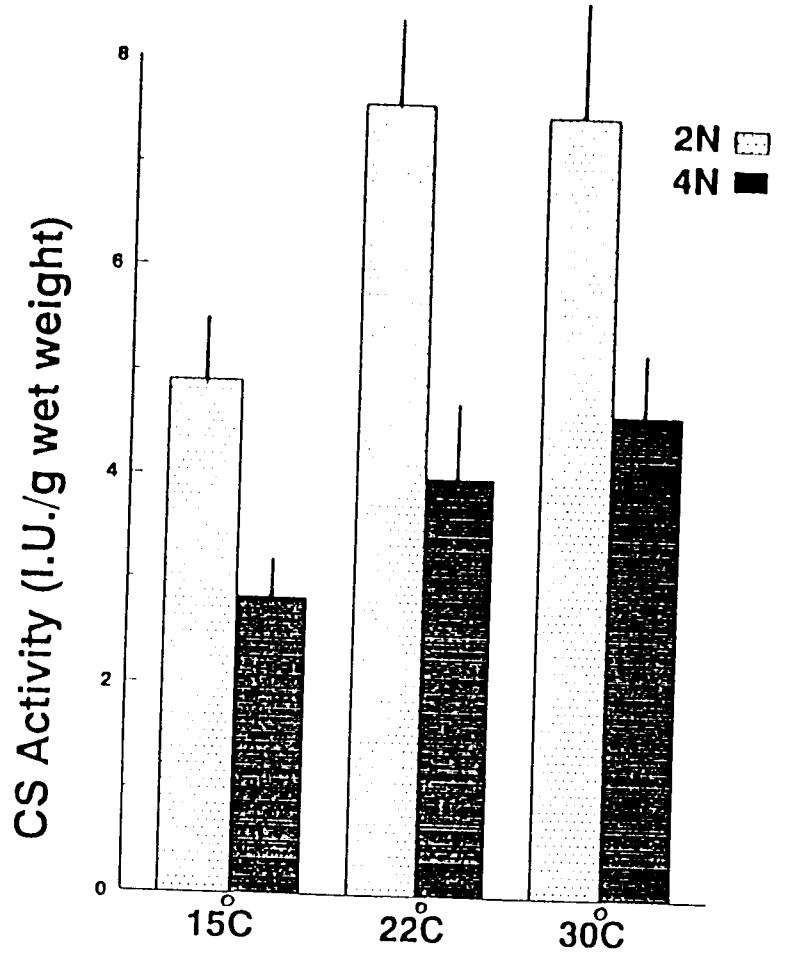

Fig 5-1c. Citrate synthase (CS) activities of animals cultured at different temperatures.

