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The Graduate School
Eberly College of Science

POSSIBLE ADAPTIVE SIGNIFICANCE OF THE VISIBLE POYMORPHISM OF SPOTTED SALAMANDER EGG MASSES

A Thesis in

Biology

by

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Abstract

The spotted salamander, *Ambystoma maculatum*, has three types of egg masses. Two of these types, a white, opaque phenotype, and a clear, transparent phenotype, are widely distributed and often sympatric, whereas masses that appear grey, or intermediate between the two, are either scarce or absent in local populations. The degree of opacity of the egg mass depends on the concentration of white crystals (1-3 μm in length) of a small molecular weight hydrophobic protein (15,400 kD). These crystals are present in the outer jelly layer of the white and intermediate egg masses, but are absent in clear masses. Clear egg masses, and all inner jelly capsules of the three types of egg masses, contain a slightly lower molecular weight water soluble protein (14,400 kD). This protein is conspicuously absent from the outer jelly layers of white egg masses, but is found in outer layers of the intermediate masses. Glycosidic residues appear on the hydrophobic protein crystals of white egg masses from northwestern Louisiana, but not North Carolina or central Pennsylvania. The lowest molecular weight tissue polypeptide detected in females laying clear egg masses is slightly lower than that of females laying white egg masses indicating genetic differences exist between the morph types.

The frequency of the grey, intermediate egg masses over two years in central Pennsylvania, and the amount of crystalline protein they contained, was far lower than would be expected if they represented heterozygotes of a two alleles at one locus trait. The total number of egg masses and the frequency of the morph types per

pond was similar over two years in central Pennsylvania. The proportion of clear egg masses in ponds across portions of the range of *A. maculatum* has been shown to be positively correlated with the concentrations of some pond cations at the time the egg masses are laid. Pre-hatching survival and mean time to hatching were similar for embryos in white and clear egg masses exposed to low levels of light, the herbicide atrazine, partial freezing of the egg mass, temporary desiccation, low pH, and control conditions. The one exception was a lower mean time to hatching for embryos from white egg masses exposed to both low light and atrazine. Larvae from white egg masses were smaller in eight out of nine experimental trials, and this difference was significant for those egg masses incubated at low light, although a direct difference due to the presence of a symbiotic alga was not demonstrated.

This visible egg mass polymorphism involves two forms of one protein: one clear and readily soluble, the other crystalline and very hydrophobic. This trait should be considered as a significant variable in any studies that use developmental stages of the spotted salamander. Further experiments are needed to determine what if any adaptive significance this trait has. I postulate that it represents a means of adjusting larval size to compensate for heterogeneity in pond stress (i.e., bet hedging by producing either smaller, slower growing morphs more tolerant of stressful conditions, or larger, and faster growing morphs when conditions are less rigorous).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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INTRODUCTION

A striking polymorphism occurs in the egg jellies of the spotted salamander, *A. maculatum,* caused by the presence or absence of white crystals in the outer jelly of egg masses of this species. There are three identifiable types: (1) clear egg masses, where embryos (usually between 50 - 200) are clearly visible through the jelly matrix; (2) intermediate or grey egg masses, where the embryos are faintly discernable in a cloudy grey jelly matrix; and (3) white egg masses, in which the outer egg jelly is so opaque that embryos are rarely discernable. The coloration of the masses is most easily classified during the first week after laying. After extended incubation, algal growth and continued swelling of the jelly can obscure the distinction between white and intermediate morphs. The egg mass morph has been described briefly in some other papers (Banta and Gortner, 1914; Breder 1927; Bishop 1941; Salthe 1963; Tyning 1990). Hardy and Lucas (1991) were the first to clearly recognize the significance of this polymorphism, and identified the crystals as proteinaceous with approximately 1% glycosidic residues. Blem and Blem (unpub. obs.) and other authors sometimes refer to white egg masses but they incorrectly attribute the opaque coloration to fungal infestation.

The genetic basis of this phenomena is supported by two observations: (1) females in captivity never lay more than one type of egg mass (J. Bogart, per. comm.); and (2) the egg mass types are often sympatric in ponds, suggesting that environmental conditions are not the immediate cause. The phenotypes of the three morphs

(two extreme and one intermediate) suggest a simple co-dominant genetic model of two alleles at one locus; the white and clear egg masses could represent the two homozygotes and the intermediate type the heterozygote. Elucidation of any adaptive significance of the trait is complicated by the apparent lack of regional differences in distribution of white and clear egg masses, both morphs of which occur from Louisiana to Nova Scotia. Murphy (1961) noted that a caddis fly larva ate eggs of the spotted salamander. However, choice trials using this predatory larva, *Ptilostomis sp.*, did not demonstrate any preferences (W. Sadinski, B. Ruth, and W. Dunson, unpub. obs.). There are no other reliable accounts of predators that may consume eggs of the three morphs differentially.

A chlamydomonad algae, *Oophila amblystomatis*, is found within the inner jelly capsules of egg masses of several ambystomatid salamanders, and sometimes the frog *Rana sylvatica* (Gilbert, 1942, 1944; Hammen and Hutchison 1962; Biebal 1969; Gatz, 1973; and Goff and Stein, 1978). It has been shown to have a beneficial role on the development of larvae of *A. maculatum* (Breder, 1927; Gilbert, 1942, 1944; Hutchison and Hammen, 1958; and Hammen and Hutchison 1962). This may occur through the production of oxygen above the respiratory needs of the larvae (Bachmann *et al.*, 1986) although Hutchison and Hammen (1958) claimed earlier that oxygen has no role in the symbiosis. The algae is thought to benefit from nitrogenous excretion from developing embryos (Gilbert, 1942; Goff and Stein, 1978). If the amount of light reaching this algae is diminished by the opacity of the egg mass's

surrounding jelly to levels that might limit its photosynthetic activity, then the quality of the symbiosis could also be affected.

The spotted salamander has been recommended for use as a pollution bioindicator for the northeastern deciduous forest ecosystem (Freda *et al.* 1990). Its embryos have often been used in studies on the effects of atmospheric acidification on temporary ponds (for example, Pough, 1976; Nielsen *et al.*, 1977; Pough and Wilson, 1977; Tome and Pough, 1982; Clark and Hall, 1983; Cook, 1983; Dale *et al.*, 1984; Clark, 1986; Freda and Dunson, 1986; Ling *et al.*, 1986; Clark and LaZerte, 1987; Freda *et al.*, 1990; Ireland, 1991; Portnoy, 1990). In addition, the life history of this species has been studied repeatedly (for example, Stenhouse *et al.*, 1983; Walls and Altig, 1986; Sexton *et al.*, 1986; Stenhouse, 1987; Ireland, 1989; Philips and Sexton, 1989; Sexton *et al.* 1990). Thus it is surprising that the striking visible polymorphism of the egg masses has received such limited notice and it is important that further studies of the spotted salamander take it into account.

I have here estimated the total amount of protein in the jelly layers of the three egg mass types based on their nitrogen contents, and verified Hardy and Lucas's (1991) account of the causative agent of this polymorphism. I have also tested a series of hypotheses delineating the adaptive significance of this unusual trait. I have made, and solicited from others, egg mass counts and frequencies of the three types, both in central Pennsylvania and across portions of the range of *A. maculatum,* which we correlated with several pond water chemistry parameters. I made an indirect test of the two alleles at one locus model by measuring the amount of crystals in

white and intermediate egg masses. Morphometric characteristics of females (gravid and post gravid fresh mass, and length) that laid white or clear egg masses were compared. Properties of intact egg masses (hydration rates, number of embryos, wet volume, and cooling and heating rates) were measured. The effects of a symbiotic algae, light, and simulated episodic environmental variables on embryonic survival, time to hatching, and larval length were also determined.

MATERIALS AND METHODS

Determination of the amount of nitrogen in the egg jellies

Using Nessler's technique (as modified by Jensen, 1962), nitrogen concentrations were calculated as percentages of the egg jelly dry mass for four 10 mg samples of dried outer egg jelly from three egg masses of each morph type. White egg masses were collected near the university from two ponds in a valley area called the Barrens and one from a mountainous area in Rothrock State Forest, intermediates from three different ponds in the Barrens, and clear egg masses from one pond in Rothrock. All egg masses were collected during the last week of April.

Identification of the causative agent of the visible polymorphism

Samples of clear and white outer egg jelly were viewed under a light microscope at 400x. Samples of white egg jelly were stained separately using Fast Green (FCF), fluoro-dinitrobenzene, and Periodic Acid Schiff (PAS) reagent. Samples of acetone dried outer jelly (400 mg per egg mass) and inner jelly (< 50 mg per egg mass) from white, intermediate, and clear egg masses were re-hydrated in 30 ml of deionized water. The jelly was sonicated using a broad probe (tip diameter 0.75 cm) attached to a Heat Systems model W225 Sonicator, at power setting 8 for 0.5 to 2 minutes. After an

initial low speed centrifugation of 1,500 rpm for 5 minutes to remove debris, crystals from white and intermediate egg masses were purified and isolated using centrifugation at 15,000 rpm for 30 minutes, followed by rinses of the pellet with deionized water, re-suspension, and re-sonication. This cycle was repeated four times. Emulsified jellies from white, intermediate, and clear outer and inner egg mass layers along with centrifuged crystals from white and intermediate outer egg jelly layers were dissolved in 2 x SDS sample buffer (1:1 by volume) containing 2-mercaptoethanol and tracer dye, and run on 15% polyacrylamide thick slab gels (1.75 mm) against standard molecular weight markers following the procedure of Laemmli (1970). Modifications included the use of 50 µl of TEMED (N,N,N',N'-tetramethylenediamine) and 250 µl of ammonium persulfate for both stacking and separating gels, and the use of the Coomassie Blue staining solution to fix, as well as stain, the gels. Destaining solution was not used once deionized water was found to be adequate for this purpose. Samples of the white crystals extracted from the white and intermediate egg masses were sent to the Penn. State Biotech. Institute for determination of amino acid composition and verification of estimates of molecular weight based on polyacrylamide gel electrophoresis (PAGE). The Biotech. Institute used high performance liquid chromatography (HPLC) and generated a partial amino acid sequence of the white crystalline protein using an Applied Biosytems 477A Protein Sequencer. To correctly distinguish intermediate egg masses from white, samples of fresh outer jelly from all egg masses used in experimental trials were removed and placed in 1.5 ml microfuge tubes for subsequent PAGE.

Spermatophores of male spotted salamanders taken from four different locations in central Pennsylvania were also run on 15% PAGE.

Determination of the amount of white protein present in white versus intermediate egg masses

Crystals were isolated from 800 mg of dried powder from each of nine white and nine intermediate egg masses following the procedure used above, with the exception that only one centrifugation of 20,500 rpm for 1 hour followed the initial debris removing low speed spin. After pouring away the supernatant, the pre-weighed centrifuge tubes, with the pellet, were oven dried at 60^0C for one day, before re-weighing. The amount of the crystals was calculated as the percentage of the mass of the dried jelly.

