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EXTRACTABLE LIPOFUSCIN IN LARVAL MARINE FISH

The "age pigment", lipofuscin, is thought to be a biochemically heterogeneous byproduct of the peroxidation of polyunsaturated lipids which accumulates in dense, intracellular inclusions called ceroid bodies. The material has been studied both microscopically and biochemically in tissues of several species (Miquel et al. 1977; Shimasaki et al. 1980; Dowson 1982), and some portion of it is quantitatively extractable with organic solvents (Fletcher et al. 1973).

Flies prevented from flying by putting baffles in the bottles in which they were raised, accumulated lipofuscin (as assessed by solvent extraction) more slowly than did free-flying flies but had a longer lifespan, so that at the ends of the respective lifespans the body contents of lipofuscin were similar in the two groups (Sohal and Donato 1978). Extractable lipofuscin thus appears to accumulate as a function of cumulative oxidative metabolism; it could be an indicator of physiological (rather than strictly chronological) age.

Additionally, if lipofuscin represents an integral of oxidative metabolism since birth and weight represents an integral of growth over the same period, the ratio of lipofuscin to organic weight should be proportional to the reciprocal of cumulative net growth efficiency [K_2 = growth/assimilation = growth/(growth + respiration), hence $1/K_2 = 1$ + respiration/growth].

Ettershank (1984a) introduced the fluorometric measurement of extractable lipofuscin as a measure of physiological age in growing marine crustaceans, based on the work with insects, and (1984b) recommended a simple method for routine use in marine work. He also argued (without presenting extensive evidence) that preservation

of tissue in formalin-seawater did not invalidate the analysis; thus, historical samples of zooplankton (and, presumably, larval fish) appeared to be usable for study of intraspecific geographic or interannual variability.

Although most studies of lipofuscin (especially by histology) have concerned postmitotic cells, such as nervous tissue, we evaluated the assay as an estimate of the relative efficiency of growth of larvae analyzed whole. That is, we were less interested in lipofuscin as an indicator of age (for which, as we show, other measures of mass are useful) than as an indicator of health whose relation to mass would reflect environmental conditions over time. We therefore investigated the importance of interfering fluorescing pigments (Csallany and Ayaz 1976), tested the effect of preservation in formalin (Nicol 1987), and measured the accumulation of extractable lipofuscin in three species of larval fish reared in the laboratory—California grunion, *Leuresthes tenuis*; white seabass, *Atractoscion nobilis*; and California halibut, *Paralichthys californicus* (hereafter referred as grunion, seabass, and halibut respectively).

Analytical Considerations

We analyzed lipofuscin by a method first described by Fletcher et al. (1973), as modified by Ettershank (1984b). The tissue to be analyzed (usually whole larvae) was frozen (−15° or −70°C) and later freeze-dried, and a 1–5 mg sample was homogenized in at least 2 mL of 2:1 chloroform:methanol in a Wheaton glass tissue homogenizer. After extracting for 3–4 hours at 4°C, 100 mM $MgCl_2$ (25% of the solvent volume) was added, and the solutions were thoroughly mixed and then centrifuged for 20 minutes at 3000 rpm at −4°C. The lower, chloroform layer was withdrawn for fluorometric analysis after reaching 20°C in a water bath, and the fluorescence was measured on a Turner 111[1] fluorometer using a CS 7–60 filter (approximately 360 nm) for excitation and a CS 47B filter (approximately 430 nm) for emission. A known concentration of quinine sulfate was the standard, and results are therefore reported as "fluorescence units" or FU/mg.

A stock solution of quinine sulfate (2 mg/L in 1 N H_2SO_4) was stored in a light-tight reagent bottle. To compare analyses done at different times, sets of standards were prepared from this stock in distilled water (0.02, 0.04, 0.06, 0.08, and 0.10 μg/mL). Some of the sets prepared over a year's time are shown in Figure 1; the overall reproducibility is good.