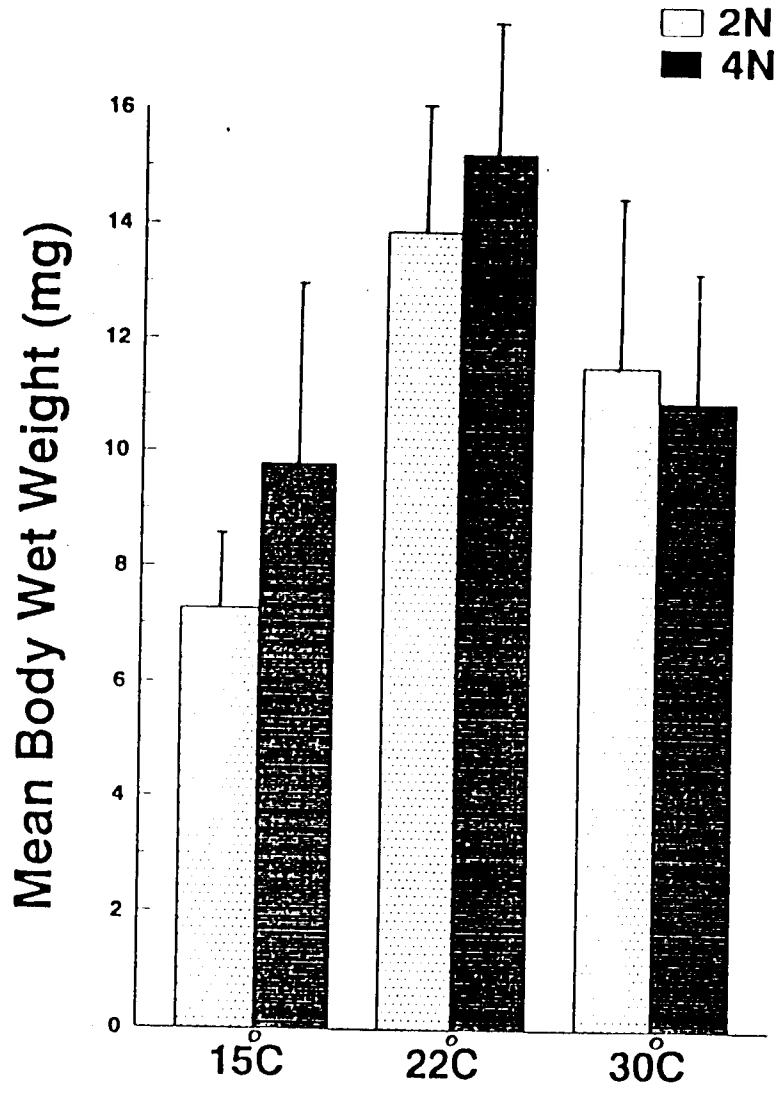

Fig 5-1d. Mean body wet weight (mean ± SD) of the animals used for enzyme assay.

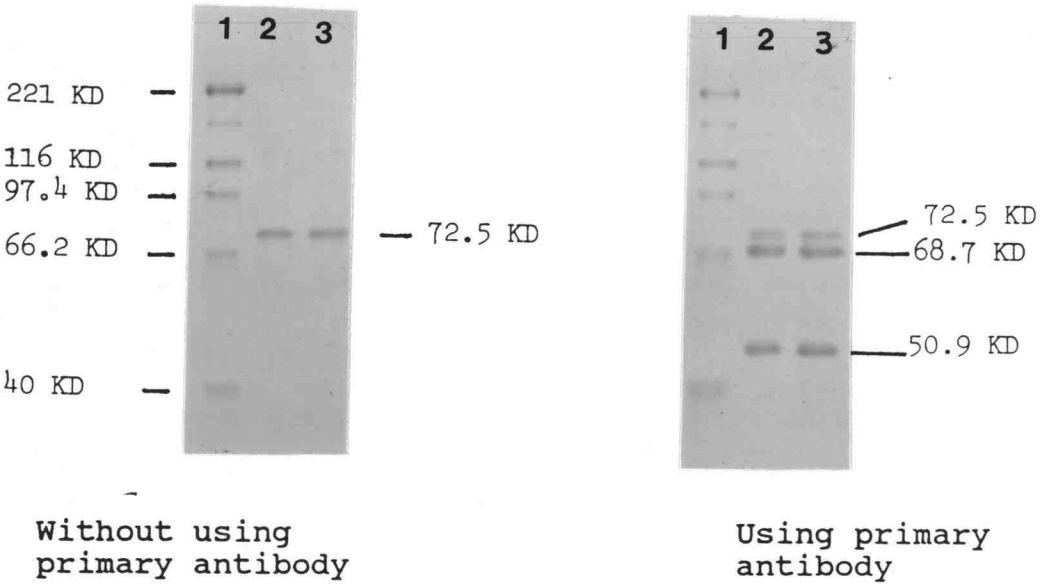

Fig 5-2a. Protein band patterns detected using immunoblotting techniques. Lane 1: biotinylated molecular weight standard. Lanes 2 and 3: each contains 20 ug protein sample from a 30°C-acclimated Artemia adult. Each lane involves different animals.

