DIFFERENTIATION OF *PRIONOTUS CAROLINUS* AND *PRIONOTUS EVOLANS* EGGS IN HEREFORD INLET ESTUARY, SOUTHERN NEW JERSEY, USING IMMUNODIFFUSION

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ABSTRACT

Immunochemical techniques were used to classify the planktonic eggs of *Prionotus carolinus* (northern searobin) and *Prionotus evolans* (striped searobin) collected from a southern New Jersey estuary. Results of immunochemical identifications were compared with identifications based upon the commonly used morphological character of egg oil globule distribution. An average identification error of 22.3% was found when results using this conventional morphological characteristic were compared with immunodiffusion results. Improved accuracy of searobin egg identification can be achieved in future ichthyoplankton studies by using immunochemical techniques. A similar application of immunochemical identification techniques should also better resolve classification uncertainties among other morphologically similar co-temporal and co-spatial planktonic fish eggs.

The accuracy of ichthyoplankton analysis is often limited by the lack of reliable, distinguishing, morphological characteristics that are useful for identifying fish eggs and larvae. Conventional characteristics used to identify fish eggs include egg and oil globule diameters; number, distribution, and pigmentation of oil globules; and pigmentation patterns on developing embryos. However, overlapping diameters of eggs and a similar if not identical number of oil globules with comparable pigmentation and size among closely related species impose a relatively high degree of uncertainty concerning the identity of planktonic fish eggs from many areas. Increased accuracy has been more recently achieved through the analysis of fish eggs using biochemical, immunological, and ontogenetic methods. Morgan (1975) examined electrophoretic patterns of white perch and striped bass egg extracts and found differentiation was possible on this basis. Orlowski et al. (1972) differentiated cunner, *Tautogolabrus adsperus*, from tautog, *Tautoga onitis*, eggs using monospecific antisera in microimmunodiffusion analyses. The technique was especially useful with early stage eggs which were morphologically identical. Ontogenetic methods allow careful study of laboratory-reared eggs and larvae of known parentage to document species-specific developmental histories. These studies may provide new distinguishing morphological features for future egg identifications. However, additional means are required where well-documented features shared with other species do not provide adequate differentiation of field-collected eggs.

This paper is a report on the results obtained from a microimmunodiffusion analysis which successfully differentiated the planktonic eggs of the northern searobin, *Prionotus carolinus*, from those of the striped searobin, *Prionotus evolans*, which were collected from the Hereford Inlet estuary, southern New Jersey, between May 1973 and September 1974 (Keirans 1977). Identifications based separately upon immunochemical and morphological evidence were also compared to evaluate the reliability of differentiations based entirely upon conventional morphology. *Prionotus* spp. were selected in our study first because the searobins represent a large breeding population which appears co-temporally and co-spatially near shore to provide an abundant source of gravid adults. Eggs of known parentage became readily available for preparation of experimental reagents and specimens. Secondly, this study would expand the application of microimmunodiffusion analysis to species differentiation as an extension of the study of Orlowski et al. (1972), which documented differentiation of eggs from two genera. Finally, the identification of *Prionotus* spp. ova has never been properly resolved.

Prionotus carolinus ova were described by Kuntz and Radcliffe (1918) as highly transparent but slightly yellowish spherical eggs ranging from 1.0 to 1.15 mm in diameter. The yolk sphere contained a

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variable number of 10 to 25 unequal-sized oil globules scattered over the yolk surface which showed some tendency toward aggregation with progressing development. The diameter range was extended from 0.94 mm to 1.20 mm by Bigelow and Schroeder (1953) and Wheatland (1956), respectively. The upper diameter limit extension was verified by Herman (1963). *Prionotus evolans* ova have never been positively identified. Perlmutter (1939) made a tentative identification, later accepted by Marshall (1946), from ripe ova stripped from gravid females collected in Long Island Sound and described as having similar appearance and diameter as northern searobin eggs, but with oil globules clustered at one pole rather than dispersed across the yolk sphere surface. This singular observed morphological difference of oil globule distribution pattern has been used as the primary distinguishing characteristic between ova of *Prionotus carolinus* and *Prionotus evolans*.

MATERIALS AND METHODS

Conventional Identifications

Field-collected, buffered Formalin[3]-preserved plankton samples were physically sorted for all ichthyoplankton using forceps under a dissecting microscope, and the criterion of oil globule distribution differences established by Perlmutter (1939) was used to tentatively separate *P. carolinus* from *P. evolans* eggs. The annual cycle and species composition aspects of the field-collected samples using conventional means for egg and larval identifications have been submitted elsewhere for publication.

