Two new species of *Sphaerodactylus* from eastern Cuba (Squamata: Gekkonidae). Herpetologica 48: 358-367.

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APPENDIX 1

Specimens Examined

Sphaerodactylus oliveri.—Cuba: Sancti Spíritus Prov., Trinidad, Loma del Niarin (MCZ 19568); 7 mi. W Trinidad (USNM 140430-39); Finca Morales, 8 mi. NW Trinidad (AMNH 78350); 10 mi. W Trinidad (138018-20); Cienfuegos Prov., near Soledad (MCZ 19901); Soledad (MCZ 22717); Rancho Gavilán, near Cienfuegos (MCZ 52210).

S. scaber.—Cuba: Camagüey Prov., Sierra de Najasa (MCZ 21673), Sierra de Najasa, 3.8 mi. S, 5.1 mi. W Ecuador (AMNH 95982); Ciego de Ávila Prov., Sierra de San Juan de los Perros (MCZ 12304, holotype); Finca La Concepción, near Jicotea (MCZ 57354-58); Sancti Spiritus Prov., Sierra de Jatibonico (MCZ 7952).

S. storeyae.—Cuba: Isla de la Juventud, Punta del Este (AMNH 81191-96); Cueva No. 6, Punta del Este (IZ 4280, 4281, 4298); Archipiélago de los Canarreos, Cayo Ingles (MNHNCU 5-8); Cayo Largo del Sur (MNHNCU 63).

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A Molecular and Functional Evaluation of the Egg Mass Color Polymorphism of the Spotted Salamander, *Ambystoma maculatum*

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ABSTRACT.—**The spotted salamander, *Ambystoma maculatum*, has three types of egg masses; white, clear, and intermediate. White and clear forms are widely-distributed and often sympatric, whereas intermediate forms may be scarce or absent in local populations. Opacity of the egg mass depends on the concentration of hydrophobic protein crystals (1-3 μm in length, 15,400 kD in molecular size) in the outer jelly layers of white and intermediate egg masses. A water soluble protein (14,400 kD) replaces the hydrophobic crystals entirely in outer jelly layers of clear egg masses and partly in intermediate egg masses, and is found in the inner jelly of all three egg mass types. A mitochondrial DNA sequence analysis supports the hypothesis that these variants represent a simple polymorphism of a single gene, rather than the presence of a cryptic species complex. Over a two-year period, the number of total egg masses and the percentage that were clear remained relatively constant within individual ponds. The proportion of total egg masses that was clear was correlated (positively and negatively) with the concentration of some pond cations, although there was considerable annual variation in this relationship. The body lengths, wet masses prior to and after oviposition, and times of egg deposition were similar for females depositing white or clear egg masses. Embryos in white and clear egg masses exposed to low levels of light, simulated episodic environmental changes, and control conditions exhibited similar mean times to hatching and larval sizes. Survival was lower for both morphs in pH 5.2 than at a higher pH, and larvae of both morphs hatched under low light conditions were larger than those under high light conditions. There is as yet no definitive evidence that the "white" protein has a functional role in embryonic development. We suggest that the presence of this polymorphism may be related to differential fitness of the two morphs under low and high nutrient levels.**

A striking polymorphism occurs in the egg jellies of the spotted salamander, *Ambystoma maculatum*, caused by the presence or absence of white crystals in the outer jelly layer. There are three identifiable types: (1) clear egg masses, where embryos (usually between 50 and 200) are clearly visible through the jelly matrix; (2) intermediate or grey egg masses, where the embryos are faintly discernible in a cloudy grey jelly matrix; and (3) white egg masses, where the outer egg jelly is so opaque that embryos are rarely discernible, at least during early stages of development. Hardy and Lucas (1991) were the first to clearly recognize the significance of this polymorphism, and identified the crystals as protein. The genetic basis of this polymor-

phism is suggested but not proven by two observations; (1) females in captivity never lay more than one type of egg mass over successive years (J. Bogart, pers. comm.), and (2) the egg mass types are often sympatric in ponds, suggesting that environmental conditions are not the immediate cause.

A chlamydomonad algae (*Oophila amblystomatis*) is found within the inner jelly capsules of egg masses of several ambystomatids and sometimes those of the frog *Rana sylvatica* (Gilbert, 1942, 1944; Hammen and Hutchison, 1962; Biebel, 1969; Gatz, 1973; Goff and Stein, 1978). This alga 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; 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 that oxygen has no role in the symbiosis. The alga is thought to benefit from nitrogenous excretion of the developing embryos (Gilbert, 1942; Goff and Stein, 1978). If the amount of light reaching the algae is diminished by the opacity of the egg mass to levels that might limit its photosynthetic activity, then the quality of the symbiosis could be related to the egg mass type.