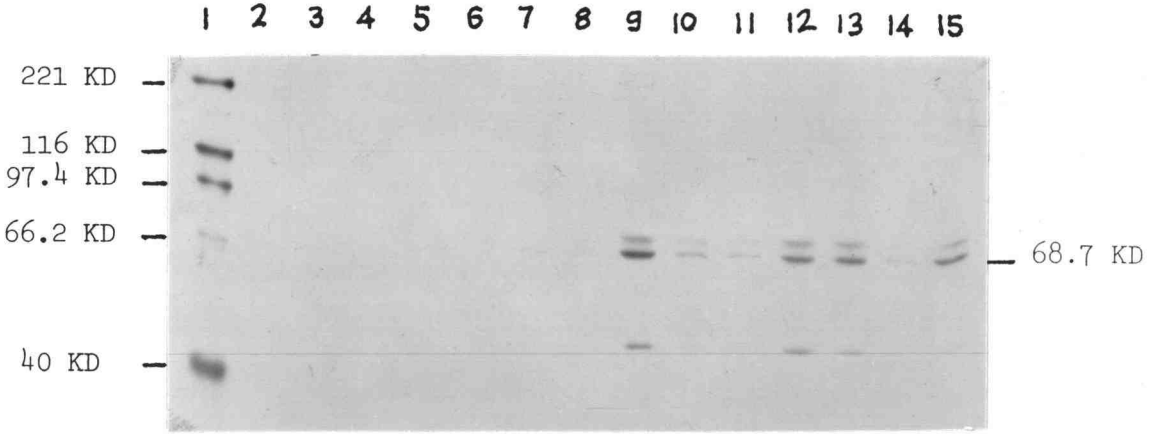

Fig 5-2b. Hsp70 levels of Italian diploid and tetraploid individuals cultured at 25°C. 20 ug protein was loaded into each lane for each Artemia sample (one individual is one sample). Lane 1: biotinylated molecular weight standard. Lane 2 to 8: diploid individuals. Lane 9 to 15: tetraploid individuals.

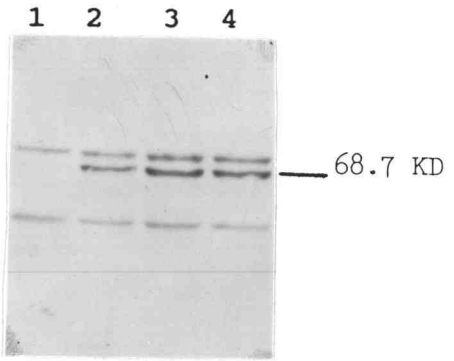

Fig 5-2c. Hsp70 levels of Chinese and French diploid cysts (lanes 1 and 2, respectively), and Indian and Chinese polyploid cysts (lanes 3 and 4, respectively). 30 ug protein was loaded into each lane for each cyst sample.

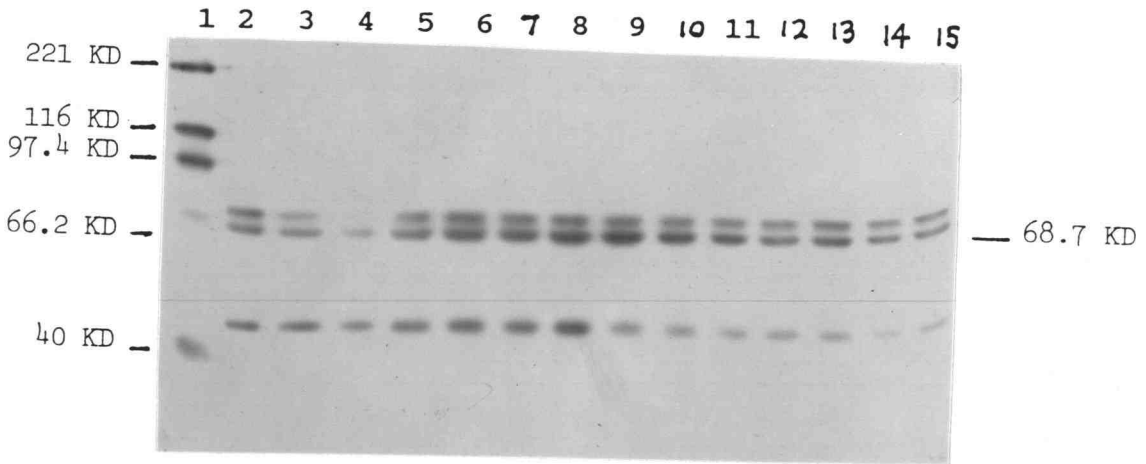

Fig 5-2d. Hsp70 levels of Italian diploid and tetraploid individuals cultured at 30°C. 20 ug protein was loaded into each lane for each Artemia sample (one individual is one sample). Lane 1: biotinylated molecular weight standard. Lanes 2 to 8: diploid individuals. Lanes 9 to 15: tetraploid individuals.