Egg mass counts

The numbers of, and when possible, the time of laying of white, clear, and intermediate spotted salamander egg masses were enumerated in temporary ponds in two topographically dissimilar areas in Centre County, Pennsylvania, during the 1990 and 1991 breeding seasons. One area is located in the limestone valley of the Julian and Port Matilda 7.5' topographic USGS quadrangles (the Barrens - Gamelands 176) and comprised 25 study ponds in 1990

and 32 in 1991. Pond pH's were generally higher (Freda and Dunson 1986) than those of ponds in the sandstone ridges of the second area, Rothrock State Forest (Sadinski, 1991). In the latter area there were 24 study ponds in 1990 and 35 in 1991 in the Barrville quadrangle. Counts were made visually throughout the breeding period. In some cases there was doubt that all egg masses in a given pond had been counted and this was noted. Survey forms and water sample bottles were sent to herpetologists in New York, Massachusetts, North Carolina, and Virginia during the Spring of 1991. A visit to northwestern Louisiana to count egg masses and collect water samples was also conducted in early March

Pond water chemistry

To determine if correlations existed between pond chemistry and the percentage of different egg mass types (percentage of clear egg masses was arbitrarily chosen), water chemistry parameters were measured at the start of the amphibian breeding season in 27 ponds in the Barrens. Samples were taken throughout the breeding season in 32 ponds in Rothrock State Forest by C. Rowe and W. Sadinski for a related project. Water samples were collected in nitric acid washed polyethylene containers rinsed three times in the field, filled 3 cm below the surface, and then stored in an ice chest for transport back to the laboratory. Pond samples were replicated twice. An Orion model SA720 meter was used to measure the pH of water samples within 48 hours of collection. Conductivity was

recorded using a YSI model 32 Conductance Meter. A Perkin-Elmer model 2280 Atomic Absorption Spectrophotometer was used to measure the concentrations of Ca, Na, K, and Mg in samples filtered through 0.45 μm nitrocellulose paper. Dissolved organic carbon (DOC), PO_4, SO_4, NO_3, alkalinity (as bicarbonate), and Al, were determined in the Rothrock samples by EPA approved methods of the Water Analysis Laboratory of the Environmental Resources Research Institute at Penn State. Water samples from North Carolina, Louisiana, and the Pennsylvania Barrens area were analyzed with the following procedure: Tannin - lignin was estimated using a HACH Model TA-3 kit with a colorimeter wheel. Total inorganic phosphate was estimated using HACH 25 ml sample PhosVer 3 reagent pillows calibrated with 0, 1, 5, 10, and 25 μg/l PO_4 standards. Sulfate was measured using 10 ml sample HACH SulfaVer 4 powder pillows calibrated with 0, 1, 5, 15, and 30 mg/l standards. Solutions for both procedures were measured on a Beckman model 25 spectrophotometer. Values of R^2 were 0.977 to 1.000, and 0.986 to 0.999 for the PO_4, and SO_4 calibrations, respectively.

Characteristics of the female parent

Twenty-seven gravid females were collected from the two study areas in central Pennsylvania and allowed to deposit their eggs in captivity. Females were placed in 3 L plastic containers with water from one pond in the Barrens (J36) and a length of dowel rod on which to lay their egg masses. The first seven females were kept at

5^{0}C until laying; subsequent animals were kept in a 12:12 light/dark cycle in a cold room maintained at 11 - 12^{0}C. Females were weighed before and after laying and their length measured from snout to the anterior edge of the vent (SAVL). Time of laying of the egg mass, and in most cases gravid versus post laying wet body mass, were recorded for each female. In addition, the tissue proteins from a piece of tail taken from three frozen females that laid white and three that laid clear egg masses, were resolved on 15% PAGE.

Egg mass hydration, size of egg mass and number of embryos

The time since laying can be judged by the superficial appearance of the mass; smaller masses where little swelling has occurred and where the embryos are close together are younger. Within a few hours of being laid, egg masses obtained in the laboratory, or freshly laid in the field, were kept in pond water (J36) for five days to facilitate inoculation with the symbiotic alga, *Oophila amblystomatis*. For the next five days the egg masses were placed in artificial soft water (ASW) comprising 1 mg/l each of $CaCl_2$, KCl, $MgCl_2$ and NaCl (as in Freda and Dunson, 1985, plus $MgCl_2$). ASW had a conductivity of about 11 μ S/cm, compared to 16 μ S/cm for the J36 pond water. The volume of white and clear egg masses after five days in J36 water was compared to their volume after the additional five days in ASW to determine if any differences existed in hydration rates between white and clear egg masses. A sub-

sample of these, and other egg masses, were dissected to count the number of embryos per mass.

Effect of symbiotic algae and light levels on embryonic development

The effects of light, and the presence of the algal symbiont, *O. amblystomatis*, on larval development in white and clear egg masses, was investigated. The herbicide, atrazine, was used to inhibit algal growth (Pratt *et al.* 1988). Freshly laid egg masses (24 white and 24 clear) were collected from ponds in central Pennsylvania, or directly from females that laid in the laboratory. The design comprised two factors (light and atrazine) each with two levels and replicated three times. Two egg masses that had been laid on the same day were placed in each replicate in 5 L of ASW. Data from each replicate pair were pooled for analysis. To facilitate the counting and removal of hatching larvae, the egg masses were suspended in individual slings made from four ~2 x 20 cm strips of greenhouse shading cloth stapled to two pieces of dowel rod rested across the container. The containers were placed in a cold room maintained at 11-12^{0}C in a 12:12 light/dark cycle.

Low light (2.7 ± 0.4 µmol for both clear and white egg masses; mean ± SD) was achieved by interposing layers of greenhouse shading cloth held in place over the containers with clothes pins. Unshaded containers had a light level of 33.0 ± 7.1 µmol (mean ± SD). Light was provided by two 1 m long "cool" 40cW fluorescent strip lights for each of three tables of eight 7 l containers. Light level was

measured using a Li Cor Model LI-189 photometer held at the water surface of each egg mass container. Technical grade atrazine (96.8% pure), donated by Ciba-Geigy, was introduced into half the low light and half the high light containers, at a dose of 500 µg/l, a day before the egg masses were added. Hatching larvae were counted and removed daily to holding tanks (three per treatment). Time to hatching was expressed as the mean hatching day (MHD) calculated by multiplying each day since the egg masses had been laid by the number of larvae hatched on that day. Two weeks after the last larvae had been observed to hatch, the remains of the egg masses were dissected to obtain an estimate of both the total number of embryos per container and the pre-hatching mortality. Five days before the last larvae had hatched random sub-samples of larvae were taken from the holding tanks and their snout to vent length recorded using a digital micrometer.

Egg mass cooling and heating rates

Eight white and eight clear egg masses from one pond (J8) were placed in separate 350 ml plastic containers in ASW and randomly arranged on the bottom shelf of a -15^{0}C chest freezer. Temperatures in the center of four masses of each type were measured using 8 copper/constantan Type T 0.09 mm diameter insulated welded thermocouples connected to a Bailey model Bat 9 thermocouple monitor. All thermocouples were checked against boiling water and melting ice and found to deviate no more than 0.2^{0}C. This was

accounted for when recording the data. Temperature was recorded on the hour for three hours by which time the center of the mass was lower than 5^0C and ice had formed over the outside of the mass. Differences in cooling rate of white and clear egg masses were estimated by comparing the rate of change of temperature at each hour. All 16 egg masses were later incorporated into the environmental stress trial (see below). Dissection of the egg masses after the latter trial enabled estimates of embryonic survival before hatching.

Five white, and five clear egg masses from one pond (N) in Rothrock were paired according to approximate wet volumes (150, 175, 250, 325, and 375 ml), placed in a 3 L plastic container with 2 L of ASW, 30 cm underneath a Sylvania 250 W, 115-125 V, IR heat lamp. Temperature was recorded every five minutes with the same thermocouples used above placed at the center of the egg mass until a temperature of 40^0C was reached. The time taken to reach 40^0C and embryonic survival (determined by egg mass dissection one day after the trial) were compared to wet volume and egg mass morph.

Effect of extreme environmental parameters on embryonic development

Egg masses (40 white and 40 clear) were collected from pond J8. These egg masses were not freshly laid when collected so that an estimate of their time of laying was based on the mean laying time of the freshly collected egg masses that were used in the previous

experiment (noon of 26th March ± 1 day; mean ± SD). The experiment was designed to test the effects of episodic environmental extremes such as freezing and desiccation, as well as the chronic stress of acidification, on the development of larvae in white and clear egg masses. Egg masses were kept suspended in slings, described above, in 2 L of ASW. Five, non-factored treatments, each replicated eight times, comprised; (1) freezing (see the method above for cooling rates); (2) desiccation (selected egg masses were held at 11-12^{0}C for ten days in their containers without water); (3) low pH (pH 5.2 ± 0.8 and 5.1 ± 0.8, mean ± SD for white and clear egg masses respectively) measured weekly over four weeks; (4) a duplicate of the atrazine treatment used in the previous trial; and (5) a control. Acidification was achieved using diluted sulfuric acid added to each container once a week. If the pH were observed to rise 0.1 above that of the initially acidified ASW more sulfuric acid was added. Light was provided by one 0.5 m "cool" 40cW fluorescent tube for each shelf of ten containers (eight shelves, each treatment represented once on each shelf). The mean light level for each container was 7.0 ± 2.0 µmol, much lower than the high light treatment used above, but more than sufficient to allow the symbiotic algae to develop. The egg masses were kept in a cold room at between 11 and 12^{0}C at all times. Measurements of MHD, total embryo number per egg mass, survival, and larval length were recorded in the same manner as above, with the exception that the first sample of larvae was taken nineteen days before the last larva hatched.

RESULTS

Determination of the amount of nitrogen in the egg jellies

There were no significant differences in nitrogen as a percentage of dry outer egg mass jelly among the morphs (white 9.59 ± 0.96%, intermediate 8.81± 0.31%, and clear 9.02 ± 0.35%; mean ± SD; n=9), though the variance associated with white egg masses is around three times as large as that of intermediate and clear masses.

Identification of the causative agent of the visible polymorphism

Microscopic examination revealed the presence of many small crystals (1-3μm) scattered throughout the outer jelly of white and intermediate egg masses. These crystals were not observed in the inner jelly capsules of these egg masses, or in any of the jelly layers of clear egg masses. Staining of dried jelly with fluoro-dinitrobenzene, Fast Green FCF, and periodic acid Schiff Reagent, suggested the crystals were proteinaceous and non-glycosidic. PAGE showed two apparently related proteins in the outer and inner egg jelly layers of the three morphs (Fig. 1). The crystalline protein had a molecular weight of around 15,400 kD, and was found only in the outermost jelly layer of white and intermediate egg masses. Another protein of about 14,400 kD, absent only in the outer jelly layers of the white egg masses, was present in preparations of inner jelly

layers and all clear egg mass jelly layers (Table 1). The outer jelly layers of intermediate egg masses contained both of these proteins (see Fig. 1 and Table 1). The outer egg jelly of white egg masses used in the gel in Fig. 1 represent concentrates of the crystalline protein, all subsequent gels of non-treated white and intermediate outer jelly, however, showed the fixed difference indicated. Amino acid composition of the white crystalline protein is similar for samples from Pennsylvania and Louisiana (Table 2). Partial amino acid sequences of the N-terminal end of the white crystalline protein from both localities, however, are the same for the first three residues only: ALA PRO VAL GLY(?) ALA PRO PHE(?) PRO ALA ALA for the Pennsylvania sample and ALA PRO VAL TYR SER ALA ISO THR PRO GLY, for the northwestern Louisiana sample. No low molecular weight proteins were detected from male salamander spermatophores using PAGE.