We carried out a series of investigations to determine whether there are any functional differences between egg masses of the clear and white morphs. These investigations fall into four main categories: (1) Properties of DNA and the protein- DNA sequence analysis to determine whether the morphs represent cryptic species; separation of the white protein crystals from the jelly matrix; characterization of their structure through gel electrophoresis; determination of amino acid composition and partial sequence; measurement of the amount of crystalline protein and total nitrogen of the outer jelly layer; (2) Field distributions-observations of the occurrence of the morphs over two years in two different regions (ridge and valley sites); correlation of the proportions of morphs in each pond with water chemistry variables; (3) Characteristics of the female parent-body mass before and after egg laying, body length, time of laying, gel electrophoresis of tissue proteins; and (4) Properties of the egg mass-relation between egg mass volume and the number of embryos; the rate of initial hydration or swelling; effect of different light levels; effect of other abiotic stresses (freezing, desiccation, low pH). The basic hypothesis was that the presence of the protein crystals in the white/opaque morph would be associated with some differences in functional response(s). The nature of such differences might then be used to predict the possible adaptive role of the presence of the protein (or its absence), leading to further tests of more specific hypotheses.

Materials and Methods

Properties of DNA and the Protein.—We examined the possibility that the two egg mass morphs represent cryptic species by DNA sequence analyses. DNA was extracted from liver samples of eight females of known egg mass type collected in ponds of two areas (J and PM) that are approximately 5 km apart in the barrens region (State Game Lands 176) of Centre County, Pennsylvania. The white morph was represented by two females from each of the areas, whereas the clear morph was represented by four females from a single pond (J1). The DNA extraction, amplification, and sequencing of a 307 base pair fragment of the mitochondrial cytochrome *b* gene followed methods detailed elsewhere (Hedges et al., 1991, 1992).

Nitrogen concentrations were measured using Nessler's technique (as modified by Jensen, 1962) and expressed as a percentage of the egg jelly dry mass. For these measurements, four 10 mg samples of dry outer egg jelly were taken from three masses of each morph type.

Outer and inner jelly layers from white, clear, and intermediate egg masses were acetone-dried, stored at −20 C, and later rehydrated and emulsified by sonification. We then obtained crystals from white and intermediate outer egg jelly by centrifugation. We dissolved samples in 2× SDS sample buffer (1:1 by volume) and ran them on 15% polyacrylamide thick slab gels (1.75 mm) against standard molecular weight markers, following the procedure of Laemmli (1970). Samples of white crystals centrifuged from white and intermediate egg masses were stained with periodic acid Schiff reagent (PAS) or sent to the Pennsylvania State University Biotechnology Institute for determination of amino acid composition and verification of molecular weight estimates made previously by polyacrylamide gel electrophoresis (PAGE) using high performance liquid chromatography (HPLC). An Applied Biosystems model 477A Protein Sequencer was used to generate a partial amino acid sequence of the white crystalline protein. To correctly distinguish intermediate egg masses from white, we removed samples of fresh outer jelly from all egg masses used in experimental trials and dissolved them in 2× SDS sample buffer for subsequent PAGE. We also ran gelatinous spermatophores of male *A. maculatum* from four different ponds in central Pennsylvania on 15% PAGE to determine whether the jelly capsule might contain a protein similar to that found in egg jelly.

We isolated crystals from 800 mg of acetone-

dried outer jelly layers from each of nine white and nine intermediate egg masses that we rehydrated, emulsified and centrifuged (20,500 rpm for 1 h after a low speed spin to remove debris). The tared centrifuge tubes containing the pellet were oven dried at 60 C for one day before reweighing. We calculated the amount of the crystals as the percentage of the mass of the original dried jelly.

Field Distribution.—We enumerated and recorded the time of laying when possible of the white, clear, and intermediate egg masses in temporary ponds in two topographically dissimilar areas in Centre County, Pennsylvania (a valley area named the Barrens—Game Lands 176), and a ridge area in Rothrock State Forest, throughout the 1990 and 1991 breeding seasons. We also sent survey forms and water sample bottles to out-of-state herpetologists during spring, 1991. A visit to northwestern Louisiana to count egg masses and collect water samples was also conducted in early March, 1991.