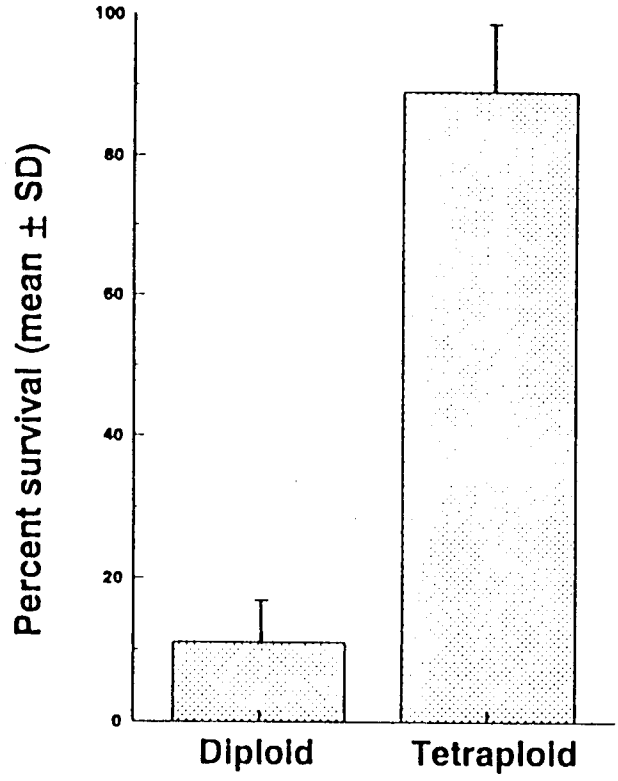

Fig 5-2e. Percent survival of Italian diploids and tetraploids that had been acclimated to 30°C for 2 weeks and were then exposed to lethal high temperature of 39°C for 5 minutes.

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APPENDICES

Appendix 1. Protocol for examining *Artemia* Chromosome

Immediately after hatching, *Artemia* nauplii are put into tap water with a colchicine concentration of approximately 0.1% and kept at room temperature for 1-2 hours. After fixation with Carnoy's fluid for 20 minutes, the nauplii are hydrolyzed in 1N HCl at 60°C for 15 minutes and then rinsed in distilled water for several minutes. Hydrolyzed nauplii are stained in 1% acetic-orcein (1 gm orcein in 100 ml 50% acetic acid) for a minimum of 30 minutes and are then transferred into 30% acetic acid for storage. Stained nauplii are mounted on microscope slides, covered with cover glass and squashed. Chromosome numbers of nauplii are counted under the microscope.

Appendix 2. Protocol for allozyme electrophoresis.

Cellulose acetate plate electrophoretic equipment was purchased from Helena Laboratories (P.O. Box 752, Beaumont, Texas, USA 77704-0752). The equipment kit includes a sample well plate (which has wells for 12 samples, Cat. No. 4096); cellulose acetate plates (onto which samples are loaded for electrophoresis; Cat. No. 3033); a sample application device (used to simultanously transport the 12 samples from the well plate onto a cellulose acetate plate; Cat. No. 4090); an aligning base (which supports the cellulose acetate plate when samples are being loaded, Cat. No. 4094), and an electrophoresis chamber (where the sample-loaded cellulose acetate plate is mounted and electrophoresis is run; Cat. No. 1283;). This technique was first designed for clinical use. I found that it works equally well with Artemia with only minor modifications from the protocol of Weider and Hebert (1987). The following protocols present the specific technical conditions used to resolve Artemia allozymes:

Running buffer, voltage, time and stain recipes:

Running buffer:

CAAPM: 10.5 g citric acid H_2O, 12.5 ml N(3-aminopropyl)-morpholine, make up to 1 L.

Tris Glycine: 15 g trizma base, 72 g glycine, make up to 1 L.

Stain buffer: 11.1 g trizma base, 87.5 ml 1 N HCl, make up to 1 L.

