

Acknowledgments

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HISTOCHEMICAL INDICATIONS OF LIVER GLYCOGEN IN SAMPLES OF EMACIATED AND ROBUST LARVAE OF THE NORTHERN ANCHOVY, *ENGRAULIS MORDAX*

On the basis of histological criteria (O'Connell 1976), 8% of northern anchovy, *Engraulis mordax*, larvae from special net tows taken in the Southern California Bight in March 1977 were found to be in starving condition (O'Connell 1980). Almost three-quarters of the larvae that showed signs of starvation were concentrated in 4 of the 64 net tow samples. The present report compares the amount of glycogen in livers of additional larvae drawn from these four samples to that in the livers of larvae from samples taken in the same area, which contained robust larvae almost exclusively.

Glycogen, which is stored in the liver and transformed to glucose as needed to maintain an adequate blood sugar level, is the most immediately available of the three energy sources, glycogen, lipid, and protein (Love 1974). It is known to virtually disappear from the livers of many teleosts after only a few days of starvation (Black et al. 1966; Inui and Ohshima 1966; Bellamy 1968), but fish generally live long beyond the depletion of liver glycogen, maintaining the blood sugar level by gluconeogenesis (Love 1974; Cowey and Sargent 1979). However, there are also teleosts in which liver glycogen does not decline sharply at the onset of starvation, although gluconeogenesis does increase (Cowey and Sargent 1979). Thus abundance of liver glycogen cannot be considered a dependable indicator of starvation in teleosts, at least not for adult stages.

Postyolk-sac larval stages, which first exhibit stained liver glycogen about the time yolk is depleted, are more likely to show a drop in liver glycogen at onset of starvation. First feeding northern anchovy larvae die after only a few days of starvation (O'Connell 1976), indicating that reserves are limited. Lipid reserves, for example, are known to be negligible in early postyolk-sac herring and plaice larvae (Ehrlich 1974), and even at the relatively large size of 35 mm SL northern anchovy larvae survive starvation conditions for only 2 wk, on the average, during which time lipid reserves are severely depleted (Hunter 1976). Presumably liver glycogen declines sharply before lipid reserves are depleted in these early stages.

The estimates of glycogen reserves in the works cited above, and in many others, are derived from weight-based biochemical determinations, which

is not a feasible approach for small larvae already preserved as part of plankton samples at sea. A histochemical approach is feasible, however, and we elected the periodic acid-Schiff (PAS) procedure, which has largely superseded other histochemical tests for glycogen (Davenport 1960).

Cardell et al. (1973) showed good correlation of PAS staining reactions with biochemical determinations of liver glycogen for the rat. Glycogen decreased from about 9% of liver wet weight to 0.7% after 1 d of fasting and to 0.4% after 3 d. At the start of the experiment all hepatocytes had dense masses of intensely stained glycogen. As the fasting period lengthened to 2 and 3 or more days, the masses decreased in size, number, density, and stain intensity, and the number of cells showing glycogen decreased markedly. Presumably such differences in staining reaction in livers of northern anchovy larvae would be an indication of differences in glycogen reserves.

Materials and Methods

In the laboratory, 49 larvae in the size range 3.5-11 mm SL were selected by random dipping from about half of the 37 nearshore net tow samples that showed larvae of generally good histological condition from the March 1977 cruise (O'Connell 1980). Forty-one larvae were selected, also by random dipping, from three of the four nearshore tows containing abundant larvae in generally poor histological condition. An additional dozen larvae were selected from three of the offshore tows.

All larvae had been fixed at time of capture in Bouin's fluid and stored in 70% ethyl alcohol. They were subsequently dehydrated in *n*-butyl alcohol, embedded in paraffin, sectioned sagittally and stained by the PAS method (Preece 1965). We did not subject the material used in this study to diastase digestion tests but feel confident that the red coloration in the livers indicates glycogen. Glycogen gives one of the stronger reactions to the PAS stain (Lillie and Fullmer 1976), and Cardell et al. (1973) established with diastase controls that the PAS-positive material in the livers of rats was glycogen. The diastase test essentially distinguishes between glycogen and mucins (Preece 1965), some of which show strong PAS reactions, but these occur primarily in the integument and epithelia of the digestive tract and various glands of animals (Lillie and Fullmer 1976).

After staining, slides were randomized with

their identities concealed and then rated by microscope examination. Each of two observers rated each specimen as High, Medium, or Low, depending on the degree and extent of red coloration in the liver. The Low grade was assigned when livers showed virtually no red color, the High grade when color was strong and widespread. The Medium grade was assigned when color was light and scattered, or irregular. The two readers disagreed on a little >12% of the larvae but never by more than one grading step. These differences were reconciled by reexamination and discussion.