We measured several water chemistry parameters at the start of the 1990 and 1991 amphibian breeding seasons in 27 ponds in the Barrens. Samples were taken throughout the 1990 and 1991 breeding seasons in 32 ponds in Rothrock State Forest for a related project (unpubl. obs.). An Orion model SA720 meter was used to measure the pH of water samples held on ice within 48 h of collection. Samples from North Carolina and Louisiana were mailed to us at ambient temperatures and were measured within one week of collection; we did not analyze these samples for pH. We measured conductivity with a YSI model 32 conductance meter. We analyzed samples for concentrations of Ca, Na, K, and Mg on a Perkin-Elmer model 2280 atomic absorption spectrophotometer following filtration at 0.45 μm. Dissolved organic carbon (DOC), PO_4, SO_4, NO_3, alkalinity (as [$CaCO_3$]), and total dissolved Al were determined for the Rothrock samples by EPA approved methods at the Water Analysis Laboratory of the Environmental Resources Research Institute at Penn. State University. We estimated tannin/lignin using a Hach Model TA-3 kit with a colorimeter wheel. We measured total inorganic phosphate and sulfate for samples from the Barrens with Hach PhosVer 3 and Sulfaver 4 reagent pillows (Hach Co., Loveland, CO). Solutions for both procedures were measured on a Beckman model 25 spectrophotometer.

Characteristics of the Female Parent.—We collected gravid females (N = 27) from the two study areas in central Pennsylvania and allowed them to deposit their eggs in individual tanks held at 11–12 C. We weighed females before and after laying and measured their length from the tip of the snout to the anterior edge of the vent (SVL). Tissue proteins from a piece of the tail taken from frozen females that had laid either white or clear egg masses (three of each) were resolved on 15% PAGE.

Properties of the Egg Mass.—The approximate 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 deposited in the laboratory or freshly collected in the field, were placed in water from a single pond (J36) for five days. For the next five days we maintained the egg masses in artificial soft water (ASW) made up of 1 mg/L each of NaCl, KCl, $CaCl_2$, and $MgCl_2$ (as used by Freda and Dunson, 1985, except for the addition of Mg). ASW had a conductivity of about 11 μS/cm, compared to 16 μS/cm for J36 water. We compared the volumes of white and clear egg masses after 5 days in J36 water to volumes after the additional five days in ASW to determine if any differences existed in hydration rates between white and clear egg masses. We dissected a subsample of these, and other egg masses, and counted the number of embryos per mass.

We also investigated the effects of light on larval development in white and clear egg masses. We collected freshly laid egg masses (24 of each) from ponds in central Pennsylvania, or directly from females that laid in the laboratory, and kept them in pond water (J36) for 5 days to facilitate inoculation with the alga *O. amblystomatis*, identified by light microscopic examination (R. Purcell, pers. comm.). For the next 5 days we kept the egg masses in ASW. Each replicate contained two egg masses that had been laid on the same day; we placed them together in 5 L of ASW at 11–12 C (12L:12D).

We maintained low light (2.7 $\pm$ 0.4 μmol at the water surface for both clear and white egg masses; mean $\pm$ SD) by using layers of greenhouse shading cloth placed over the containers. Unshaded containers had a light level of 33.0 $\pm$ 7.1 μmol (mean $\pm$ SD). Light was provided by two 1 m long "cool" 40 cW fluorescent strip lights for each of three tables of eight containers. We measured light level with a Li Cor Model LI-189 photometer held at the water surface of each container. We counted recently hatched larvae and removed them daily to holding tanks (three tanks per treatment). We expressed time to hatching 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, and dividing by the total number of larvae. Two weeks after the last larva hatched, we dissected the remains of the egg masses to obtain an estimate of both total number of embryos per container and prehatching mortality. Five days before the last larvae had hatched, we collected random sub-

samples of larvae from the holding tanks and measured their SVL with a digital micrometer.

We additionally collected 40 white and 40 clear egg masses of roughly the same age (±1 day) from one pond in order to test the effects of episodic environmental extremes (freezing, desiccation, and chronic acidification) on development in white and clear egg masses. The non-factored treatments, each replicated eight times for both clear and white masses, comprised: (1) Freezing—We arranged egg masses randomly, each in 350 ml of ASW, in a chest freezer (−15 C). We measured temperatures in the center of the egg masses with copper/constantan Type T thermocouples connected to a Bailey model Bat 9 monitor. We estimated differences in cooling rate of white and clear egg masses by comparing rates of hourly temperature change. (2) Desiccation—We maintained egg masses at 11–12 C for 10 days in their containers without water. (3) Low pH—We maintained a pH of approximately 5.2 by adding diluted sulfuric acid weekly to each container. (4) Control—We maintained egg masses at 11–12 C in ASW. For all treatments except freezing, light was provided by one 0.5 m "cool" 40 cW fluorescent tube for each shelf of ten containers in a cold room maintained at 11–12 C. The mean light level for each container of 7.0 ± 2.0 μmol was sufficient to allow the symbiotic algae to develop. Measurements of MHD, total embryo number per egg mass, survival, and larval length were recorded in the same manner as above. We sampled larvae 19 days before the last larva hatched.