Gel Running Voltage, time and stain recipes:

LDH -- 150 V, 30 minutes. 1.0 ml tris HCl, 1.5 ml NAD (2 mg/ml), 10 drops 0.5 M Na lactate solution, 1 drop MTT (10 mg/ml), 1 drop PMS (2 mg/ml, added immediately before applying the stain to the plate), 2 ml of 1% agar (prepared with boiling water and held at 60°C during use).

MDH -- 280 V, 30 minutes. Stain in LDH staining solution except use 13 drops malate substrate (18 ml H_2O; 2 ml tris HCl, PH = 9.0; 0.4 g L-malic acid) to replace Na lactate solution.

ME -- 150 V, 60 minutes. Stain in MDH staining solution except use 1.5 ml NADP (2 mg/ml) and 2 drops of $MgCl_2$ (20 mg/ml) to replace NAD.

PGI -- 150 V, 120 minutes. Stain in LDH staining solution except use 5 drops fructose-6-phosphate (F6P, 20 mg/ml) and 5 ul G6PDH (about 270 units/mg) to replace Na lactate solution.

G6PDH -- 200 V, 60 minutes. 1.0 ml tris HCl, 1.5 ml NADP, 1.0 ml D-glucose-6-phosphate (20 mg/ml), 6 drops $MgCl_2$, 1 drop MTT, 1 drop PMS, 2 ml agar.

6PGDH -- 150 V, 120 minutes. Stain in G6PDH staining solution except use 6-phosphogluconic acid (50 mg/ml) to replace D-glucose-6-phosphate.

IDH -- 150 V, 60 minutes. Stain in G6PDH stain solution except use 15 drops isocitric acid (100 mg/ml)to replace D-glucose-6-phosphate.

GOT -- 150 V, 40 minutes. 3 ml solution A, 10 drops solution B, 2 ml agar. A: 10 ml 100 mM phosphate, pH = 7.0; 1 mg pyridoxal-5-phosphate; 46 mg L-aspartic acid; 26 mg ketoglutaric acid; adjusted to pH = 7.4 with NaOH. B: Saturated solution of Fast Blue BB salt.

One adult Artemia female was homogenized in 100 ul CAAPM running buffer solution by using a 1.5 ml Eppendorf tube and an epon pestle. Samples were stored on ice from the time of homogenization until they were used. The homogenized sample was centrifuged to

sediment detritus and the supernatant was removed and then held at -70°C for long-term storage. Frozen samples were thawed at 5°C, 9.5 ul of each sample was transferred to the sample well plate, and, finally, loaded onto a cellulose acetate plate with the sample application device. Prior to sample loading, the cellulose acetate plate was soaked before use for at least 20 minutes in the same buffer as was placed in the electrophoresis chamber. The sample-loaded cellulose acetate plate was mounted inside the electrophoretic chamber and was then run inside a 5°C refrigerator. Running time varied from 60 to 90 minutes, depending on specific enzymes and voltage. Following electrophoresis a volume of about 2 ml stain solution was mixed with 2ml 60-70°C 1.4% agar solution at 40°C. The resulting staining mixture was poured onto the cellulose acetate plate immediately after its removal from the electrophoretic chamber. Staining was conducted at room temperature in the dark to prevent photo-oxidation of the MTT and PMS. It usually took about 10 to 30 minutes for the stain solution to work. The agar overlay was then rinsed away with running hot water and the cellulose plate was dried in 50-70°C oven for at least 10 minutes for a long-term storage. The dried cellulose acetate plates can be clearly scored with the help of light viewing box.

The cellulose acetate plate can also be cut into one third or fourth of the original size to examine just one or two samples. The volume of staining solution can be reduced accordingly. Once the approximate position of the isozyme bands on the cellulose acetate plate has been known, the volume of the stain solution can be reduced to one half.