[3]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Immunochemical Identifications

Antigens and Immunizations

Antigen preparations from both species of searobin eggs were generated using the techniques developed by Orlowski et al. (1972) with ovarian tissue from ripe adults and immature individuals. The four antigen preparations presented in detail in Table 1 were each used to elicit immune responses in at least two New Zealand white rabbits to improve the probability of obtaining useful antisera. Preimmune serum samples were obtained from each animal to establish that no reactivity with antigen existed prior to immunization.

The soluble protein antigens of *Prionotus evolans* (PeSP) and *Prionotus carolinus* (PcSP) were injected intravenously in 4.7 and 4.8 mg protein doses (standard biuret analysis), respectively, to begin the immunization program. Maintenance injections of 2 mg protein followed on a weekly basis. Blood samples were obtained by cardiac puncture 3 wk following the first injection and the presence of precipitating antibody was demonstrated by the standard precipitin ring test (Abramoff and LaVia 1970). Additional monthly cardiac puncture samples were monitored by quantitative double diffusion (Feinberg 1957) until after about 12 wk; a titer of 32 was reached in all animals receiving soluble antigens when sera were tested with 40 μg homologous antigen.

Particulate protein antigens from macerated ovarian tissue of northern (PcPP) and striped (PePP) searobins were prepared in a 1:1 emulsion with Freund's complete adjuvant (Cappell Laboratories). PcPP (8 mg) and PePP (10 mg) protein preparations were injected subcutaneously along several bilateral dorsal sites on New Zealand white rabbits. Rabbits injected with Freund's complete adjuvant developed

TABLE 1.—Antigen characterization and nomenclature.

Species	Antigen source and designation	Range protein concentration (mg/mL)	Method of determination	Immunization route and dose		Titer	
				Initiation	Maintenance	Double-diffusion	Complement fixation
Prionotus carolinus Northern searobin	Mature ova (PcSP)	8-15	Biuret	Intravenous (4.7 mg)	Intravenous (2 mg)	32	
	Immature follicular material (PcPP)	15-40	Microkjeldahl	Subcutaneous (8 mg)	Intravenous (2 mg)		1,280
Prionotus evolans Striped searobin	Mature ova (PeSP)	8-15	Biuret	Intravenous (4.8 mg)	Intravenous (2 mg)	32	
	Immature follicular material (PePP)	15-40	Microkjeldahl	Subcutaneous (10 mg)	Intravenous (2 mg)		1,280

Arthus reactions following a single dose. Subsequent injections were accomplished intravenously using Millipore (0.45 μm) filtrates of PcPP and PePP. Titers were monitored utilizing the standard complement fixation assay because of the particulate consistency of the macerated antigen preparation (Kabat and Mayer 1961). Maximum titers of 1,280 were obtained after about 10 wk with immunizations using PcPP or PePP.

Antiserum Specificity

Antisera elicited in response to both soluble and particulate antigens were multicompetent and exhibited cross-reactions with heterologous antigens. The presence of common antigens between the northern searobin and striped searobin ovarian material preparations required the specific adsorption of antisera with these shared antigens to render a given antiserum monospecific (Eisen 1974). Although antisera elicited in response to particulate protein antigens exhibited precipitation reactions in agar with both soluble antigens and extracts of particulate antigens from the two species under consideration, they were not competent in reactions with homologous fish eggs. Therefore, since the selected method for analysis of planktonic eggs was immunodiffusion, only antisera elicited in response to soluble antigens were used in all analyses of unknowns. Specific adsorption of common antigens shared by northern and striped searobins was accomplished by adding PcSP to antisera elicited in response to PeSP and vice versa. Adsorption lots of 1.5 mL anti-PeSP antisera combined with 70 μL PcSP (0.65 mg protein) were incubated at 4°C for 48 h prior to use. This adsorption eliminated all reactivity of anti-PeSP antisera with both PcSP and known ova of *P. carolinus*, without significantly reducing activity with ova of *P. evolans*. This specifically adsorbed anti-PeSP, which reacted solely with known homologous ova of *P. evolans* under controlled conditions, was used as the basis for differentiation of northern and striped searobin eggs. Species-specific anti-PeSP antisera capable of 100% accuracy in differentiating known ova of both searobins was the reagent selected for use in all immunodiffusion analyses.