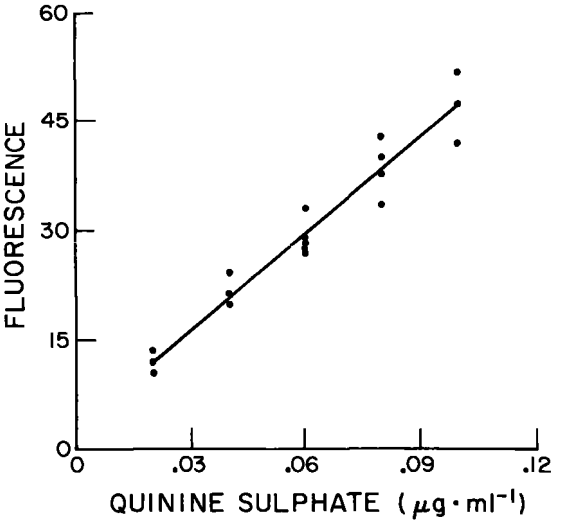


FIGURE 1.—Reproducibility of different sets of quinine sulfate standards prepared from a stock solution over a year.

Precision of the method, including extraction from tissue, was estimated by comparing replicate samples of liver and white muscle from adult halibut. The coefficients of variation for 5 determinations on each of 3 sets of tissue were for muscle, 0.2, 0.35, and 0.57, and for liver, 0.1, 0.15, and 0.18. The difference in variability between the two kinds of tissues is probably due to the greater difficulty in homogenizing muscle tissue.

Quinine sulfate standards were compared on a Farrand Spectrofluorometer, with excitation at 350 nm and emission at 420 nm, and on the Turner 111 fluorometer. The correlation coefficient for measurements on the two instruments was 0.97. We therefore used the Turner 111 routinely, so that analyses could be done easily in a hood.

Csallany and Ayaz (1976) described interference by retinol in the analysis of organic-solvent-soluble lipofuscin in mammalian tissues, and recommended a chromatographic step to remove this contaminant. We extracted a variety of fish tissues in 2:1 chloroform:methanol, and after addition of $MgCl_2$ and centrifugation, dried the chlo-

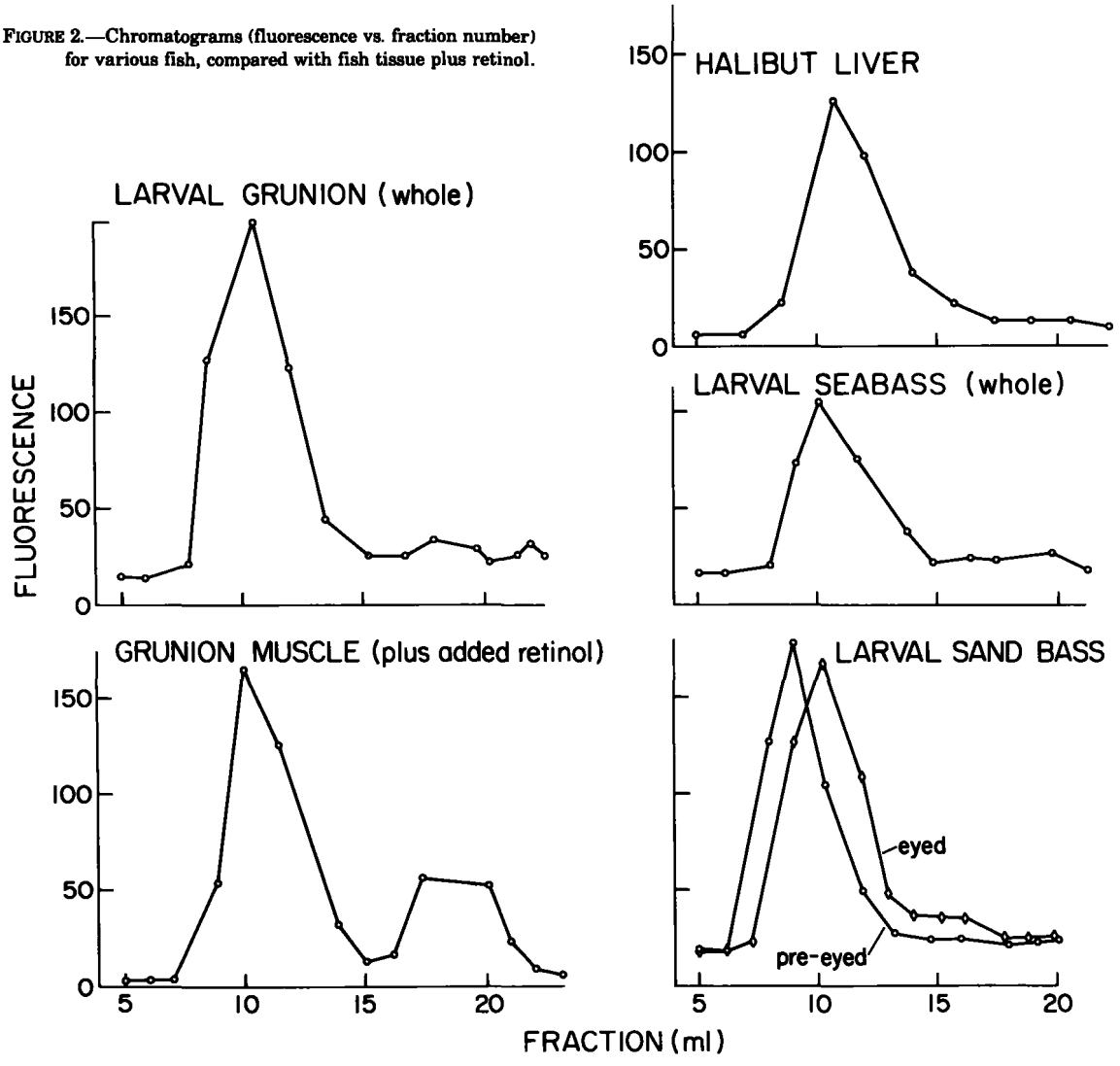
[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA

roform layer gently (35°C) in an open vial overnight in a hood on a sand bath. The dried extract was reconstituted with 1:9 chloroform:methanol and chromatographed on a 30 cm Sephadex LH20 column at a flow rate of 3–4 mL/hour. A fraction was collected by hand every 20 minutes, its volume measured, and its fluorescence determined on the Turner 111 at the same wavelengths used for routine analysis of lipofuscin. To determine where a retinol peak would appear, we chromatographed a tissue extract to which a commercial preparation (Sigma) of retinol had been added. Also, extracts of larval fish in the pre-eyed stage were compared with extracts of larvae that had pigmented eyes, assuming that the eyed larvae would have more retinol.

A UV irradiation step had been proposed to degrade retinol where it may interfere with measurement of extracted lipofuscin, but Csallany and Ayaz (1976) reported that this procedure was ineffective. We therefore determined the time course of degradation of commercial retinol in 1:9 chloroform:methanol by UV irradiation in quartz tubes.

At the wavelengths we used, retinol was not an important interfering substance in a variety of larval fish and in the adult grunion muscle and adult halibut liver tissues (Fig. 2). There also was little difference between extracts of eyed and pre-

FIGURE 2.—Chromatograms (fluorescence vs. fraction number) for various fish, compared with fish tissue plus retinol.



eyed larvae. We therefore did not use UV irradiation routinely. If UV irradiation is used to degrade retinol, the exposure must be kept short (1 or 2 minutes), or else another compound fluorescing at these wavelengths appears (Fig. 3), giving the spurious impression that UV did not affect retinol; this may explain the negative results of Csallany and Ayaz (1976).

To evaluate the effect of preservation in formalin, we froze subsamples from stocks of larvae, and preserved other subsamples in 10% formalin in a glass container, using formalin from a glass reagent bottle. We analyzed frozen and preserved larvae after 3 months, and after an additional year we analyzed more preserved larvae.