I initially looked for variations in 21 enzymes: lactate dehydrogenase (LDH), malic enzyme (ME), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glutamate oxaloacetate transferase (GOT), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucoseisomerase (PGI), aconitase (ACON), tetrazolium oxidase (TO), hexokinase (HEX), adenylate kinase (AK), phosphoglucomutase (PGM), glycerol-3-phosphate dehydrogenase (GPDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), mannose phosphate isomerase (MPI), fructose-1,6-diphosphatase (FRUCT), fumarate hydratase (FUM), aldehyde dehydrogenase (AO), esterase (EST) and alkaline phosphatase (ALP). Two running buffer solutions (tris glycine and CAAPM as suggested by P.D.N. Hebert) were tried. Among these enzymes, FRUCT, FUM and AO could not be detected and EST and ALP could not be adequately resolved in either of the the two buffer solutions. Except for AK and HEX, the remaining

enzymes were best resolved in the CAAPM buffer solution. AK and HEX were best resolved in tris glycine buffer solutions. The first 12 enzymes gave consistently good resolution. 9.5 ul homogenate is enough to make at least 5 electrophoretic samples. PGM, G3PDH, PGDH and MPI have very low activity when assayed by this technique and thus were not selected for further study. We selected the first 8 enzymes (table 1) to examine allozyme variation among the 106 Artemia in our sample. The following three pairs of enzymes can be stained together on the same cellulose acetate plate: MDH and LDH and ME were stained together using the MDH stain solution to which Na-lactate solution and NADP had been added; IDH and G6PDH were stained together using the G6PDH stain solution with the addition of isocitric acid; 6-PGDH and PGI were stained together using the 6PGDH stain solution with the addition of both NAD and F-6-P.

Appendix 3. Techniques used to measure enzymatic activities

(the following protocols are Somero lab's general protocol and were used with minor modifications with Artemia. (Somero and Childress 1980)).

a. remove the gut of an adult Artemia (41-day old) immediately before weighing the wet body weight.
b. after quickly removing the body surface water by drying the animal on a filter paper, obtain its wet body weight.
c. homogenize one animal in 10 volumes ice-cold 10mM-Tris/Cl (pH=7.5 at 20°C).
d. centrifuge for 5 minutes in microfuge (4°C) at top speed.
e. obtain supernatant and maintain on ice.
f. add 10 ul (25ul for CS) supernatant of each sample to 2 ml enzyme assay cocktail in a cuvette being held at the assay temperature of 20°C in a vv/vis spectrophotometer. A constant-temperature water bath that was connected to the cuvette-holder was used to maintain a constant temperature of 20°C at which enzyme activities were measured.
g. to measure activity of each enzyme for each animal, two measurements were conducted to get the average.

For malate dehydrogenase and lactic dehydrogenase, the activities were determined by measuring the disappearance of NADH at 340 nm via chart recorder and then translated to International Units. For citrate synthase, the activity was determined by measuring the increase of absorbance at 412 nm and then translated to International Units. Chart paper runs at a speed of 30 mm/min, and scale is 0-1. So change in Absorbance/min is calculated as 0.15*slope.

$$\text{MDH or LDH Rate (I.U)} = \frac{(\text{Change in Absorbance/min}) * 2 * 100}{6.22 * (\text{g wet weight of the animal})}$$

$$\text{CS Rate (I.U.)} = \frac{(\text{Change in Absorbance/min}) * 2 * 250}{13.6 * (\text{g wet weight of the animal})}$$

Recipes for each enzyme cocktail:

Malate dehydrogenase cocktail:

20 ml 0.2M Imidazole, pH =7.2 at 15°C

5ml 1M KCl

0.0053g NADH

0.0013g Oxaloacetic Acid

add Milli-Q water to a final volume of 50 ml.

Store on ice in a light-tight container.

Lactic dehydrogenase cocktail:

20 ml 0.2 M Imidazole, pH =7.2 at 15°C

5 ml 1M KCl

0.0053 g NADH

0.0275 g pyruvic acid

add Milli-Q water to a final volume of 50 ml.

Store on ice in a light-tight container.

Citrate synthase cocktail

25.5 ml 50 mM imidazole/HCl buffer (PH=8.0 at 10°C)

3 ml 15 mM $MgCl_2$ in 50 mM imidazole/HCl

0.0012 g 0.1 mM DTNB [5'5'-Dithiobis-(2-nitrobenzoic acid)]

0.003 g Acetyl-CoA

adjust to 30 ml with Milli-Q water

To measure activity of citrate synthase, put 2ml of assay cocktail in cuvette. Add 25ul protein homogenate to start reaction. Let this run for 2-3 minutes to measure background activity of DTNB. Then add 25ul oxaloacetic acid (fresh daily) solution (0.0053g/10ml 50mM Imid-Cl) to assay cocktail in cuvette.