Microimmunodiffusion Analysis

Unknown planktonic fish eggs were analyzed with monospecific anti-PeSP antiserum in a micromodification of the immunodiffusion technique (Ridgeway et al. 1962). Microscope slides (2.5 × 8 cm) were washed, rinsed first in distilled water and then methanol, and wiped dry. Two milliliters of 1% Noble Agar (Difco) in FA-Bacto buffer (Difco), pH 7.2, were applied across each slide on a leveling table and allowed to harden. Slides were then placed over a template and wells cut using a Brewer needle with beveled inner surface (Ridgeway et al. 1962).

Agar plugs were removed from wells by aspiration. Reagents were applied with either 1 mL syringes (Burron) or sterile capillary pipettes, and 0.005 to 0.01 mL was required to fill each well. A typical testing array appears in Figure 1, where corner wells contain unadsorbed antiserum, the central well contains adsorbed or monospecific antiserum, and remaining wells contain individual fish eggs which have been broken using jeweler's forceps. FA-Bacto buffer was applied to each well following egg disruption, and slides were allowed to incubate in moist chambers for 18 h at 20°C. Slides were then washed for 24 h in FA-Bacto buffer, and stained according to the method of Crowle (1958). Results were always recorded at a fixed time interval following slide preparation to insure comparability from one determination to another.

RESULTS AND DISCUSSION

A total of 732 searobin ova were recovered from plankton samples collected in the 1973-74 period. The combined morphological characteristics of egg diameter, number, color, and distribution of oil globules, and embryo pigmentation when present, allowed the separation of searobin eggs from those of other species with reasonably high confidence. Preliminary classifications of *Prionotus* ova into either *evolans* or *carolinus* species was based upon differential oil globule distribution patterns reported by Perlmutter (1939). Striped searobin, *P. evolans*, eggs were placed into one grouping based upon a polar or clustered oil globule distribution, and northern searobin, *P. carolinus*, eggs placed into a second group having oil globules generally dispersed across the yolk sphere.

Each tentatively classified egg was then analyzed in the microimmunodiffusion method illustrated in Figure 1, to establish the immunochemical reactivity of soluble egg antigens with adsorbed and unadsorbed anti-PeSP antisera. When soluble *P. evolans* egg antigens were sufficiently concentrated, a classical line of identity was observed with fusion of precipitin bands between adsorbed and unadsorbed anti-PeSP wells. Identification of *P. carolinus* eggs was based upon reactivity with unadsorbed anti-PeSP antiserum and no reactivity with adsorbed anti-PeSP. Previously established reactivity of unadsorbed anti-