Determination of the amount of white protein present in white versus intermediate egg masses

Expressed as percent of dry masses, outer jelly of white egg masses was shown to have about five times as much crystalline protein as that of intermediate egg masses (6.14 ± 3.19%, and 1.17 ± 0.39%, respectively). Again, the variance of the white egg masses was considerably more than that of the intermediate forms suggesting variation in total protein in white egg masses may be attributed to variation in the amount of

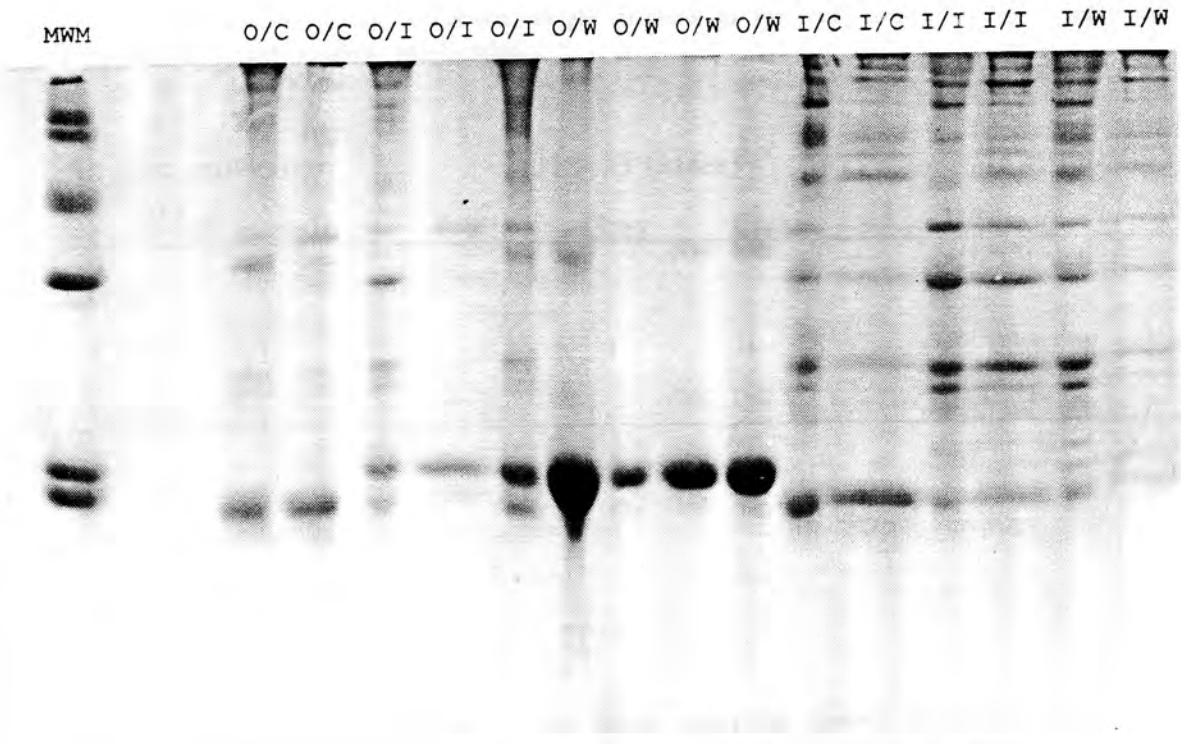


Fig. 1. PAGE of the outer and inner jelly layers of clear, intermediate, and white egg masses of the spotted salamander.

KEY: MWM = molecular weight markers
O/C = outer egg jelly of clear egg masses
O/I = outer egg jelly of intermediate egg masses
O/W = outer egg jelly (sonicated and centrifuged to concentrate crystals) of white egg masses
I/C = inner egg jelly of clear egg masses
I/I = inner egg jelly of intermediate egg masses
I/W = inner egg jelly of white egg masses.

Table 1. Occurrence of low molecular weight proteins in two jelly layers of three egg mass phenotypes of the spotted salamander.

Approx. molecular	J1 - J3 (inner)			J5 (outer)		
weight of protein	White	Grey	Clear	White	Grey	Clear
14,400	+	+	+	-	+	+
15,400	-	-	-	+	+	+

Table 2. Amino acid composition of the white crystalline protein detected using HPLC. Values on the left are those from central PA samples analyzed by the Biotech Institute. Values in parentheses are those of Hardy and Lucas for white egg masses from Louisiana.

Residue	Frequency		Residue	Frequency	
ASP	19	(12)	GLY	15	(19)
GLU	6	(8)	ALA	14	(18)
LYS	12	(7)	VAL	6	(8)
ARG	4	(6)	LEU	9	(8)
HIS	1	(1)	ISO	6	(10)
SER	13	(15)	MET	1	(1)
THR	10	(9)	PHE	7	(8)
PRO	9	(10)	TYR	8	(7)
CYS	0	(0)			
			TOTAL	142	146

crystalline protein. Denaturing (SDS) PAGE verified that their was no confusion in identifying the two phenotypes so that no intermediate egg masses were mistakenly identified as white egg masses.

Egg mass counts

In a given pond the total number of egg masses and the percentage of clear egg masses were highly correlated in 1990-1991 (Fig.s 2.A and 2.B). Out of 2,292 egg masses counted in the Barrens during the spring of 1990, 33% were clear (no distinction had been made between white and intermediate egg masses in 1990). At the same time in Rothrock State Forest, 35% of 1,263 egg masses were clear. Of 4,715 egg masses counted in the Barrens in the spring of 1991, 71% were white, 3% were intermediate, and 26% were clear. Out of 4,047 egg masses counted in Rothrock State forest in the spring of 1991, 59% were white, 15% were intermediate, and 26% were clear. Thus white egg masses were the predominant morph type in central Pennsylvania, although there were a few ponds with the proportion of clear egg masses above 50% (Table 3).

Based on a simple genetic model of two alleles at one locus, and a population in Hardy-Weinberg equilibrium the expected number of intermediate masses (hypothesized heterozygotes) out of the total for the Barrens in 1991 would be 1,867, which is significantly higher than observed (χ^2=3,974, p O=E, <0.0001). For the 1991 Rothrock counts, the expected number of intermediate egg masses would be 1,804, also higher than observed (χ^2 = 1861; p O=E, <0.0001).

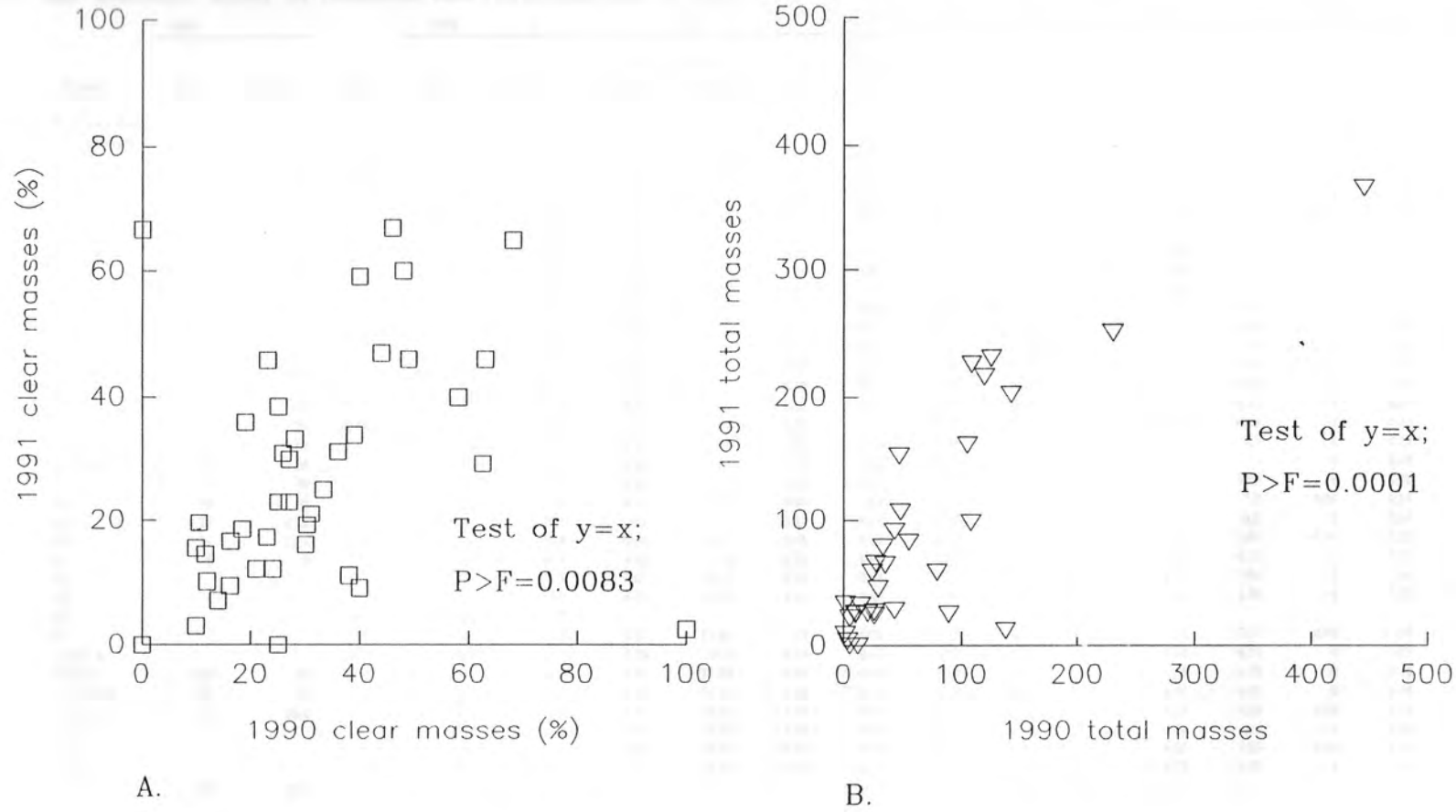


Fig. 2. Egg mass counts from 37 ponds in central Pennsylvania from 1990 to 1991; A. Percentage of clear egg masses; B. Total number of egg masses.

Table 3. Spotted salamander egg mass counts in central Pennsylvanian temporary ponds along with water chemistry parameters and additional counts in Louisiana and North Carolina in 1991.