Appendix 4. Protocol used to quantify heat shock proteins.

1. Artemia proteins of different molecular weights were separated using 10% SDS-PAGE as follows.
   a. Remove the gut of Artemia.
   b. homogenize individual Artemia in 100ul homogenizing buffer in a 1.5ml-microtube. This gave a total volume of about 120ul homogenates.
      homogenizing buffer:

      0.0625 M Tris-Cl (pH 6.8)
      2% SDS
      10% glycerol
      5% 2-Mercaptoethanol
      1mM PMSF.

   c. boil in 100°C water bath for 3 mins.
   d. centrifuge at 14000 rpm for 10 mins.
   e. obtain 70 ul of supernatant from each microtube. Protein concentration of each sample was determined using Coomassie Protein Assay Reagent (a commercial product of PIERCE). Protein samples were then kept at -70°C for storage. These samples were boiled in 100°C water bath for 3 mins before used for SDS-PAGE.

f. equal amount of protein (20 ug) was loaded into each lane of the 0.75mm-thick 10% polyacrylamide gel. In order to quantify and compare level of HSP70 in different samples, 1ug biotinylated molecular weight standard was also loaded into one lane. The SDS gel and running buffer were prepared according to manufacture's protocol (Hoefer Scientific Instruments). A constant current of 20 mA/gel was used. Electrophoresis was ended when the tracking dye just runs out.

2. **HSP70** was detected using immunoblotting technique.

Primary antibody (Monoclonal anti-**HSP70** gene family antibody IgG) was purchased from Affinity Bioreagents. This primary antibody was originally made in rat against Drosophila **HSP70** gene family and the cognates. It also recognizes **HSP70** gene family and cognates in yeasts, trypanosomes, mice, soybeans and mice (introduced by Affinity Bioreagents; also by Kurtz et al 1986). Biotinylated secondary antibody (Rat IgG, Cat No. PK-4004) was purchased as VECTASTAIN ABC Kit from Vector Laboratories, together with Avidin DH and Biotinylated Horseradish Peroxidase.

a. transfer the resolved proteins from gel to ImmobiLon membrane (constant current of 185 mA; 4°C; 1.5 hour)

Transfer buffer: 39 mM Glycine

48 mM Tris base

0.0375% SDS

20% Methanol

b. Immerse the membrane in 0.1% (v/v) Tween 20 in Tris-buffered saline (100 mM Tris, 0.9% NaCl, adjust to pH=7.5). This buffer is called TTBS hereafter. Incubate for 30 mins with gentle agitation.

c. Transfer the membrane to a solution of primary antibody (produced in rat) in TTBS. A dilution of 1:2000 was used because it gave the lowest non-specific bindings in my system. Incubate for 30 mins with gentle agitation.

d. Wash with 3-4 changes of TTBS, 10 mins each with gentle agitation. This step was to wash away the residual primary antibody that was not bound to **HSP70** and cognates on the membrane.

e. Transfer the membrane to a 2.5 ug/ml solution of biotinylated secondary antibody in TTBS. Incubate for 30 mins with gentle agitation. In this step, biotinylated secondary antibody (rat IgG)

recognized and bound to the primary antibody that had been bound to **HSP70** and cognates.

f. Wash with 3-4 changes of TTBS, 10 mins each with gentle agitation. This process was to wash away residual biotinylated secondary antibody that had not bound primary antibody.

g. Transfer the membrane to avidin-enzyme conjugate solution and incubate for 30 mins with gentle agitation. Avidin-enzyme conjugate solution was prepared according to Vector's Laboratories' instructions: mix Avidin DH and biotinylated horseradish peroxidase and allow 30 mins for complex formation before use. In this step, the biotinylated secondary antibody was connected to this avidin-enzyme conjugate.

h. Wash with 3-4 changes of TTBS, 10 mins each with gentle agitation. In this step, unbound horseradish peroxidase was washed away.

i. **HSP70** band was visulized using enhanced chemiluminancent method, a commercial product of Amersham company. The intensity of the band on the X-ray film can be used to quantify **HSP70**.