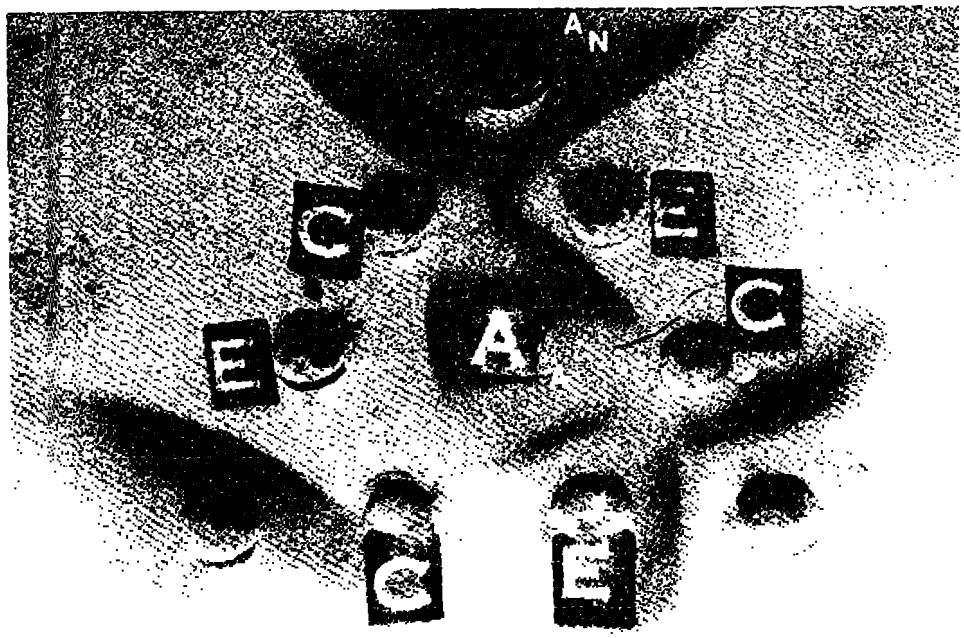

FIGURE 1.—Testing array (10×). C: *Prionotus carolinus* ovum (1.00 mm); E: *Prionotus evolans* ovum (1.00 mm); A_A: Anti-PeSP antiserum (adsorbed: 0.20 mL antiserum: 0.11 mg PeSP protein); A_N: Anti-PeSP antiserum (unadsorbed). Specific adsorption of cross-reactive antibodies has occurred with PeSP, rendering anti-PeSP antiserum (A_A) incompetent to react with antigens of *Prionotus carolinus* ova (C), indicated by the lack of precipitin bands about the central well adjacent to (C) egg wells. Corner wells contain multicompetent, unadsorbed anti-PeSP antisera.

PeSP with known *P. carolinus* eggs was considered sufficiently definitive for its use in differentiating *P. carolinus* from *P. evolans* ova.

The immunochemical classifications derived from this analysis indicated that an average 22.3% misclassification error had been made when eggs were differentiated solely on the basis of oil globule distributions. An approximately equal number of both northern and striped searobin eggs had been mistakenly identified, based upon oil globule distribution patterns. The final classification based upon immunochemical data was 406 ova of *P. carolinus* and 326 ova of *P. evolans*.

It was confirmed that egg diameters could not serve as a reliable characteristic for species classifications by retrospectively analyzing diameters of immunochemically classified eggs according to the period of field collection. The data presented in Table 2 illustrate that no statistical difference exists in the diameter ranges of *P. carolinus* and *P. evolans* eggs for the collection period of this study. However, the trend of declining egg diameters over the spawning season previously documented by other workers is

TABLE 2.—Immunochemical classification of *Prionotus* spp. eggs collected in plankton samples.

Date	Average diameter (mm)	Range (mm)	*n*
Prionotus carolinus			
1973			
May	1.16	1.02-1.24	4
June	1.06	1.00-1.21	10
July	1.08	1.05-1.10	3
August	1.02	0.92-1.18	312
September	0.99	0.90-1.05	32
1974			
July	0.98	0.95-1.02	13
August	0.96	0.92-1.02	3
September	0.99	0.92-1.02	29
Prionotus evolans			
1973			
May	1.12	1.00-1.25	10
June	1.06	1.00-1.12	35
July	1.08	1.00-1.15	2
August	1.03	0.95-1.12	225
September	0.98	0.90-1.08	26
1974			
July	0.97	0.95-1.00	6
August	1.02	1.02	1
September	0.99	0.92-1.02	21

confirmed. The data also show that in 1973 and 1974, the ratios of eggs collected in plankton samples and identified based upon morphology and immunochemical reactions for nothern and striped searobins were 1.1:1 and 1.6:1, respectively. These ratios are similar in magnitude to the ratio of northern and striped searobin adults observed by Marshall (1946). Finally, the data indicate that egg diameter and oil globule distribution cannot serve to reliably distinguish northern from striped searobin eggs. An immunochemical distinction can be made that suggests morphology alone is inadequate to provide a positive identification of *P. evolans* eggs.

The course of future research in immunochemical taxonomy of fish eggs should emphasize an increase in sensitivity, as well as automation of the analysis. At present, the utility of the immunodiffusion method is limited by its labor-intensive nature. Initial stages of the analysis require manual sorting of ova from plankton samples that is tedious, time-consuming, and subject to error. Bowen et al. (1972) initiated studies in which a moderate degree of success was achieved in sorting fish ova from pelagic plankton samples on sucrose density gradients. However, estuarine plankton samples that contained a wide range of particulate materials characterized by different sizes, densities, and shapes, and that also included high levels of detrital materials, disturbed the gradients sufficiently to destroy separation potential. Despite the recognized limitations, there is currently no practical alternative to manual sorting of plankton samples.

Immunodiffusion analysis requires that individual fish eggs be subjected to several manual manipulations, with the final determination in solid media requiring the careful applications of reagents. Screening large numbers of planktonic ova with several different antisera becomes impractical on a large scale. A more rapid and potentially more specific approach to immunochemical ichthyoplankton identifications might employ monoclonal antibodies coupled to fluorescent indicator molecules. The antibody products of fused mouse lymphocytes and myeloma cells may be screened and selected for exquisite specificity to single antigenic determinants or epitopes using egg antigens of known origin, preferably those associated with the chorion surface, to procure a reagent that would specifically label ova without requiring that each egg be mechanically ruptured. Identifications might be based upon the differential fluorescence characteristic of a particular fluorescent label associated with a selected antibody and labelled eggs might be isolated using a fluorescence-activated cell sorter.