	1990			1991													
Ponds	Total	%Clear	Date	Total	%Clear	pH microS/cm	Cond. microS/cm	Ca mg/l	Na mg/l	K mg/l	Mg mg/l	T/L mg/l	DOC mg/l	SO4 mg/l	PO4 mg/l	Al mg/l	Alk CaCO3mg/l eq.
Rothrock State State Forest																	
A'	1	100	4/24/91	33	2.5	5.78	26.62	1.12	0.4	0.82	2.69		3.61	8.03	0	0.04	7.27
A	29	48	4/23/91	45	60	6.05	50.73	4.54	0.45	0.8	2.39		2.17	10.33	0.01	0.01	13.47
B'	3	33.3	"	4	25	4.17	38.47	0.29	0.29	0.64	0.51		5.72	7.43	0	0.6	0
B	444	54.3	"	366	26.2	4.35	28.43	0.42	0.26	0.47	0.43		8.38	4.7	0.01	0.32	0
B-1			-----	74	39.2												
N			4/23/91	154	24.7	4.42	30	0.31	0.53	0.28	0.67		2.86	6.17	0	0.39	0
O	105	16.2	"	162	9.3	4.32	32.22	0.36	0.48	0.32	0.77		3.78	6.3	0	0.33	0
P	33	30.3	"	78	19.2	4.39	30.83	0.27	0.5	0.42	0.69		3.24	6.7	0	0.59	0
Q	120	11.7	"	216	14.4	4.61	23.35	0.55	0.48	0.36	0.83		2.71	4.97	0.01	0.29	0
R	27	18.5	"	65	18.5	4.29	32.8	0.22	0.47	0.16	0.59		3.13	6.83	0	0.38	0
S	35	22.9	"	64	17.2	4.4	29.58	0.22	0.46	0.26	0.72		2.7	8	0	0.73	0
T	43	16.3	"	91	16.5	4.35	30.08	0.27	0.46	0.34	0.68		3.45	6.5	0	0.47	0
U			"	30	13.3	4.27	35.45	0.38	0.41	0.42	0.85		4.34	6.07	0	0.4	0
U'	5	0	"	0	0	4.22	36.6	0.25	0.46	0.42	0.85		4.72	8.57	0	0.67	0
CROW 1	12	25	4/24/91	0	0	4.3	32.07	0.28	0.4	0.32	0.57		4.53	6.53	0	0.43	0
CROW 2	10	10	"	26	15.4	4.44	28.47	0.38	0.42	0.38	0.72		3.56	6.1	0	0.25	0
CROW 3	14	36	"	32	31.3	4.38	30.23	0.4	0.43	0.34	0.67		3.76		0	0.27	0
C1	5	40	"	22	59.1	4.52	31.5	0.86	0.76	0.4	0.7		2.89	7.43	0	0.21	0
C2	10	30	"	25	16	4.37	35.17	0.86	0.4	0.31	0.99		4.69	7.8	0.01	0.14	0
C3	24	62.5	"	58	29.3	4.5	33	1.17	0.32	0.52	0.61		4.6	8.5	0	0.44	0
C4	26	23.1	"	24	45.8	4.45	34.92	0.41	0.39	0.43	0.57		3.5	9.03	0.01	0.82	0
I	1	0	"	9	66.7	4.38	27.2	0.24	0.47	0.22	0.64		3.56	4.17	0	0.21	0
H			"	387	41.9	4.67	21.2	0.34	0.41	0.39	0.74		2.53	5.03	0	0.16	0
H'			"	337	23.7	6.14	12.23	0.41	0.44	0.49	0.99		2.28	1.53	0	0.02	2.07
D			-----	25	72												
E			"	456	45.8	5.16	25.03	1.8	0.35	0.37	0.74		4.52	7.67	0.01	0.118	0.01
MAC 6			"	108	24.1	4.38	27.2	0.24	0.47	0.22	0.64		3.56	4.17	0	0.21	0
BAR 1	143	28		202	33.3	4.93	25.28	0.81	0.44	0.56	1.59		3.64	6.37	0	0.16	0
CROW 0	20	25		26	38.5	4.24	35.35	0.31	0.43	0.18	0.64		4.44	6.43	0	0.34	0
BAR 2	47	10.6		153	19.6	5.13	25.64	1.19	0.37	0.63	1.65		5.57	7.03	0.01	0.19	0.27
BAR 3				105	16.2	6.21	34.45	1.86	0.49	0.45	4.08		6.47	5.6	0	0.09	9.9
BAR 4				75	32	4.66	28.22	0.67	0.44	0.66	1.59		4.52	7.23	0.01	0.24	0
BAR 5				38	28.9	4.83	26.63	0.78	0.49	0.54	1.93		3.57	7.47	0	0.13	0
BAR 6	109	35.8		226	31												
Barrens																	
PM1	55	14	4/16/91	82	7	5.7	13	0.91	0.38	1.05	0.69	3		0	2		
PM2	154	12	4/16/91	39*	10	5.3	20	1.69	0.41	1	1.24	3.1		38	0		
PM4	299	10	4/16/91	87*	3	5	22	1.33	0.3	2.15	0.79	4.1		32	10		
PM15			4/16/91	38	21	5.9	41	1.02	0.45	3.26	0.76	3.2		38	13		
PMC			4/16/91	50	22	5.5	17	1.21	0.54	1.14	0.88	2.8		35	5		
PMD			4/16/91	106	20	5.6	18	1.59	0.32	1.53	0.77	4		33	2		
PMG			4/16/91	81	21	4.7	22	0.59	0.44	0.71	0.6	2.8		42	3		
PMH			4/16/91	375*	18	5.3	15	0.53	0.55	0.83	0.53	3.1		37	4		
PMI			4/16/91	327	13	5	18	1.26	0.33	1.16	1.26	3.2		36	3		
PMJ			4/16/91	160	13	4.7	19	0.48	0.57	0.53	0.48	3.2		38	2		
PMK			4/16/91	176	11	5.2	15	0.84	0.29	0.81	0.84	2.2		36	4		
PM13			4/24/91	110	11	8	17	1.21	0.29	1.78	0.69	1.7		41	6		
J1	284	33	4/24/91			6.4	87	8.74	0.34	1	5.6	1.2		37	2		
J2	44	27	4/24/91	43*	30	6	22	2.3	0.35	0.82	1.3	3.1		11	3		
J5	48	25	3/21/91	107	23												

Table 3 contd.

	1990			1991										
Ponds	Total	%Clear	Date	Total	%Clear	pH microS/cm	Cond. microS/cm	Ca mg/l	Na mg/l	K mg/l	Mg mg/l	T/L mg/l	SO4 mg/l	PO4 mg/l
J6	108	27	3/21/91	98	23									
J7	171*	39	4/24/91	140*	34	5.8	20	2.5	0.32	1.4	0.94	2.7	10	4
J8	230	49	3/21/91	251	46									
J10	79	58	4/24/91	58	40	5.9	20	2.3		1.25	0.82	2.5	23	3
J19	58	19	4/24/91	97*	36	6.1	16	1.58	0.8	0.88	0.9	2.5	28	0
J22	25	44	4/24/91			6	22	3.31		1.14	1.11	3.2	44	2
J23	37	38	3/27/90											
J14	26	31	4/24/91	27	21	6	19	1.4	0.38	0.95	1.1	2.3	30	1
J16	89	24	4/24/91	25	12	6.1	19	1.7	0.22	1.13	1.03	2.1	31	2
J17	22	32	3/24/90											
J21	138	46	4/24/91	12	67	6.1	21	2.5	0.7	0.83	1.53	3	50	2
J24	43	44	4/24/91	74*	47	6	34	4.24	0.81	2.16	1.1	1.5	51	3
J27	43	63	4/24/91	28	46	6.2	13	2.3	0.6	0.83	0.62	1.1	26	1
J30	30	40	4/24/91	57*	9	6.2	14	2.8	0.63	1.14	0.6	2.1	37	1
J36			4/24/91	922*	27	5.8	16	3.1	0.62	0.3	0.99	3.2	34	2
J38a	126	21	4/24/91	231	12	6.1	13	3.53	0.7	0.43	0.87	0.1	35	2
J38b			4/24/91	128	36	6.1	19	3.96	0.67	0.69	1	0.5	32	2
J39			4/24/91	163	47	6.2	54	7.7	1.2	0.52	2.36	1.4	47	2
J40	59	66	4/24/91			6.4	54	7.2	1.1	1.43	3.5	1.2	47	4
J41			4/24/91			6.3	20	3.45	0.99	0.75	1.17	2	38	4
HV1	53	68	4/24/91	158	56	5.8	32	3.02	0.98	0.57	0.75	1	77	4
HV2	32	38	4/24/91	74*	65	5.6	26	3.1	1.01	0.74	0.63	1.6	64	4
GK1			4/24/91	19*	11	5.6	32	3.6	1.2	1.13	1.45	0.6	59	3
GK2	39	23	4/24/91			5.6	33	3.42	1.4	1.18	1.45	2	49	4
Louisiana														
L1a			3/5/91	19	100	6.2	56	0.94	1.39	7.91	1.5	1	4	3
L1b			3/5/91	0	0	6	32	3.03	1.39	3.12	1.85	1.4	1	2
L2			3/5/91	25	100	5.9	31	1.45	1.94	3.59	1.7	2	5	3
L3			3/5/91	15	100	5.8	30	3.26	3.03	3.96	2.07	1.2	2	2
L4			3/5/91	20	100	5.9	30	2.95	1.31	2.81	1.93	1.4	2	1
L5			3/5/91	17	52	6.2	36	1.11	2.53	2.18	2.28	0.6	3	1
L6			3/5/91	19	37	6.7	82	3.19	3.62	3.79	4.76	0	24	1
L7			3/5/91	36	33	6.4	30	1.08	2.67	1.87	1.71	1.2	16	2
L8			3/5/91	3	0	6.2	28	1.02	13.42	1.97	1.65	2.2	7	1
L9			3/5/91	7	0	6.8	67	2.93	4.09	4.57	3.1	1.4	17	1
L10			3/5/91	22	0	5.9	40	2	1.13	4.72	2.11	2.2	1	1
L11			3/5/91	23	0	6.2	52	1.63	2.72	3.87	2.47	1.4	2	1
North Carolina														
SM1			3/21-3/91	62	100	6.2	59	2	2.29	4.79	4.02	0	16	14
SM2			3/21-3/91	57	37	5	34	1.35	2.33	4.04	1.02	2.8	0	6
SM3			3/21-3/91	50	76	6.1	45	1.2	2.83	6.75	2.24	0	9	4
SM4			3/21-3/91	11	82	6.2	50	1.85	1.63	6.61	2.03	2	8	2
JIM1			1/3/91	13	0	6.5	99	1.57	11.64	5.03	2.46	1.8	18	6
JIM2			1/3/91	43	33	6.8	44	1.07	2.85	5	1.82	0.6	23	4
JIM3			1/3/91	7	100	6.7	56	1.77	3.08	3.05	3.57	1.2	25	5
JIM4			2/3/91	20*	75	6.6	82	3.56	4.42	3.39	5.37	0.6	34	5
JIM5			2/3/91	26*	61	6.5	41	1.53	2.11	4.42	1.85	1.8	20	2

* Incomplete counts.