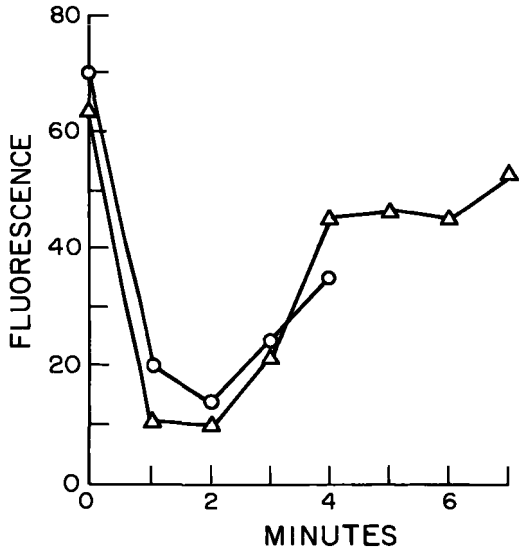


FIGURE 3.—Time course of fluorescence resulting from UV irradiation of duplicate batches (two symbols) of retinol.

Nicol (1987) recently demonstrated that formalin preservation significantly increases the fluorescence of lipofuscin extracted in chloroform-methanol, and our results show similar analytical problems (Table 1). There was significant enhancement in fluorescence in the larvae preserved for over a year, when compared with those preserved for 3 months. Quinine sulfate standards run at the same time as the samples for different periods were very similar (Fig. 4), indicating real changes with time of preservation. Thus, this method will not permit using historical collections of formalin-preserved animals to determine their relative physiological states until more is known about the time course and nature of the effect of formalin on the extract of preserved tissue.

Larval Growth Experiments

Seabass larvae were obtained from Hubbs Research Institute and halibut larvae from the Los Angeles County Natural History Museum; larval grunion were obtained by stripping adults and bringing fertilized eggs into the laboratory for hatching in an aerated, 10 L container of seawater at room temperature.

Larval grunion were reared in 40 L Nalgene tubs with spigots at the bottom (which facilitated emptying and cleaning), initially stocked with 100–120 newly hatched grunion per tub, with replicated "high" and "low" food concentrations. Larvae were fed newly hatched *Artemia* nauplii and the rotifer, *Brachionus*, which was cultured on *Dunaliella tertiolecta* in 100 L, lighted

TABLE 1.—Lipofuscin (as fluorescence units, FU) per animal and per unit dry weight (DW) of frozen vs. formalin-preserved larval fish. N = number of larvae per analysis.

	Frozen (3 months)		Preserved (3 months)		Preserved (1 year)	
	FU (larva)$^{-1}$	FU (mg DW)$^{-1}$	FU (larva)$^{-1}$	FU (mg DW)$^{-1}$	FU (larva)$^{-1}$	FU (mg DW)$^{-1}$
Grunion	16.0	47.4	24.3	60.1	44.3	98.1
N = 15	16.7	47.3	31.0	76.7	45.0	93.1
	17.3	47.7	23.6	60.4	48.0	100.1
	17.0	49.7	24.0	63.0	54.3	112.7
			26.0	65.3	51.0	106.2
$\bar{X}$ =	16.8	48.0	25.7	65.3	48.5	102.0
Seabass	0.68	18.6	0.82	13.6	1.72	22.3
N = 30	0.47	13.0	0.71	12.0	2.05	26.3
	0.50	14.5	0.46	8.0	2.03	27.2
	0.43	12.7	0.52	8.7	1.63	22.2
	0.33	9.4	0.60	10.5	2.05	28.9
$\bar{X}$ =	0.48	13.6	0.62	10.6	1.89	25.4

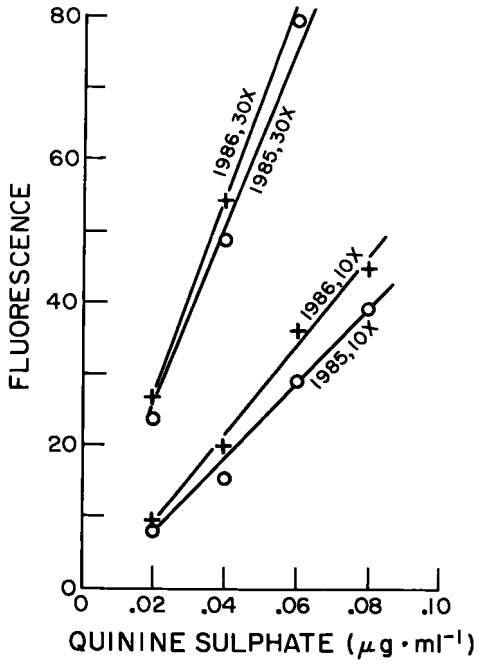


FIGURE 4.—Fluorescence of various concentrations of quinine sulfate standards read at 10× gain (lower pair of lines) and 30× gain (upper pair) in 1985 (lower line of each pair) and 1986 (upper of each pair).