Statistical Analyses.—We employed stepwise linear regression to investigate relationships between pond water chemistry and the type and total number of egg masses laid. We checked data for normality by comparing correlations between normal scores and raw and transformed data (similar to the Shapiro-Wilk test; Minitab, 1989). In order to satisfy this assumption, we transformed data for proportion of clear masses with the arcsine transformation ($Y' = 2 \cdot \arcsin\sqrt{Y}$); data for total number of egg masses were transformed to their square roots ($Y' = \sqrt{Y}$). Since we tested two responses to pond chemistry (mass type and number of masses) for two separate years (1990 and 1991), we adjusted our initial type I error rate ($\alpha = 0.05$) downward by dividing by the number of individual statistical analyses (2 responses · 2 years = 4 separate analyses). This resulted in a per-comparison type I error rate and minimum critical P-value of $0.05/4 = 0.0125$. Thus, only analyses that resulted in P-values <0.0125 were judged significant for this set of tests.

We used analyses of covariance (with mass and total number of embryos per egg mass as covariates) to test the effects of light and environmental extremes on MHD, survival, and length of larvae from white and clear morphs following tests for normality as described above. We transformed the proportion that survived with the arcsine transformation. We adjusted our minimum critical values as above, which resulted in $P = 0.05/(3 \text{ responses} \cdot 2 \text{ levels}) = 0.008$ for the light experiment and $P = 0.05/(3 \text{ responses} \cdot 3 \text{ treatments} \cdot 2 \text{ levels}) = 0.003$ for the environmental extremes experiment. We used Tukey's pairwise comparisons procedure following analyses of variance that were judged significant to identify the particular treatment levels that differed. We used a two sample t-test to compare rates of hydration between egg mass types. No transformation was necessary on this response.

Results

Properties of DNA and the Protein.—DNA sequences of the 307 base pair fragment of the cytochrome *b* gene from eight *A. maculatum* are shown in Fig. 1, along with a sequence of the same region in a congeneric species, *A. jeffersonianum* (Hedges et al., 1992), for comparison. Only five sites are variable within *A. maculatum*, and all of the substitutions are synonymous. There are three clusters of identical sequences: J1-1 and J1-3; J1-2, J1-6, and J1-7; PM-11 and PM-14. Such a low level of sequence variation is typical of individuals within the same species of *Ambystoma* (Fig. 1 in Hedges et al., 1992) and the few variants observed here do not correspond to the two egg mass morphs.

Polyacrylamide gel electrophoresis (PAGE) revealed two apparently related proteins in the outer and inner egg jelly layers of the three morphs (Fig. 2). Outer jelly layers of white egg masses contain a crystalline protein (15,400 kD). Those of clear egg masses contain a slightly smaller water soluble protein (14,400 kD), that is present in the inner jelly layers of all three egg masses. The outer jelly layers of intermediate egg masses contain both proteins. Staining of the crystals with PAS reagent did not reveal any glycosidic residues. Amino acid composition of the white crystalline protein is similar for samples from Pennsylvania and Louisiana (Table 1). 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; ALA PRO VAL TYR SER ALA THR PRO GLY for the northwestern Louisiana sample (C. Lucas and L. Hardy, pers. comm.). We detected no low molecular weight proteins from the spermatophores of male *A. maculatum* using PAGE.

We found no large differences in total nitrogen of the outer jelly layers as a percentage of

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1 A.jeff          C TTC GGT TCT CTA CTA GGA CTG TGT TTA ATT ACA CAA ATC TTA
2 J1-1   (W)      . ..T ... ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
3 J1-2   (W)      . ..T ..C ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
4 PM-11  (W)      . ..T ... ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
5 PM-14  (W)      . ..T ... ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
6 J1-3   (C)      . ..T ... ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
7 J1-6   (C)      . ..T ..C ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
8 J1-7   (C)      . ..T ..C ..C ... T.. ... T.A ..C ... ... ... ... ..T ...
9 J1-8   (C)      . ..T ... ..C ... T.. ... T.A ..C ... ... ... ... ..T ...

1 A.jeff        ACA GGA CTA TTT CTA GCT ATA CAT TAT ACA GCT GAC ACA TCA TCA
2 J1-1   (W)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
3 J1-2   (W)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
4 PM-11  (W)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
5 PM-14  (W)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
6 J1-3   (C)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
7 J1-6   (C)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
8 J1-7   (C)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...
9 J1-8   (C)    ... ... T.. ... ... ... ... ... ... ... ..A ... ..G ... ...