The ratio of clear to white plus intermediate egg masses in central Pennsylvania was 1:3 in 1990, and 1:4 in 1991. Counts collected from North Carolina were 1:1.5 (out of 289 egg masses), and were 1:2 from Louisiana (out of 206 egg masses, Table 3). Additional information from correspondents in New York, Massachusetts, Virginia, and Nova Scotia suggested that ponds with breeding populations of spotted salamanders may contain from 0 to 100% clear egg masses. Since the total numbers of egg masses recorded were low in these cases the data were omitted from any further analysis.

Pond water chemistry

Multiple regression of the percentage of clear egg masses found at the time of water sample collection against pond chemistry, yielded correlations which varied with sites surveyed. The combined data from the two central Pennsylvania locations showed that the percentage of clear egg masses was positively correlated with [Na] ($p>F=0.018$), [Ca] ($p>F=0.072$), and [Mg] ($p>F=0.0018$). Although the percentage of clear egg masses in a small sample of ponds from the North Carolina piedmont, the North Carolina coastal plain, and north western Louisiana didn't correlate positively with any chemical parameters (after allowing for nine comparisons of interest) when data from all of these regions are pooled other correlations become apparent (see Figs. 3-5). The percentage of clear egg masses was positively correlated with [K] ($p>F=0.009$), and nearly so with [Ca]

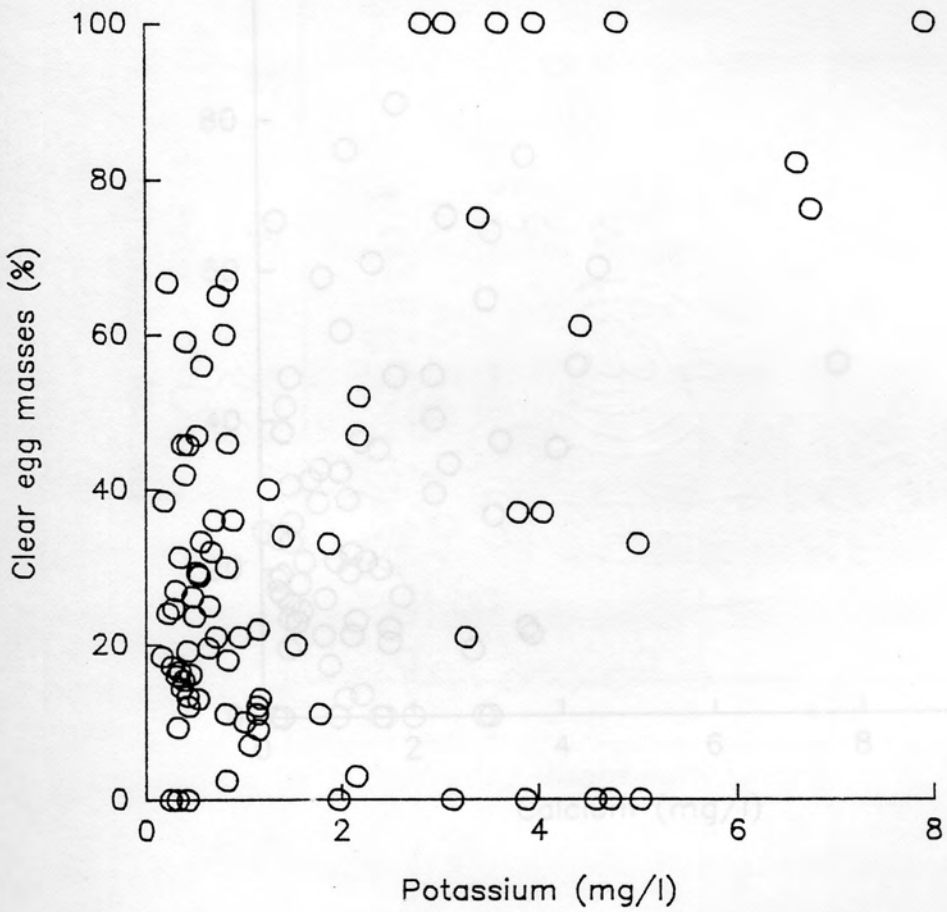


Fig. 3. The correlation between pond [K] and the percentage of clear egg masses in ponds from central Pennsylvania, North Carolina, and Louisiana.

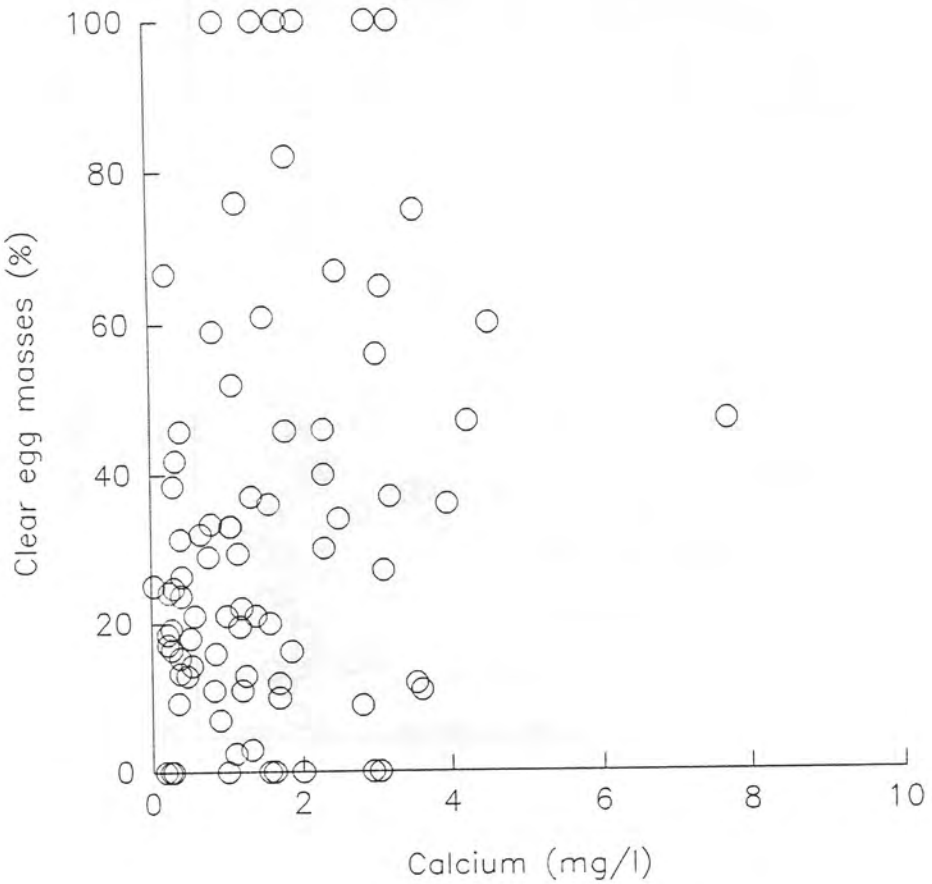


Fig. 4. The correlation between pond [Ca] and the percentage of clear egg masses in ponds from central Pennsylvania, North Carolina, and Louisiana.

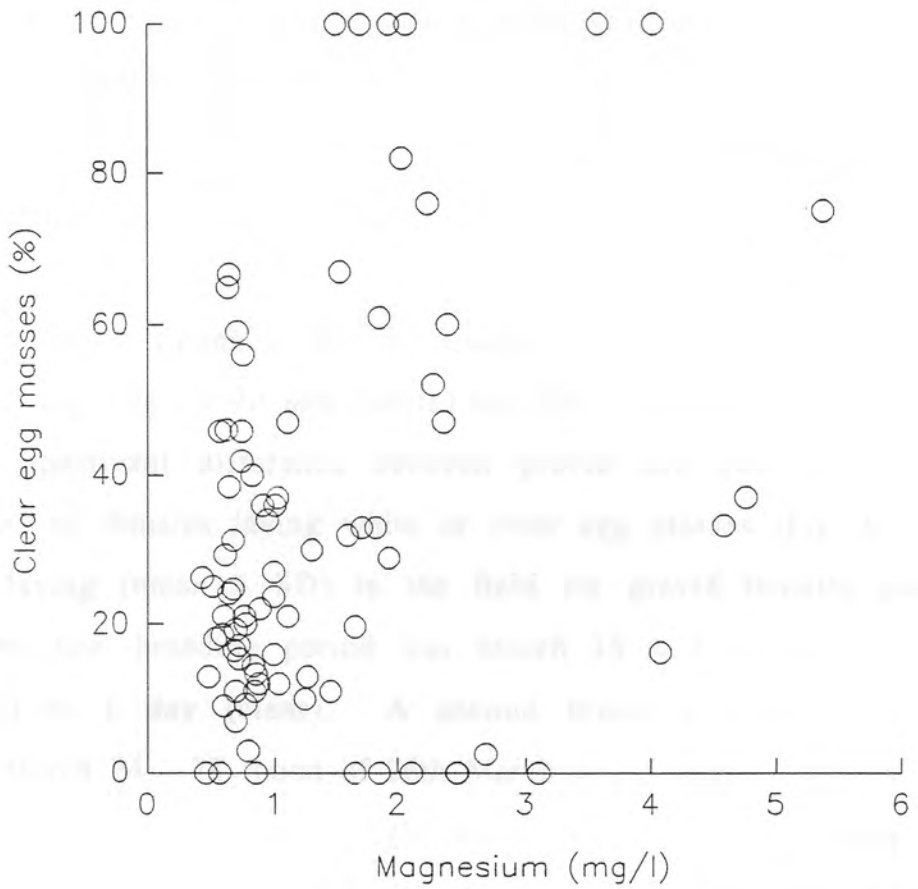


Fig. 5. The correlation between pond [Mg] and the percentage of clear egg masses in ponds from central Pennsylvania, North Carolina, and Louisiana.

and [Mg], (the latter two were not significant after allowing for nine comparisons of interest). The total number of all types was positively correlated with conductivity, [K], [Mg], and [Na], although these latter effects were not significant after allowing for the nine comparisons of interest.

Characteristics of the female parent

The SAVL (mean ± SD) of females that deposited eggs in the laboratory was 796 ± 70 mm (white) and 790 ± 80 mm (clear). There was no significant difference between gravid and post gravid wet body mass of females laying white or clear egg masses (Fig. 6). The date of laying (mean ± SD) in the field for gravid females caught during the first breeding period was March 15 ± 1 day (white) and March 11 ± 1 day (clear). A second breeding period occurred between March 24 - 28 (noon of 26th March ± 1 day; mean ± SD).

The lowest molecular weight tissue protein from females run on 15% PAGE was found to be slightly smaller in the three females that laid white egg masses (approximately 15,000 kD) compared to three that laid clear (approximately 16,000 kD).