No attempt was made to characterize the specimens stained by the PAS procedure as robust or emaciated on the basis of histological factors per se. There was the possibility that the PAS procedure would be less precise and consistent than the previously used hematoxylin and eosin stain in demonstrating cell and tissue components other than polysaccharides.

Study of material from the sea samples was preceded by analysis of 99 specimens from groups of larvae that were fed or starved in the laboratory. Fixation, staining, and microscope analysis were exactly as outlined above, except that the laboratory material was held in 70% ethyl alcohol for only a few days before dehydration and embedding. Reader disagreement was 9% on the laboratory material.

The larvae obtained from laboratory containers ranged from 5- to 26-d-old. The rotifer *Brachionus plicatilis* was introduced into containers as food at a density of 40 to 60/ml when the larvae were 3-d-old and maintained above 30/ml by additions as needed. The smaller *Gymnodinium splendens* was included as a starting food at the outset, but was not afterwards maintained. Northern anchovy larvae require *Brachionus* at densities of at least 10 to 20/ml to survive and grow well in laboratory containers for the first weeks of life (Theilacker and McMaster 1971).

Food was withheld from two containers, and specimens from these were sacrificed on the first and second day after yolk exhaustion. Starvation at more advanced ages was accomplished by removing the food from selected containers 2 to 4 d before the larvae were sacrificed. Food was removed with a siphon filter devised by P. Paloma for the purpose. A typical air-driven aquarium siphon was enclosed in an 8.89 cm (3.5 in) diameter perforated plastic cylinder covered by nylon netting with 0.333 mm mesh openings, which allowed food organisms but not fish larvae to pass

towards the siphon. The outflow tube of the siphon recurved after passing through the top stopper of the cylinder and terminated in a small perforated cylinder above the water surface. Inside the small cylinder the outflow tube opened into a bag of nylon netting with 0.024 mm mesh openings, which removed food organisms and allowed water to return to the rearing container by dropping onto a small glass plate at the water surface.

Operating at a flow rate of 3 l/h in containers of 12.6 l capacity, the filter reduced *Brachionus* populations ranging from 40 to 100/ml at the start to 7/ml in 1 d, 3/ml in 2 d, and considerably < 1/ml in 3 d. It reduced populations of *Gymnodinium* at about the same rate. At the filtering rate of 3 l/hr, larvae close to the submerged barrier cylinder did not appear to be disturbed.

Results

Laboratory material showed good association between food treatment and PAS glycogen rating over the entire length range involved, 3 to 15 mm SL. The larvae were divided into two groups, those smaller than the median (6.4 mm SL) and those larger than the median. For both size groups, as for all larvae pooled, the majority of High ratings occurred in the "fed" category and the majority of Low ratings occurred in the "starved" category (Table 1). It is noteworthy that only 4 larvae among the 45 from starvation treatments received High ratings. Three of these were from the oldest age group, 26 d (starved 3 d), and were in fact three of the four largest larvae in the entire starved contingent. Examples of stained liver sections representing High, Medium, and Low glycogen ratings are shown in Figure 1.

Larvae from the laboratory trials were fixed in Bouin's solution, as described earlier, to be comparable to the Bouin-fixed sea samples, but a few other fixatives were tested as a matter of perspective. Gendre's solution (Preece 1965) gave somewhat better results than Bouin's, but 10%

alcoholic Formalin,¹ which is theoretically a better fixative for preserving glycogen (Davenport 1960; Preece 1965), gave the best results. Some specimens fixed in alcoholic Formalin from fed containers had most hepatocytes solidly filled with deep red color (Figure 1). Presumably more of the glycogen present in the livers at termination was retained with this fixation. However, integrity of cells and tissues of the larvae was not well preserved in alcoholic Formalin.

High, Medium, and Low ratings for the ocean-caught larvae (Figure 2) reflect essentially the same levels of staining intensity as for laboratory material. Ocean samples show an appreciably higher proportion of Medium ratings than the laboratory material, but nevertheless exhibit some association between glycogen rating and histological characterization of the samples from which the larvae were drawn (Table 2). However, the association occurs only among the smaller larvae, as indicated by comparing the distributions for larvae smaller and larger than the median, 6.9 mm SL. The smaller larvae show a relatively good proportion of High ratings for robust samples and of Low ratings for emaciated samples. The larger larvae do not exhibit this association.