1 A.jeff        GCA TTT TCA TCC GTA GCA CAC ATC TGC CGA GAC GTA AAC TAC GGC
2 J1-1   (W)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
3 J1-2   (W)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
4 PM-11  (W)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
5 PM-14  (W)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
6 J1-3   (C)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
7 J1-6   (C)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
8 J1-7   (C)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...
9 J1-8   (C)    ... ... ... ..T ... ... ... ... ... ..T ..T ... ... ... ...

1 A.jeff        TGA CTT ATA CGA AAT ATT CAC GCA AAC GGA GCT TCA TTT TTT TTC
2 J1-1   (W)    ... ... ... ... ..C ... ..T ... ..T ..T ... ... ..C ... ...
3 J1-2   (W)    ... ... ... ... ..C ... ..T ... ..T ..C ... ... ..C ... ...
4 PM-11  (W)    ... ... ... ... ..C ... ..T ... ..T ..T ... ... ..C ... ...
5 PM-14  (W)    ... ... ... ... ..C ... ..T ... ..T ..T ... ... ..C ... ...
6 J1-3   (C)    ... ... ... ... ..C ... ..T ... ..T ..T ... ... ..C ... ...
7 J1-6   (C)    ... ... ... ... ..C ... ..T ... ..T ..C ... ... ..C ... ...
8 J1-7   (C)    ... ... ... ... ..C ... ..T ... ..T ..C ... ... ..C ... ...
9 J1-8   (C)    ... ... ... ... ..C ... ..T ... ..T ..T ... ... ..C ... ...

1 A.jeff        ATT TGT ATT TTT CTT CAT ATC GGC CGA GGA ATA TAC TAT GGG TCA
2 J1-1   (W)    ..C ..C ... ... ..C ..C ... ... ..G ..G ... ... ..C ..C ...
3 J1-2   (W)    ..C ..C ... ... ..C ..C ... ... ..G ... ... ... ..C ..C ...
4 PM-11  (W)    ..C ..C ... ... ..C ..C ... ... ..G ..G ... ... ..C ..C ...
5 PM-14  (W)    ..C ..C ... ... ..C ..C ... ... ..G ..G ... ... ..C ..C ...
6 J1-3   (C)    ..C ..C ... ... ..C ..C ... ... ..G ..G ... ... ..C ..C ...
7 J1-6   (C)    ..C ..C ... ... ..C ..C ... ... ..G ... ... ... ..C ..C ...
8 J1-7   (C)    ..C ..C ... ... ..C ..C ... ... ..G ... ... ... ..C ..C ...
9 J1-8   (C)    ..C ..C ... ... ..C ..C ... ... ..G ..G ... ... ..C ..C ...

1 A.jeff        TAC ATA TTT AAA GAA ACA TGG AAT ATT GGA GTT ATT TTA CTA TTT
2 J1-1   (W)    ... ..G ... ... ... ... ... ... ... ..G ... ..C C.C ... ...
3 J1-2   (W)    ... ..G ... ... ... ... ... ... ..C ..G ... ..C C.C ... ...
4 PM-11  (W)    ... ..G ... ... ... ... ... ... ..C ... ... ..C C.C ... ...
5 PM-14  (W)    ... ..G ... ... ... ... ... ... ..C ... ... ..C C.C ... ...
6 J1-3   (C)    ... ..G ... ... ... ... ... ... ... ..G ... ..C C.C ... ...
7 J1-6   (C)    ... ..G ... ... ... ... ... ... ..C ..G ... ..C C.C ... ...
8 J1-7   (C)    ... ..G ... ... ... ... ... ... ..C ..G ... ..C C.C ... ...
9 J1-8   (C)    ... ..G ... ... ... ... ... ... ..C ... ... ..C C.C ... ...

1 A.jeff        TTA GTA ATG GCA ACA GCT TTT GTA GGA TAT GTT CTT CCG
2 J1-1   (W)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
3 J1-2   (W)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
4 PM-11  (W)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
5 PM-14  (W)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
6 J1-3   (C)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
7 J1-6   (C)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
8 J1-7   (C)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
9 J1-8   (C)    ... ..T ..A ... ... ..C ... ..T ... ... ..C ..C ..A
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FIG. 1. Alignment of mitochondrial DNA sequences from a 307 base pair portion of the cytochrome *b* gene in eight female *Ambystoma maculatum* representing white (W) and clear (C) egg mass morphs. A sequence from a congeneric species, *A. jeffersonianum* (Hedges et al., 1992: their sequence 16), is included for comparison. Dots indicate identity with the first sequence.

dry outer egg mass jelly among the morphs (white 9.59 ± 0.96%, intermediate 8.81 ± 0.31%, clear 9.02 ± 0.35%). Expressed as % of dry mass, outer jelly of white egg masses has about five times as much crystalline protein as that of intermediate egg masses (6.14 ± 3.19%, and 1.17 ± 0.39%, respectively).