Egg mass hydration, size of egg mass and number of embryos

The rate of hydration in ASW appears to be the same for both white and clear egg masses (P>F=0.7051, Fig. 7). The relation between

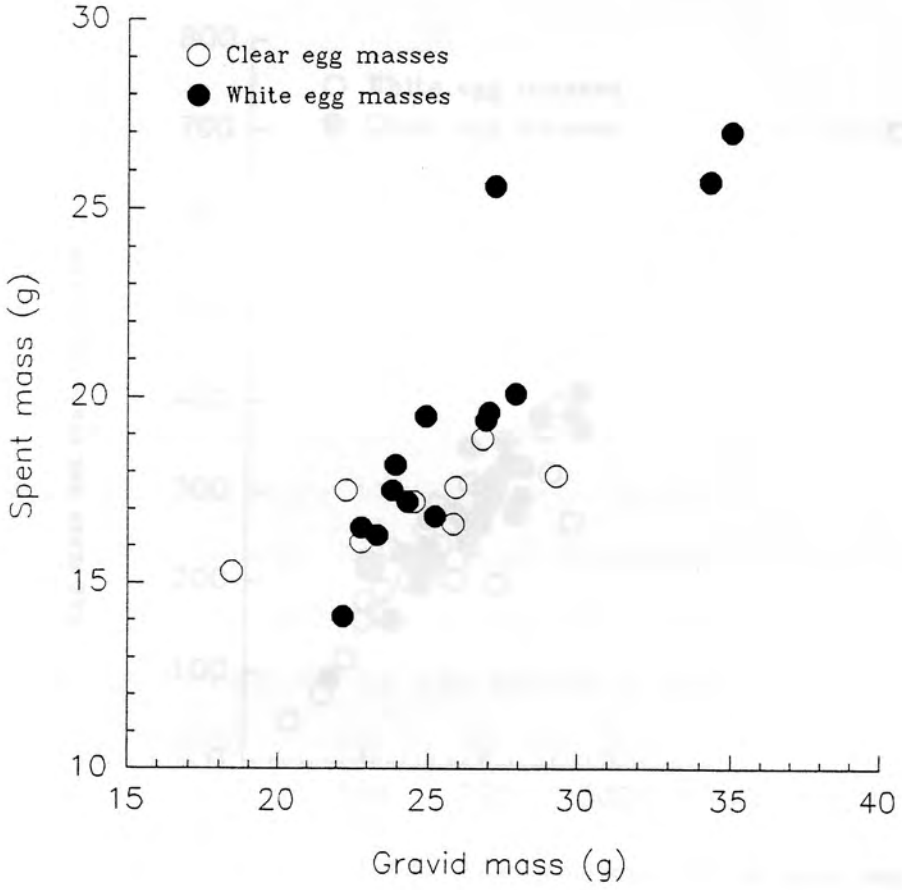


Fig. 6. Wet body mass of gravid and spent females that laid clear and white egg masses in the laboratory.

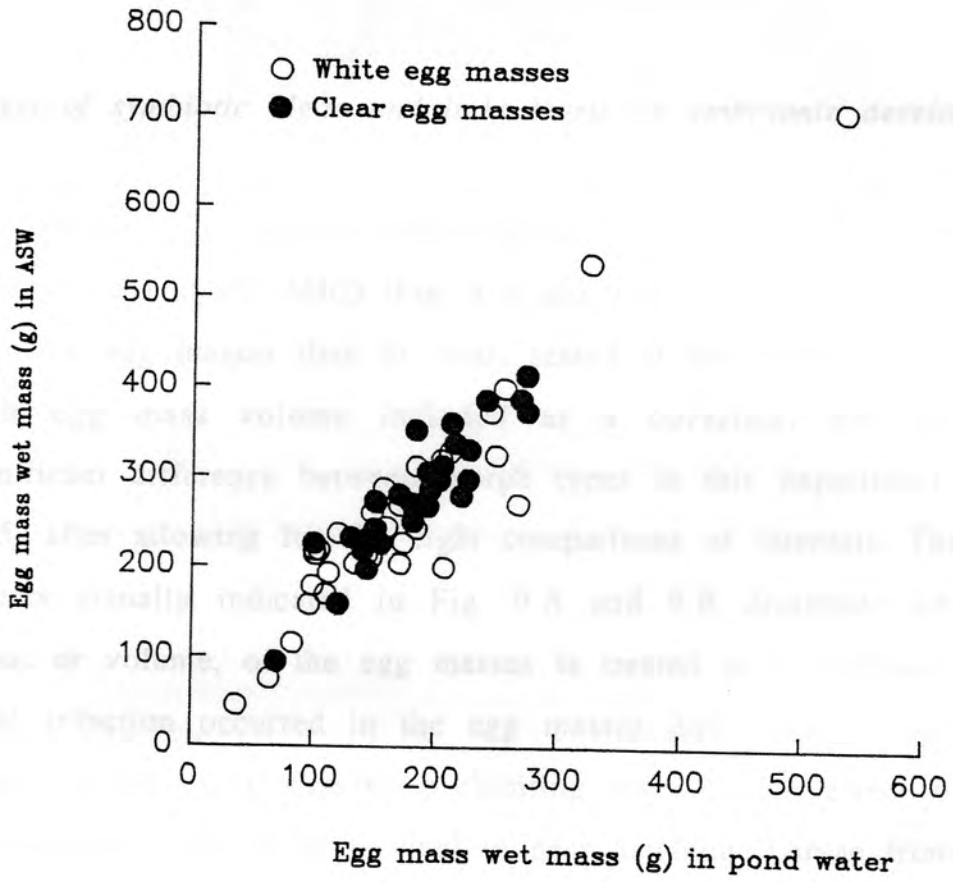
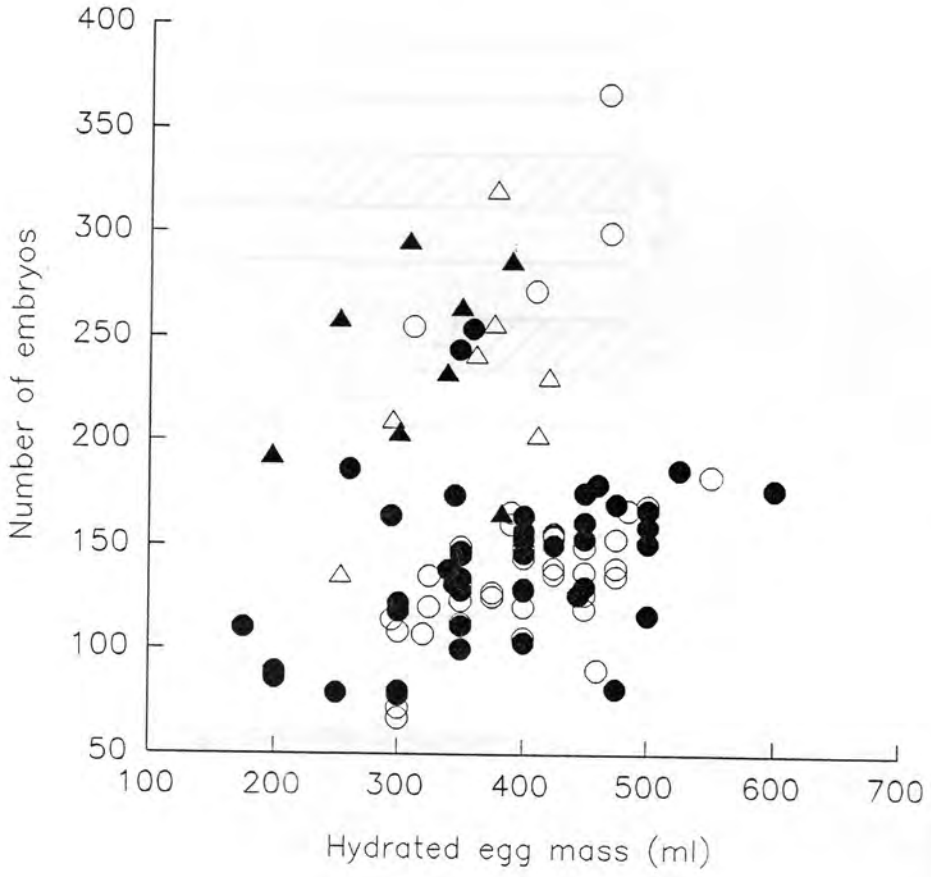


Fig. 7. Egg mass wet mass after 10 days in pond water (J36) followed by 10 days in artificial soft water.

egg mass volume and number of embryos of white and clear egg masses used in the experiment to test the effect of light, and the experiment used to test episodic environmental effects (see below) is also similar (P>F=0.8788, Fig. 8).

Effect of symbiotic algae and light levels on embryonic development

Those egg masses incubated at low light had significantly reduced survival and MHD (Fig. 9.A and 9.B). However, a lower MHD in white egg masses than in clear, reared at low light with atrazine, with egg mass volume included as a covariate, was the only significant difference between morph types in this experiment ($p < 0.05$, after allowing for the eight comparisons of interest). The other effects visually indicated in Fig. 9.A and 9.B disappear when the mass, or volume, of the egg masses is treated as a covariate. Some algal infection occurred in the egg masses dosed with atrazine and reared in the light, thereby prohibiting tests of the effect of light independent of algae on survival to near hatching. Larvae from white egg masses were significantly shorter (Table 4) than those from clear egg masses (P>F = 0.0017; all eight treatment combinations combined). This effect was largely due to the length of white larvae being unaffected by light level (P>F = 0.2610) whereas larvae from clear egg masses were significantly larger in low light than in high (P>F=0.0001). This effect withstood using egg wet mass and pre-hatching survival as covariates. Atrazine had no effect, and was also included in the ANOVA as a covariate (Table 4).



KEY:
○ Clear egg masses hydrated in 2L ASW
△ Clear egg masses hydrated in 7L ASW
● White egg masses hydrated in 2L ASW
▲ White egg masses hydrated in 7L ASW

Fig. 8. The relation between the number of embryos in an egg mass and the volume of hydrated clear and white egg masses.