polyethylene tubs augmented with f/2 phytoplankton nutrients (May 1971; Theilacker and McMaster 1971). One spring and one summer experiment were completed at ambient temperatures (spring = 17.5°–19°C, summer = 21°–23°C), with one of the "high food" containers in the second experiment kept at 26°C with an aquarium immersion heater.

We were unable to maintain absolute high and low food concentrations because of oscillations in the supply of food organisms. Thus, the high concentration was kept at 3 times the low concentration, although absolute amounts varied. The mean initial food concentrations in the spring experiment were 50 μg C/L (= "low") and 154 μg C/L (= "high"); in summer, 122 and 394 μg C/L, respectively. Tubs were censused every 2 days through each experiment to determine how much food was uneaten and how many larval fish had died (estimated by counting and removing corpses), and to add fresh food. In the low food containers, it was not unusual to find little uneaten food, particularly as the larvae grew. Once a week the tubs were emptied and the remaining larvae counted directly. It was clear from this direct census that all dead larvae were not accounted for by searching for corpses every 2 days because of cannibalism, necrophagy, or decay. Every 4 days a known number of larvae was removed; their lengths were measured and they were frozen for future analyses.

We could not estimate larval ingestion precisely because of the uncertainty in how many fish were alive through a 2-d interval. This problem was exacerbated as the larvae within each tub diverged in size, so that variance in individual ingestion increased. Although 3 times more food was offered in the high food containers, these larvae actually ingested about twice the amount of food as did those in the low food containers (Table 2). This difference was due to better survival in the high food containers, which affected the ratio between available ration and number of larvae. To compensate for this, we routinely harvested more animals from the high food containers than from the low food containers.

Freeze-dried animals or tissues were weighed on a Cahn electrobalance. Protein was determined by a method of Dorsey et al. (1977) on an aliquot of tissue homogenized in cold 1 M NaCl. DNA was measured by an ethidium bromide technique (Bentle et al. 1981, as modified by M. S. Lowrey). Basic measures of size—dry weight, protein, and DNA—were strongly and linearly correlated (Fig. 5), so that comparing lipofuscin with any of these measures would give similar patterns.

Lipofuscin accumulated as the larval fish grew (Fig. 6), but at quite different rates for the 3 species, grunion accumulating most rapidly (relative to gain in weight) and seabass much the slowest.

TABLE 2.—Estimated average ingestion, size, composition, and growth efficiency for 20-day-old, laboratory-reared larval California grunion. Compare with Figure 5.

	Spring experiment		Summer experiment	
	Low food	High food	Low food	High food
μg C ingested[1] per individual	701	1,723	1,379	2,792
μg dry weight per individual	755	1,305	901	1,895
μg DNA per mg protein	84.8	59.4	48.1	31.3
μg C[2] per individual	245	603	482	977
Growth from hatching (μg C)	104	462	341	836
Gross growth efficiency	15%	27%	25%	30%

[1]Calculated from measured carbon in *Brachionus* and *Artemia*.
[2]Estimated from literature values.