Egg Mass Counts and Pond Water Chemistry.—In a given pond the total number of egg masses and the percentage of clear egg masses were

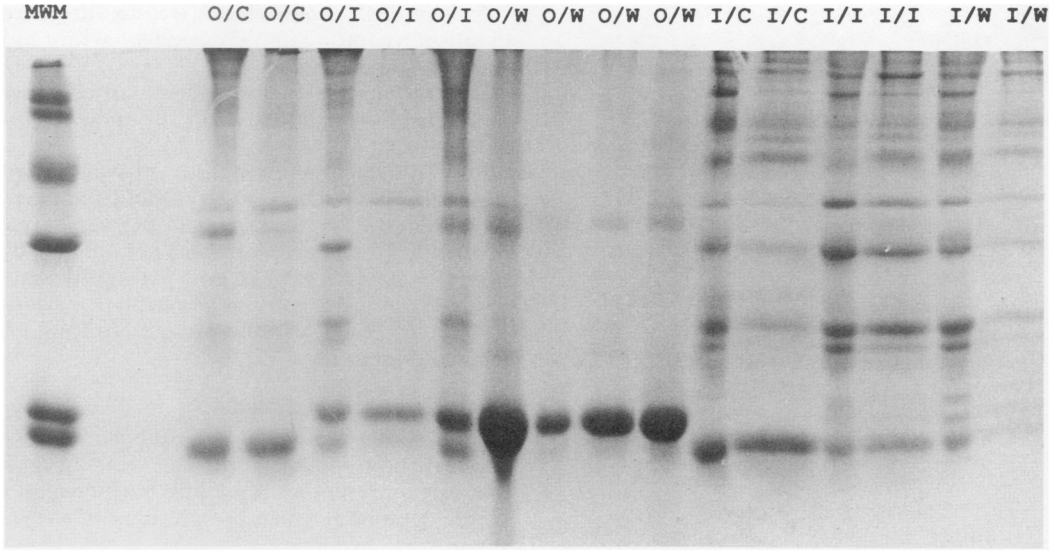

FIG. 2. PAGE of the outer and inner jelly layers of clear, intermediate, and white egg masses of the spotted salamander. Key: MWM = molecular weight markers; O/C = outer egg jelly of clear masses; O/I = outer egg jelly of intermediate egg masses; O/W = outer egg jelly (sonicated and centrifuged to concentrate 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.

very consistent between 1990 and 1991 (Fig. 3). Intermediate egg masses were infrequent in central Pennsylvania (749 out of 8762 in 1991) and have not been reliably recorded in other localities. Visual identification of intermediate masses is easiest when the egg masses are freshly hydrated; observers may not recognize them when masses are older and greatly swollen. We found the ratio of clear to white plus intermediate egg masses in central Pennsylvania to be 1:3 in 1990, and 1:4 in 1991. This ratio was 1:1.5 for ponds sampled in North Carolina (289 total egg masses), and 1:2 for those in Louisiana (206 total egg masses). 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, we did not include these data in analyses.

Stepwise linear regressions of the transformed proportions of clear egg masses against pond chemistry at the start of the breeding season yielded correlations that varied annually. In 1990 (central Pennsylvania only), no individual chemical constituents or interactions were significant predictors of the proportion of clear egg masses. In 1991 (central Pennsylvania, North Carolina, Louisiana) some relationships were significant: the proportion of clear egg masses was positively related to the [K]·[Mg] interaction, but negatively to the [K]·[Mg]·[Na] interaction ($Y' = 0.951 + 0.126[K]\cdot[Mg] - 0.192[K]\cdot[Mg]\cdot[Na]$; $R^2 = 24.5\%$, $P < 0.001$, $F = 12.50$). No main effects or other interactions were significantly related to this response. The total number of egg masses in 1990 was positively associated only with [Ca]·[Mg] ($Y' = 0.00 + 1.00[Ca]\cdot[Mg]$; $R^2 = 27.9\%$, $P < 0.001$, $F = 15.48$). This parameter in 1991 was negatively related to [K]·[Ca] ($Y' = 9.06 - 0.474[K]\cdot[Ca]$; $R^2 = 12.3\%$, $P = 0.003$, $F = 9.40$).

Characteristics of the Female Parent.—The SVL

TABLE 1. Amino acid composition of the white crystalline protein detected using HPLC. Values on the left are those from central Pennsylvania samples analyzed by the Biotech. Institute. Values in parentheses are those of Hardy and Lucas (pers. comm.) for white egg masses from Louisiana.