Significance of the various distributions described above is indicated by X^2 values (Table 3) calculated for each of the six pairs of columns (treated as 2×3 contingency tables) from Table 1 and 2. All three X^2 values for the laboratory material clearly reject the null hypothesis, i.e., that there is no association between the two classifications, food treatment and liver glycogen rating. For the ocean material the null hypothesis is rejected only for small larvae, indicating

¹Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

TABLE 1.—Distribution of High, Medium, and Low glycogen ratings, based on PAS staining intensity in livers of northern anchovy larvae fed or starved in the laboratory.

Glycogen rating	≤ 6.4 mm SL		> 6.4 mm SL		Pooled	
	Fed	Starved	Fed	Starved	Fed	Starved
High	14	1	25	3	39	4
Medium	2	7	3	4	5	11
Low	8	18	2	12	10	30

TABLE 2.—Distribution of High, Medium, and Low glycogen ratings, based on PAS staining intensity in livers of northern anchovy larvae from ocean samples showing either generally robust condition or generally emaciated condition. Condition of samples is based on a previous histological study of other specimens from the same collection.

Glycogen rating	≤ 6.9 mm SL		> 6.9 mm SL		Pooled	
	Robust	Emac.	Robust	Emac.	Robust	Emac.
High	7	2	9	8	16	10
Medium	17	5	7	10	24	15
Low	4	10	5	6	9	16

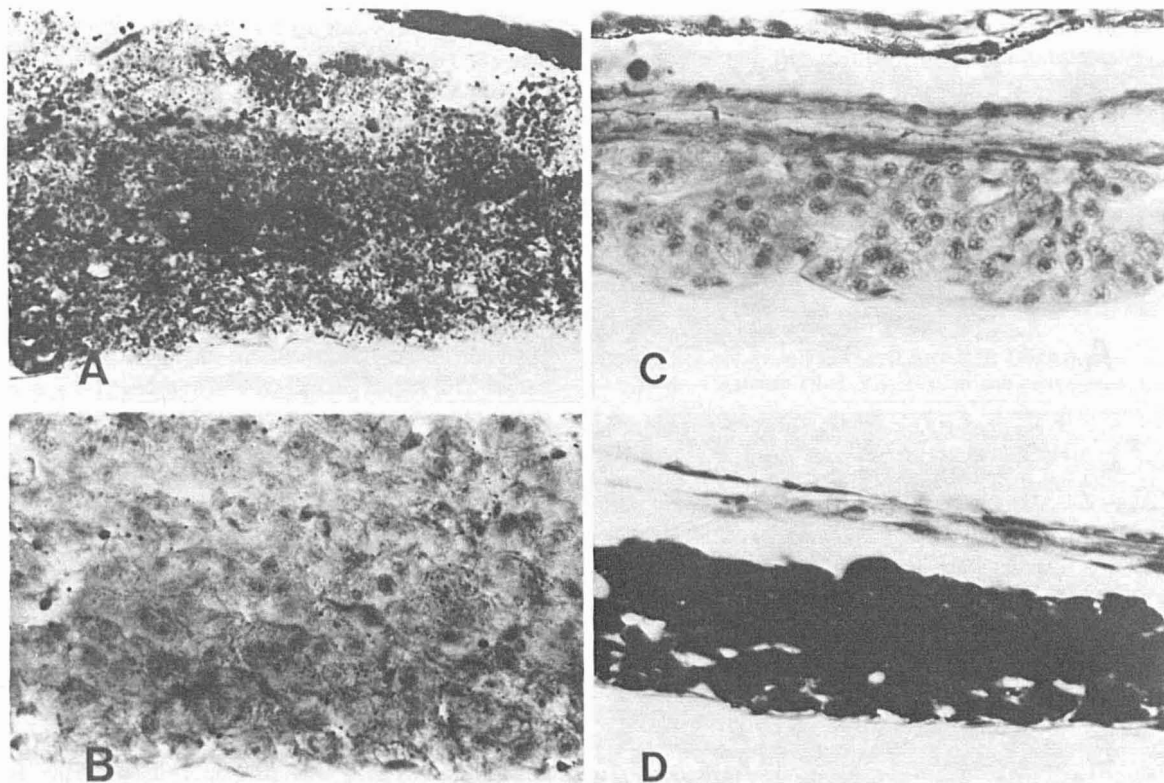


FIGURE 1.—Sagittal sections of livers of northern anchovy larvae from the laboratory, showing the three grades based on intensity of the PAS staining reaction. Except for the nuclei, and occasional portions of other organs, black and the darker shades of grey represent PAS-positive red material presumed to be glycogen. All photomicrographs were taken and processed in the same way. All were taken at $787\times$. A. 5.5 mm larva, fed, graded High. B. 5.0 mm larva, starved, graded Medium. C. 6.1 mm larva, starved, graded Low. D. 5.2 mm larva, fed, graded High. The first three were fixed in Bouin's fluid, but the fourth was fixed in 10% alcoholic Formalin and showed a very intense staining reaction.