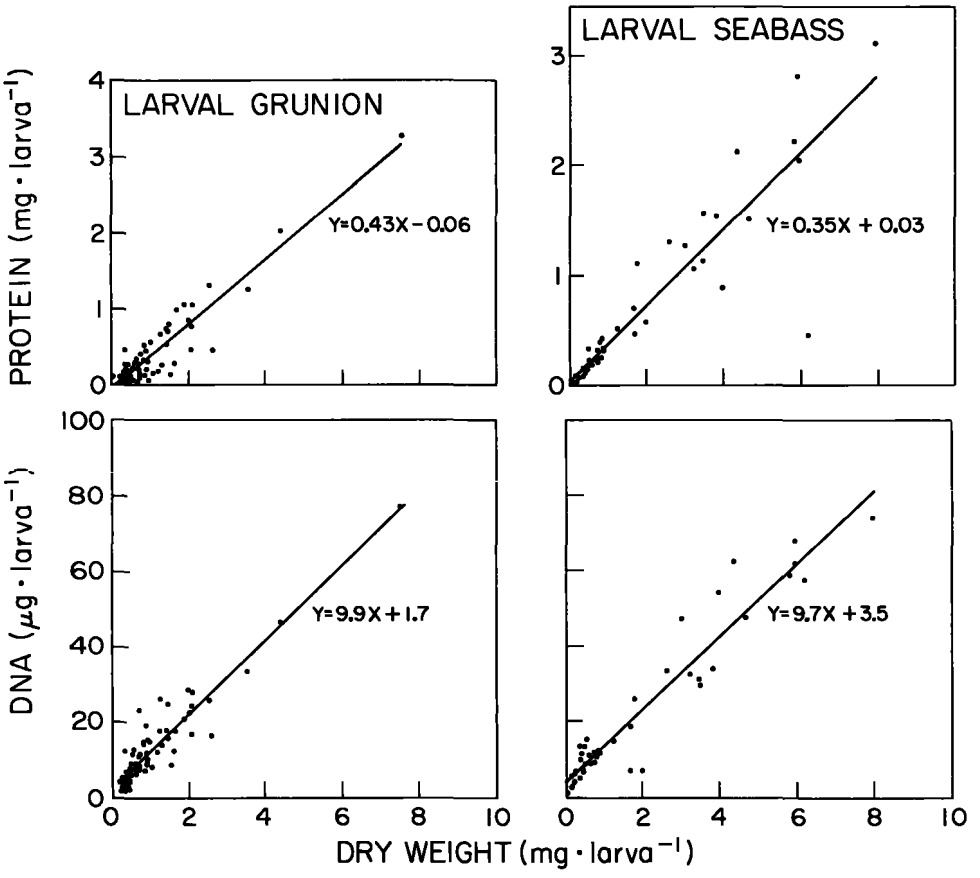


FIGURE 5.—Relations of protein (upper) and DNA (lower) to dry weight for laboratory-reared larval grunion (left) and white seabass (right).

This may reflect differences in the lifespans and metabolic rates; grunion are small fish, reaching maturity in a year and living less than 5 years (Frey 1971), while seabass and halibut grow to very much larger size and can live more than 20 years (Frey 1971; Thomas 1968).

Figure 7 shows the variability in the amount of lipofuscin in larvae of the same age. At time "0", all fish were newly hatched. The variance was low in the first few days, but increased dramatically with time because larvae within each tub grew (and, presumably, respired) at very different rates. There were differences in averages between high food and low food conditions, and between experiments (Table 2), but the variance in dry weight, protein, DNA, or lipofuscin was so large that the overlap obscured any differences between conditions of rearing.

Grunion and halibut larvae start life with greater concentrations of lipofuscin than do seabass, and though the concentration decreases rapidly as grunion and halibut age (Fig. 8), they still have almost a 10-fold greater concentration than do larval seabass when all are 20 days old. All three species increased in weight faster than they increased in lipofuscin, so the concentration of lipofuscin was "diluted" by growth. Because protein was a constant fraction of dry weight (Fig. 5, upper), this dilution was not due to skeletal growth alone. If the rate of weight-specific growth exceeds the rate of weight-specific respiration during early life, the concentration of lipofuscin should decrease, as observed, and only when growth ceases or slows considerably should lipofuscin accumulate relative to weight. Alternatively, lipofuscin could change chemically with time, becoming more difficult to extract (Vernet et al. 1988), so that the rate of accumulation of lipofuscin would be underestimated.