Residue	Frequency	Residue	Frequency
ASP	19 (14)	GLY	15 (21)
GLU	6 (9)	ALA	14 (15)
LYS	12 (6)	VAL	6 (7)
ARG	4 (5)	LEU	9 (8)
HIS	1 (1)	ISO	6 (9)
SER	13 (17)	MET	1 (1)
THR	10 (10)	PHE	7 (7)
PRO	9 (8)	TYR	8 (6)
CYS	0 (3)	TOTAL	142 (147)

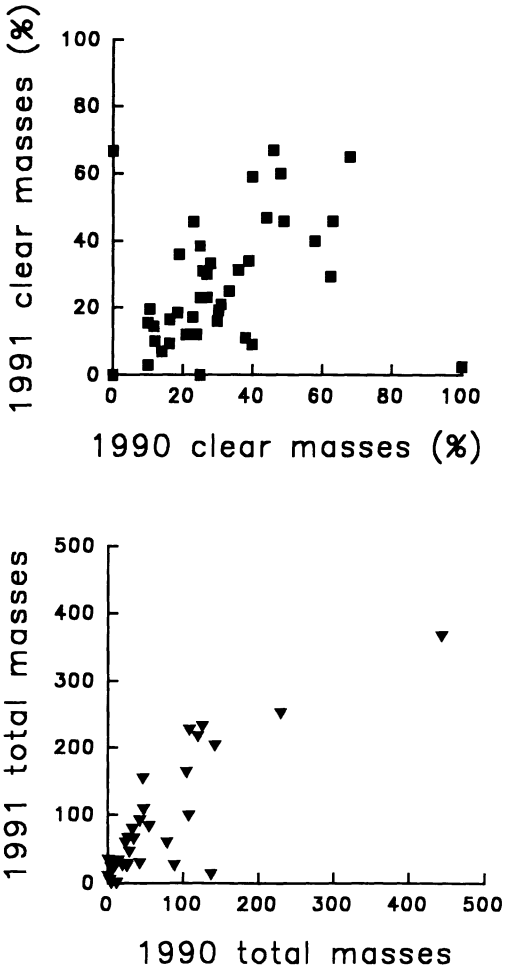

FIG. 3. The consistency of egg mass counts in 37 individual ponds in central Pennsylvania in 1990 and 1991. Top—Percentages of total masses that were clear. Regression on arcsine transformed values described this relationship by: % clear (1991) = 0.471 + 0.520[% clear (1990)], R^2 = 22.8%, P = 0.002, F = 11.51. Bottom—Total number of egg masses. Regression on square root transformed values described this relationship by: 1991 total = 1.80 + 0.889[1990 total], R^2 = 66.9%, $P < 0.001$, F = 64.65.

of females that deposited eggs in the laboratory was 796 ± 70 mm (white) and 790 ± 80 mm (clear). There was no difference between gravid and post-gravid wet body mass of females laying white or clear egg masses. The date of laying for gravid females caught during the first period of laying was March 15 ± 1 day (white) and March 11 ± 1 day (clear).

Preliminary results indicate that the lowest molecular weight tissue protein that could be visualized on 15% PAGE was slightly smaller in females laying white egg masses (approximately 15,000 kD), compared to those laying clear egg masses (about 16,000 kD).

Experimental Effects.—There was no difference in rate of hydration in ASW between white and clear egg masses (P = 0.57, T = −0.57). Egg masses in various treatments contained mean numbers of embryos between 107 and 267 (Table 2).

Low light treatments significantly decreased survival ($P < 0.001$, F = 56.78) and increased larval length (P = 0.002, F = 11.55), regardless of morph. However, MHD was not affected by light (P = 0.033, F = 5.63). Low pH significantly reduced survival regardless of morph ($P < 0.001$, F = 47.77). There were no other significant effects of treatments.

DISCUSSION

The results of the DNA sequence analysis support the hypothesis that the two egg mass variants represent a simple polymorphism of a single gene rather than the presence of a cryptic, undescribed species.

Banta and Gortner (1914) found slightly higher nitrogen levels in a white egg mass compared to a clear mass (9.09–9.27% versus 8.29–8.36%). However, our nitrogen analysis suggests that the total amount of protein in the outer jelly of the three egg mass morphs is the same. The polymorphism comprises 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. The sequence, or more simply, the amino acid composition, of these two proteins is needed to verify their likely relatedness.