TABLE 3.— χ^2 and mean contingency square (X^2/n) values for the association between PAS glycogen rating and food treatment for the arrays of larvae shown in Table 1 (laboratory) and Table 2 (ocean). Each set of values is for a 2×3 contingency table with (n) larvae and 2 df. In each group, small larvae are those below, and large larvae those above, the median standard length.

Contingency set	n	χ^2	P	X^2/n
Laboratory:				
Small larvae	50	17.84	0.001	0.36
Large larvae	49	23.28	.001	.48
Pooled	99	40.13	.001	.41
Ocean:				
Small larvae	43	9.74	.01	.22
Large larvae	43	.47	NS	.01
Pooled	86	4.73	NS	.05

that only for the lower part of the length range is there a meaningful association between treatment (histological characterization of the samples from which larvae were drawn) and liver glycogen rating. As already mentioned, the relevant asso-

ciation is High ratings with samples of generally robust histological condition, and Low ratings with samples of generally emaciated condition. The mean square contingency values (X^2/n) indicate the extent to which the association for small larvae from the ocean is weaker than those from the laboratory.

The dozen larvae from the offshore samples, not included in the above analysis of inshore larvae, showed relatively high liver glycogen ratings. They were almost equally divided among three tows, each of which was taken between 0300 and 0400 h on a different day and contained larvae in a different part of the size range. There were no Low liver glycogen ratings among them. Ratings were largely Medium for the small to medium standard lengths (3.4-7.8 mm) and High for the largest larvae (8.0-9.2 mm). These High ratings, moreover, represent the most intensely PAS