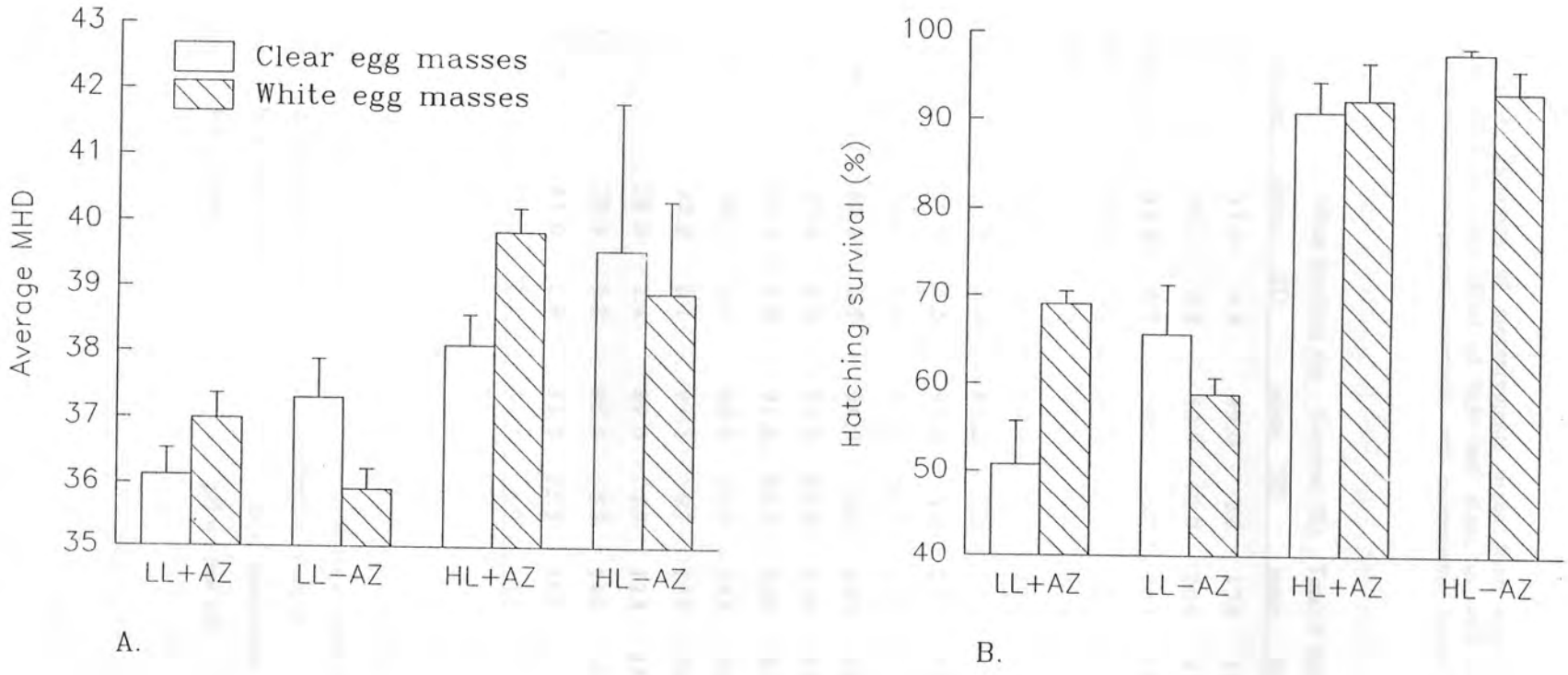


Fig. 9. Development of larvae from clear and white egg masses exposed to two levels of light and atrazine; A. Mean hatching day (MHD); B. Mean survival.

KEY: LL = low light, HL = high light, +AZ = with atrazine, -AZ = without atrazine

Table 4. Summary of measurements made from egg masses incubated under conditions designed to test the effect of light and algae, as well as episodic environmental events on embryonic development, survival, and subsequent larval length.

	Mean hatching day		Survival (%)		Total # embryos		Egg mass (g)		Length (mm)	
Treatment	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
WL+	37.0	0.8	69.0	2.9	178	18.4	325	40.1	5.0	0.8
CL+	36.1	0.8	50.7	10.0	206	41.3	335	46.2	5.6	0.6
WL-	35.9	0.7	58.8	3.7	203	19.8	265	57.7	5.0	0.7
CL-	37.3	1.2	65.6	11.3	267	71.6	433	24.6	5.7	1.0
WH+	39.8	0.7	92.3	8.5	272	17.8	338	22.2	4.9	0.5
CH+	38.1	0.9	90.9	7.1	293	26.6	407	43.8	4.9	0.5
WH-	38.9	2.8	93.2	5.2	263	17.8	329	57.9	4.6	1.1
CH-	39.6	4.5	97.6	1.4	217	58.3	341	65.2	4.8	0.6
Wctrl	39.8	1.9	87.4	16.7	125	20.4	328	98.0	4.6	0.8
Cctrl	40.2	2.7	94.8	4.2	145	20.2	421	67.1	4.6	0.8
W+	37.6	1.2	71.3	11.9	114	32.0	388	113.8	4.2	0.6
C+	37.5	1.9	71.6	22.2	129	31.5	399	62.6	4.1	1.0
WD	38.1	1.1	86.4	13.4	145	28.2	430	61.4	ND	ND
CD	39.0	2.1	93.2	6.7	159	74.0	397	57.8	3.8	1.0
WF	39.6	1.4	89.0	1.9	123	25.9	359	77.0	3.7	0.5
CF	39.8	3.2	96.1	5.5	140	16.9	411	48.5	3.6	0.5
WpH	41.0	1.9	53.2	12.3	162	16.6	428	53.6	4.3	0.9
CpH	40.3	2.1	44.9	6.9	107	20.6	350	56.5	4.5	0.6

KEY:
W = white egg mass
C = clear egg mass
L = 3µmol of light
H = 30-45µmol of light
F = frozen
+ = incubation in 500 µgrams of atrazine
- = no atrazine added
Ccrtl = control
D = desiccation
pH = low pH

Egg mass cooling and heating rates

During freezing, white egg masses appeared to cool slightly slower (Fig. 10), although the effect was not significant (P>F=0.2428). There was a significant difference in survival of these embryos to hatching (P>F = 0.0114), based on an ANOVA which incorporated the number of embryos, the temperature of the egg mass at the time of starting the experiment, and the wet mass of the egg mass as covariates. As predicted from differences in opacity, clear egg masses took less time to warm up under an IR heat lamp (Wilcoxon one tailed test; P< 0.042; fig. 11.A), and survival was less in the clear masses (Wilcoxon one tailed; P < 0.032; fig. 11.B).

Effect of extreme environmental parameters on embryonic development

Low pH significantly lowered survival in both white and clear egg masses. Atrazine had a similar effect, but like the last trial, *O. amblystomatis* was still present, making it uncertain whether the effect is due to the chemical itself, or to reduced levels of the symbiont. There was no significant egg mass morph effect: apparent differences between white and clear egg masses (fig. 12.A and 12.B) disappear when volume of the egg mass is taken as a covariate. The lengths of larvae from white versus clear egg masses did not differ for any treatment (Table 4) or all treatments combined (P>F = 0.6072), although the mean length of larvae from clear egg masses

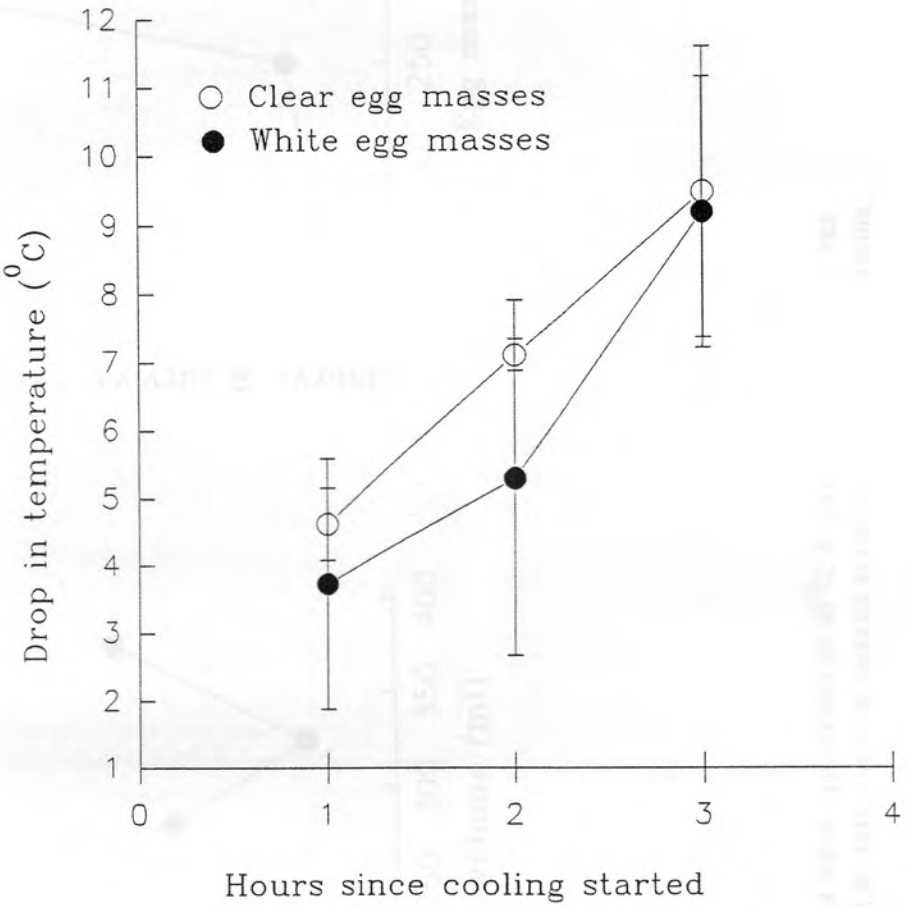
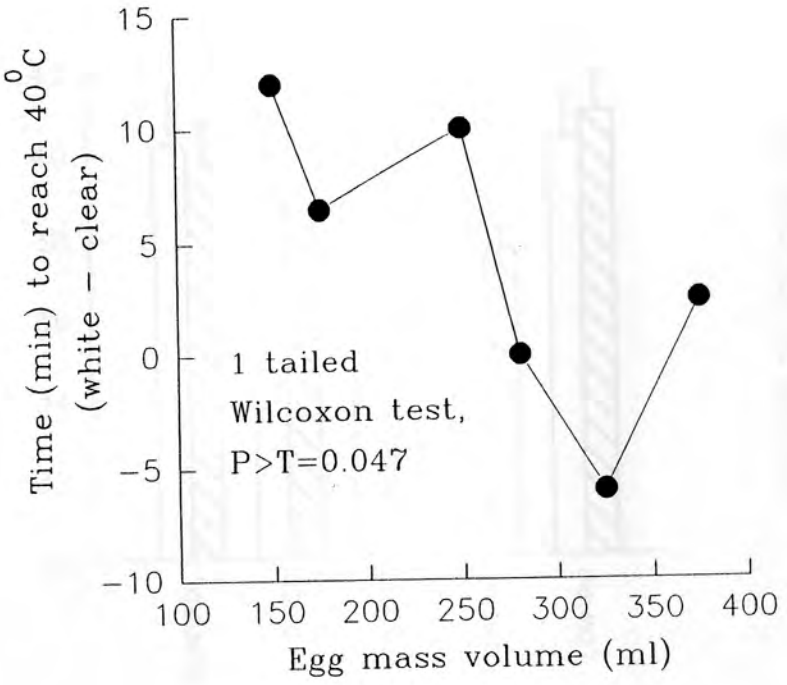
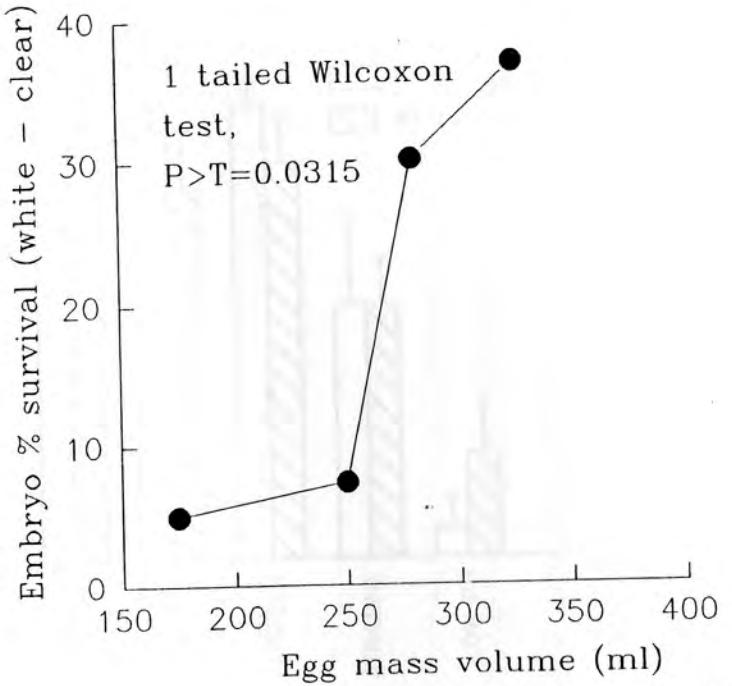


Fig. 10. Drop in temperature of clear and white egg masses placed in a freezer at -14^{0}C.