The utility of immunochemical identifications with demonstrably superior accuracy to conventional methods has been established with both intergeneric and interspecific differentiations. Several systems remain which might benefit from immunochemical differentiations, such as the complete elucidation of several sciaenid and clupeid species which occur in complex estuarine systems, such as the Chesapeake Bay and Potomac River estuary. Relationships between scombrids, bothids, and pleuronectids with more southerly distributions would serve to delineate adult ratios, population distributions, and spawning seasons. Finally, the capability of the immune system to differentiate among epitopes with relatively small structural difference (Karush 1962) might eventually be applied to the detection of racial differences or subpopulation distinctions among fish ova of the same species.

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LITERATURE CITED

ABRAMOFF, P., AND M. LAVIA.
1970. Biology of the immune reponse. McGraw-Hill, Inc., N.Y., 492 p.

BIGELOW, H. B., AND W. C. SCHROEDER.
1953. Fishes of the Gulf of Maine. U.S. Fish Wildl. Serv., Fish. Bull. 53:1-577.

BOWEN, R. A., J. M. ST. ONGE, J. S. COLTON, JR., AND C. A. PRICE.
1972. Density-gradient centrifugation as an aid to sorting planktonic organisms. I. Gradient materials. Mar. Biol. (Berl.) 14:242-247.

CROWLE, A. J.
1958. A simplified micro double-diffusion agar precipitin technique. J. Lab. Clin. Med. 52:784-787.

EISEN, H. N.
1974. Immunology: An introduction to molecular and cellular principles of the immune responses. Med. Dep., Harper & Row, Inc., Hagerstown, MD, 624 p.

FEINBERG, J. G.
1957. Identification, discrimination, and quantification in Ouchterlony gel plates. Int. Arch. Allergy 11(3-4):129-152.

HERMAN, S. S.
1963. Planktonic fish eggs and larvae of Narragansett Bay. Limnol. Oceanogr. 8:103-109.

KABAT, E. A., AND M. M. MAYER.
1961. Complement and complement fixation. Experimental immunochemistry. 2d ed. Chase Thomas Publ., Springfield, IL, 905 p.

KARUSH, F.
1962. Immunologic specificity and molecular structure. Adv. Immunol. 2:1-40.

KEIRANS, W. J., JR.
1977. An immunochemically assisted ichthyoplankton survey with elaboration on species specific antigens of fish egg

vitellins; southern New Jersey Barrier Island - Lagoon Complex. Ph.D. Thesis, Lehigh Univ., Bethlehem, PA 169 p.

KUNTZ, A., AND L. RADCLIFFE.
1918. Notes on the embryology and larval development of twelve teleostean fishes. Bull. U.S. Bur. Fish. 35:89-134.

MARSHALL, N.
1946. Observations on the comparative ecology and life history of two sea robins, *Prionotus carolinus*, and *Prionotus evolans strigatus*. Copeia 1946:118-144.

MORGAN, R. P., II.
1975. Distinguishing larval white perch and striped bass by electrophoresis. Chesapeake Sci. 16:68-70.

ORLOWSKI, S. J., S. S. HERMAN, R. G. MALSBERGER, AND H. N. PRITCHARD.
1972. Distinguishing cunner and tautog eggs by immunodiffusion. J. Fish. Res. Board Can. 29:111-112.

PERLMUTTER, A.
1939. A biological survey of the salt waters of Long Island, 1938. Section I. An ecological survey of young fish and eggs identified from tow-net collection. Suppl. 28th Annu. Rep. N.Y. Conserv. Dep. Part II:11-71.

RIDGEWAY, G. J., G. W. KLONTZ, AND C. MATSUMOTO.
1962. Intraspecific differences in serum antigens of red salmon demonstrated by immunochemical methods. Int. North Pac. Fish. Comm. 8:1-13.

WHEATLAND, S. B.
1956. Oceanography of Long Island Sound. 1952-1954. VII. Pelagic fish eggs and larvae. Bull. Bingham Oceanog. Coll., Yale Univ. 15:234-314.