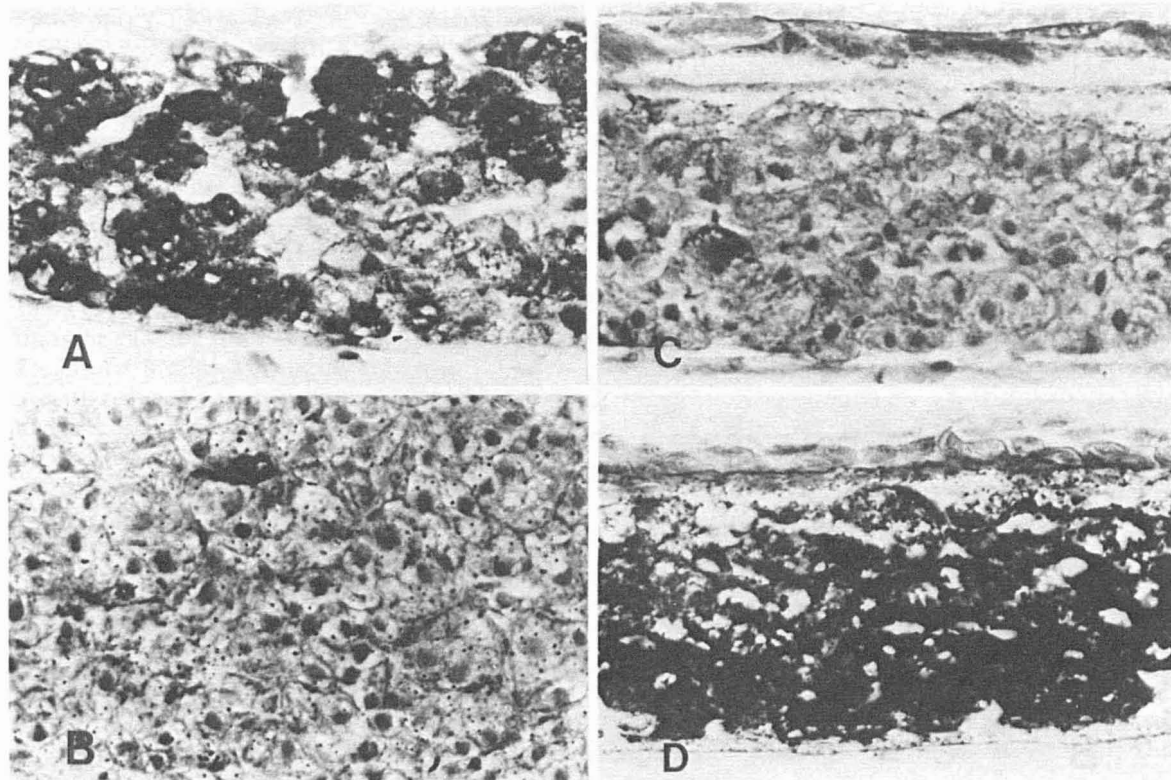


FIGURE 2.—Sagittal sections of livers of anchovy larvae from ocean samples, showing the three grades based on intensity of the PAS staining reaction. Darker shades represent PAS positive red material. Photomicrographs were taken and processed in the same way as those in Figure 1. All were taken at $787\times$. A. 6.2 mm larva, robust sample, graded High. B. 6.3 mm larva, emaciated sample, graded Medium. C. 6.0 mm larva, emaciated sample, graded Low. D. 9.2 mm larva, robust sample, graded High. Whereas the first three are from inshore samples, the fourth is from an offshore sample that produced the most intense staining reactions of all ocean samples.

stained livers in the entire set of ocean specimens (Figure 2).

Discussion

This study was undertaken to determine whether a histochemical test for glycogen in the liver would indicate the occurrence of starvation among northern anchovy larvae from the sea. The results show that variation in glycogen concentration was lower, on the average, for larvae from sea samples classified in a previous histological study (O'Connell 1980) as generally emaciated, than for larvae from samples classified as generally robust. However, the difference was not as strong as it was for larvae that were fed or starved in the laboratory.

Glycogen stored in the livers of fishes can be severely reduced by sustained swimming activity

at moderate levels (Miller et al. 1959; Pritchard et al. 1971) or by starvation (Love 1974). It may also undergo some change relative to daily cycles of feeding and nonfeeding. There is no reason to believe that undue exercise was involved in the present case, and the data do not indicate a relation between glycogen rating and hour of capture. Starvation therefore seems the most likely explanation of the low levels shown by certain samples studied here, and this is supported by the relation of glycogen level to plankton volume, a not unreasonable index of food availability in the sea. For those sea samples characterized as emaciated and showing low liver glycogen reserves, the plankton volumes were among the lowest volumes obtained on the March 1977 cruise (O'Connell 1980). These low plankton volumes can be assumed to represent reduced but not essentially nonexistent food, as does

the starvation treatment in the laboratory. This difference might account for the greater proportion of Medium ratings in the ocean samples, robust as well as emaciated. In any event, a moderate proportion of Medium glycogen ratings for the ocean samples is not inconsistent with the earlier histological assessments. The emaciated samples, for example, contained only 60% larvae showing histological signs of moderate to severe emaciation or starvation (O'Connell 1980).

The apparently higher level of liver glycogen among larvae from offshore samples further indicates that food availability is an important factor governing liver glycogen reserves. The plankton volumes for all offshore samples averaged considerably higher than the plankton volumes for inshore samples (O'Connell 1980). Moreover, the particular offshore sample that produced the larvae with the most intense PAS indications of liver glycogen had the second highest plankton volume of the entire 1977 cruise.

The fact that the association of glycogen reserves and histological condition is evident for only the smaller larvae from the sea might mean that as larvae become larger, they can better tolerate fluctuations in the plankton regime. There was a hint of this also in the laboratory results. Marine fish larvae in general select progressively larger prey and also a broader size range of prey as they grow, and engraulids tend to increase maximum prey size markedly between 8 and 12 mm SL (Hunter in press). Thus a reduction in any part of the crustacean plankton community, but particularly among the smaller organisms, could be detrimental primarily to the smaller larvae.

The PAS staining procedure appears, then, to be capable of demonstrating the presence of starvation effects in a larval fish population. On balance, it is a more readable indicator than the comprehensive histological analysis, but it also provides less information. The level of liver glycogen reflects the status of only the first line of energy reserve, whereas histological analysis, with more varied indications, is more sensitive to the extent of emaciation that has been sustained by the larva. Perhaps both approaches can be applied to the same larva, but if so, the histological indications should be interpreted cautiously. Our impression is that tissues of larvae can be moderately distorted and degraded by the PAS procedure regardless of feeding history or fixative. On the other hand, the histochemical test might

provide a sufficient characterization of the condition of larval fish samples for some purposes, especially if fixation is optimized for the preservation of glycogen.

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