A.



B.

Fig. 11. Infra-red heating of clear and white egg masses to 40^{0}C; A. Difference in time for egg masses to reach 40^{0}C (white - clear); B. differences in embryo survival 24 hours after heating.

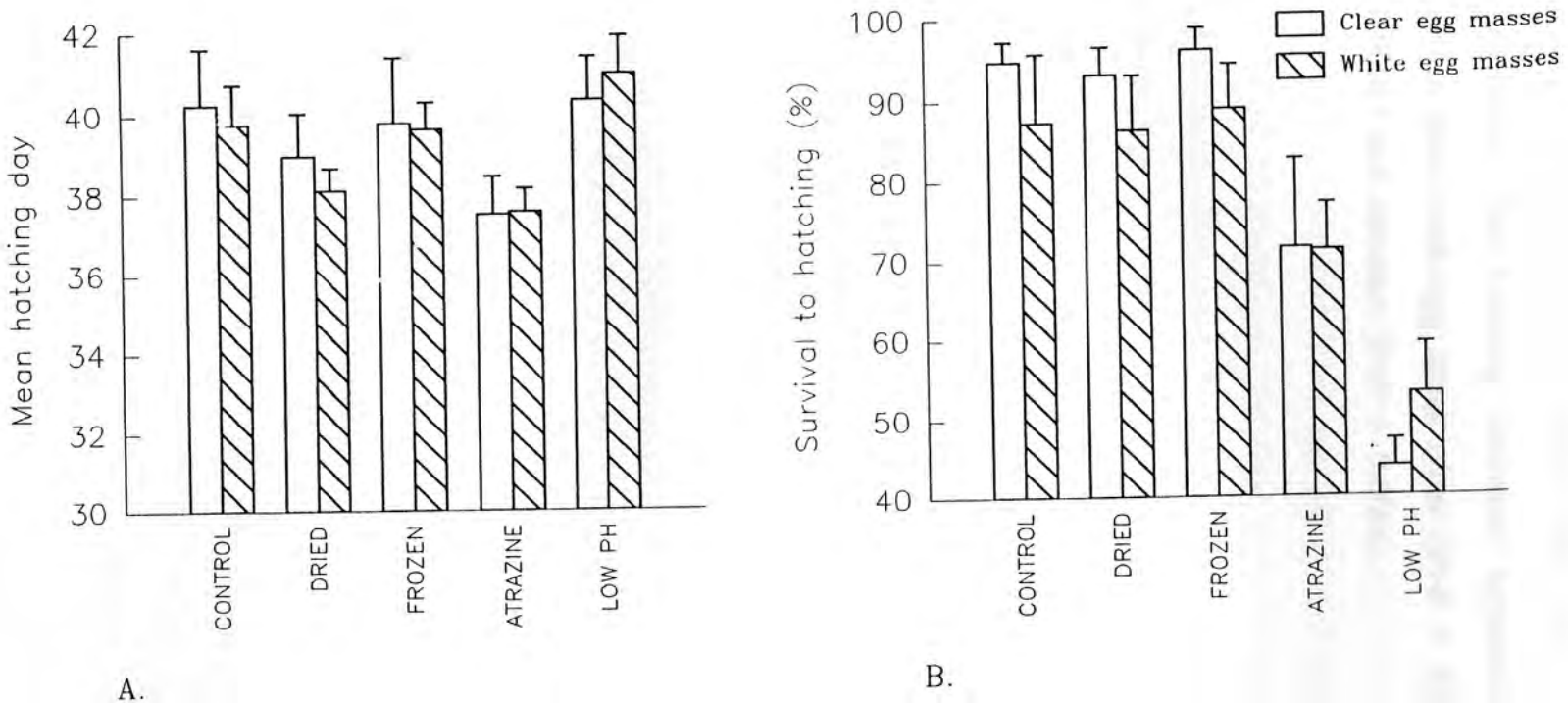


Fig. 12. Development of larvae from clear and white egg masses exposed to simulated episodic factors; A. Mean hatching day; B. Mean survival.

was greater than those from white egg masses for most of the treatments. The freezing treatment appeared to stunt the growth of larvae from both egg mass types ($P>F = 0.0001$), as did pH ($P>F = 0.0001$) and atrazine ($P>F = 0.0006$).

DISCUSSION

Banta and Gortner (1914) found slightly higher nitrogen in a white egg mass compared to a clear (9.09-9.27 versus 8.29-8.36%). However, the results of my nitrogen analysis suggested that the total amount of protein in the outer jelly of the three egg mass morphs is the same. The causative agent of the egg mass polymorphisms is probably based on two forms of one protein; a hydrophobic crystalline protein of about 15,400 kD found in the outermost jelly layer of white and intermediate egg masses and a slightly smaller water soluble protein of about 14,400 kD found in the outer jelly of clear egg masses and the capsular jellies of all three egg mass morphs. Hardy and Lucas (1991) also report these crystals in white egg masses from Louisiana and further identified them as hexagonal in cross section. Periodic acid Schiff staining of the protein crystals from central Pennsylvania samples did not reveal any carbohydrates. This observation contrasts with the 1% of glycosidic residues in crystals from Louisiana samples reported by Hardy and Lucas (per. comm.) but agrees with their assessment of white egg mass jelly from a North Carolina sample (Hardy and Lucas, per. comm.) The crystalline protein may be a modification of a slightly lower molecular weight protein (14,400 kD) already present in the inner jelly capsules of all three egg mass types, and the outer jelly of clear and intermediate egg masses. The next step would be to obtain the sequence, or more simply the amino acid composition, of both proteins to verify their relatedness. The intermediate type of egg mass may represent a heterozygote for this trait. However it is rare

in Pennsylvania populations, and apparently absent in Louisiana. Consequently the frequencies of the intermediate morph is far lower than Hardy-Weinberg equilibrium based on a simple two alleles at one locus model. If indeed the intermediate egg masses represent expression of both the egg jelly protein alleles, then it would be expected that the gene doses of each would be half that of the hypothesized homozygotes. However, the amount of crystals in white egg masses is between five and six times that found in intermediate egg masses, suggesting that the two allele model is incorrect. The scarcity of these intermediate types lends support to the idea that the egg mass trait may reveal the existence of two cryptic species. This hypothesis is supported by preliminary data revealing slightly different tissue protein moieties of female salamanders that lay white or clear egg masses (although a larger sample of females is needed to verify this result). Yet there are no apparent differences in female body size, condition, or time of laying. In all localities investigated, the overall frequencies of the white egg masses are greater than the clear ones, and this could be interpreted to imply a selective advantage for larvae hatching from a white egg mass. Indeed, there is evidence of a positive correlation of percent clear egg masses with certain pond water chemistry parameters, in particular [K], suggesting that larval development in clear egg masses may be negatively influenced by the low availability of these cations. What role these cations might have in embryogenesis is not clear; there might be a direct effect or an indirect one via the food web. The ubiquitous association of *A. maculatum* embryos and the green algae, *O. amblystomatis*, might be influenced by the availability of

these plant nutrients, as might general pond primary production and thereby zooplankton food for the larvae.

White egg masses were shown more able to withstand heating with an IR lamp than clear egg masses, and it could be conjectured that this, along with their opacity may influence the algal symbiont. Interestingly, low light levels were shown to differentially influence post-hatching larval length; in those trials involving light level, larvae from clear egg masses were significantly larger than those from white egg masses, despite similar pre-hatching survival for both types. Whether this effect was due to the absence of the symbiont, or of low light directly, could not be determined. It has subsequently been possible to culture the alga using U.S.E.P.A. algal media (U.S.E.P.A., 1989) so that egg masses can now be selectively innoculated without using the herbicide atrazine to control the growth of the symbiont. In most other trials not involving light levels, the mean SAVL of larvae from clear egg masses were greater than those from white but these differences were not significant. Rate of hydration of the egg masses, size of egg mass, number of embryos, amount of protein (as percent N), influence of freezing, desiccation, low pH, or herbicide did not appear to vary between white or clear egg masses. However, two observations: (1) that frequencies and numbers of egg mass types are correlated to varying degrees with pond cationic concentrations and (2) that a size disparity may exist between larvae from white versus clear egg masses (intermediate egg masses were too rare to use in these trials) strongly suggest selective factors are involved, both locally and regionally, across the range of *A. maculatum*. Further

experimentation is needed to test the plethora of possible explanations related to this conjecture. For instance, it could be argued that: (1) embryogenesis in clear egg masses is strongly influenced by light, cations, and the algal symbiont, (2) that the larvae from white egg masses are inherently smaller, less influenced by the algal symbiosis and all the factors that interact with it, (3) that the smaller larvae that hatch from the overall more frequent white egg masses have a selective advantage in these nutrient poor ponds over the larger larvae from clear egg masses due to lower energetic needs (thrifty versus profligate strategies). Hoffman and Parsons (1991) discuss the tradeoffs inherent to situations where environmental stress is a strong selective force. Genotypes tolerant of stress generally must pay a cost in reduced growth rate and they are less competitive than stress intolerant phenotypes when conditions ameliorate. Temporary ponds are a very stressful habitat due primarily to the short and unpredictable hydroperiod available for larval growth. In addition, low pond water nutrients and exposure to episodic freeze damage are serious abiotic stresses. There are also intense biotic interactions (competition, predation) with other amphibians and invertebrates in the ponds. It is possible that the two common egg mass types are a maternal system to program larvae alternatively to be larger for either fast growth, requiring higher nutrient levels and allowing for quicker metamorphosis (the clear phenotype), or smaller, requiring less nutrients and metamorphosing slower (the white phenotype). Pond nutrients and the role of symbiotic algae in determining hatching size of larvae may be linked directly to the opacity of the egg jelly: the

more opaque jelly may be more able to absorb lower levels of inorganic algal nutrients. Alternatively, the larval size may be determined by genes associated with the egg mass trait (hitch-hiking), and the trait itself may contribute no independent effect. Production of the two common morphs could represent "bet-hedging" within one species to allow for greater fecundity under regimes of temporal and spatial heterogeneity in stressful pond conditions. If the egg mass morphs represent two cryptic species, then they may be separated primarily by selective forces acting differentially on this egg mass trait.

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