Hardy and Lucas (1991) were the first to report crystals of hydrophobic protein in white egg masses from Louisiana, identifying them as hexagonal in cross section. Periodic acid Schiff staining of the protein crystals from our central Pennsylvania samples did not reveal any carbohydrates. This contrasts with the report of Hardy and Lucas (1991) of 1% glycosidic residues in crystals from Louisiana samples, but agrees with their assessment of white egg mass jelly from a North Carolina sample (Hardy and Lucas, pers. comm.). The intermediate type of egg mass is rare in Pennsylvania populations, and apparently absent in Louisiana. The amount of crystals in white egg masses is between five and six times that found in intermediate egg masses. The reason for the scarcity of intermediate types is unknown.

Based on two years of observations, individual ponds contained similar total numbers of egg masses and egg mass morph proportions over time. Thus although only about one-third of the female population breeds in a single pond in a given year (Husting, 1965; Phillips and

TABLE 2. Summary of measurements made from egg masses incubated under conditions designed to test the effect of light, as well as episodic environmental events, on embryonic development, survival, and subsequent larval length of white (W) and clear (C) morphs.

Treatment morph	Hatch day		% Survival		No. embryos		Egg mass, g		Length, mm	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control										
W	39.8	1.9	87.4	16.7	125	20	328	98	4.6	0.8
C	40.2	2.7	94.8	4.2	145	20	421	67	4.6	0.8
High light										
W	38.9	2.8	93.2	5.2	263	18	329	58	4.8	0.3
C	39.6	4.5	97.6	1.4	217	58	341	65	4.8	0.3
Low light										
W	35.9	0.7	58.8	3.7	203	20	265	58	5.3	0.4
C	37.3	1.2	65.6	11.3	267	72	433	25	5.5	0.8
Frozen										
W	39.6	1.4	89.0	1.9	123	26	359	77	3.7	0.5
C	39.8	3.2	96.1	5.5	140	17	411	49	3.6	0.5
Desiccation										
W	38.1	1.1	86.4	13.4	145	28	430	61	—	—
C	39.0	2.1	93.2	6.7	159	74	397	58	3.8	1.0
Low pH										
W	41.0	1.9	53.2	12.3	162	17	428	54	4.3	0.9
C	40.3	2.1	44.9	6.9	107	21	350	57	4.5	0.6

Sexton, 1989), there seems to be minimal yearly fluctuation in the proportion of breeding morphs. It would be valuable to monitor the consistency of morph proportions for a longer time, and to determine whether "non-breeding" females are actually breeding elsewhere, or are skipping years between single bouts of reproduction.

In all localities investigated, the overall frequencies of the white egg masses are greater than the clear ones. This may indicate a selective advantage for larvae hatching from a white egg mass. We realize that "neutrality" is an alternate explanation for this polymorphism, yet proof of adaptive significance is difficult when selective forces are slight, as is typically the case. Note that the clear/white morph trait consists of the presence/absence of a crystalline protein, not simply a minor amino acid substitution within a protein. It seems considerably less likely that production of such a protein is without function, than is the slight modification of a pre-existing molecule. Much better evidence for involvement of some selective force is the relationship between the proportion of clear egg masses with certain pond water chemistry parameters, in particular [K], [Na], [Ca], and [Mg]. This suggests that larval development in clear egg masses may be influenced by these cations. What mechanistic 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 embryos of *A. maculatum* and the algae *O. amblystomatis* might be influenced by the availability of plant nutrients, as might general pond primary productivity and thereby zooplankton food for the larvae. Although the overall predictive power of this association is low, this observation may be useful in developing new hypotheses.

Interestingly, light levels differentially influenced post-hatching larval length. In trials involving light level, larvae hatched from low light treatments were significantly larger than those from high light treatments, regardless of egg mass morph. Whether this indicates that even the low light treatment was near the photosynthesis versus light intensity (P vs. I) maximum of the symbiont was not determined. It has subsequently been possible to culture the alga using a standard medium (U.S.E.P.A., 1989) so that egg masses can now be inoculated selectively. Rate of hydration of the egg masses, size of egg mass, number of embryos, amount of protein (as % N), and influence of freezing, desiccation, or low pH did not appear to vary between white or clear egg masses. However the observation that frequencies and numbers of egg mass types are correlated to varying degrees with pond cationic concentrations suggests that there may be a functional difference between the morphs.

In some ponds in North Carolina (Murphy, 1961) and central Pennsylvania, the egg masses of *A. maculatum* are subjected to intense pre-

dation by a larval caddisfly (*Ptilostomis*). It appears that egg mass morph type does not influence the frequency of this predation (unpubl. obs.). However other such biotic interactions among pond inhabitants and salamander egg masses should be examined.

The spotted salamander has been recommended for use as a pollution bioindicator for the northeastern deciduous forest ecosystem (Freda et al., 1991). Any such use should take into account the occurrence of the egg mass morph, since there may be a differential effect of the phenotypes on tolerance to ecotoxicological stresses.

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