We attempted to stop growth by starving the

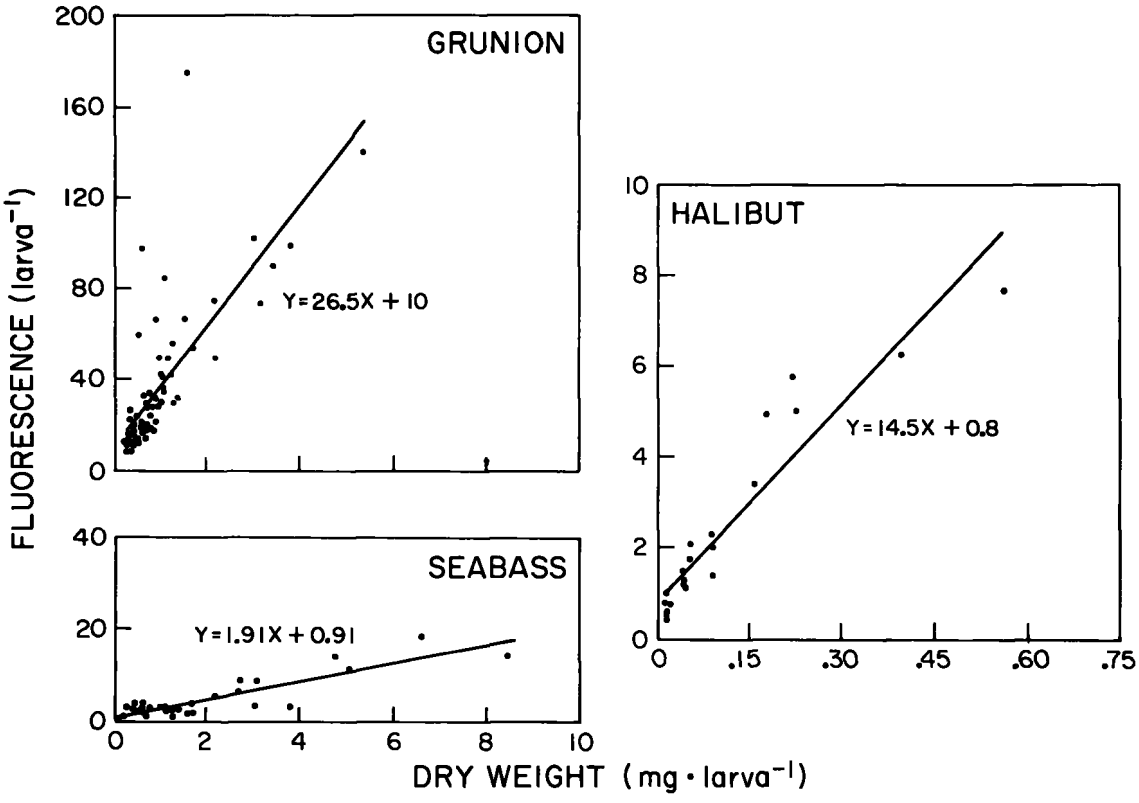


FIGURE 6.—Relation of lipofuscin fluorescence per larva to dry weight for larval grunion (upper left) white seabass (lower left), and California halibut (right). Note different axes.

larvae to see if we could detect an increase in lipofuscin. Table 3 shows results for seabass starved for the final 20% of the rearing period; there was no significant change in the concentration of lipofuscin. This is not what one would expect if accumulation of lipofuscin is proportional to physiological age, unless no lipid is metabolized during starvation. However, this result also could reflect slow transformation of lipofuscin from a more to a less soluble pool (Vernet et al. 1988).

TABLE 3.—Lipofuscin fluorescence (FU) per unit dry weight of larval white seabass starved for various periods after age 29 days. *N* = number of analyses; ranges in parentheses.

	FU (mg dry weight)$^{-1}$	*N*
Initial		
29 days old	2.4 (1.6–3.0)	4
Starved		
2 days	3.1 (2.3–4.0)	2
4 days	1.3 (0.54–1.7)	6
6 days	1.8 (1.4–2.3)	2
8 days	2.5	1

Conclusions

Our intent was to evaluate the utility of measuring extracted lipofuscin fluorometrically as an indicator of the integrated metabolic health of fish, especially preserved ones, and of relative net efficiency of growth. We conclude that this technique is unlikely to be useful in these ways, at least within the larval period. Although the accumulation of total body burden of lipofuscin was demonstrated, the variability among individuals grown under the same conditions became so large over time that we were unable to calibrate the method in an ecologically meaningful sense. The variability was evident in all measures of growth,

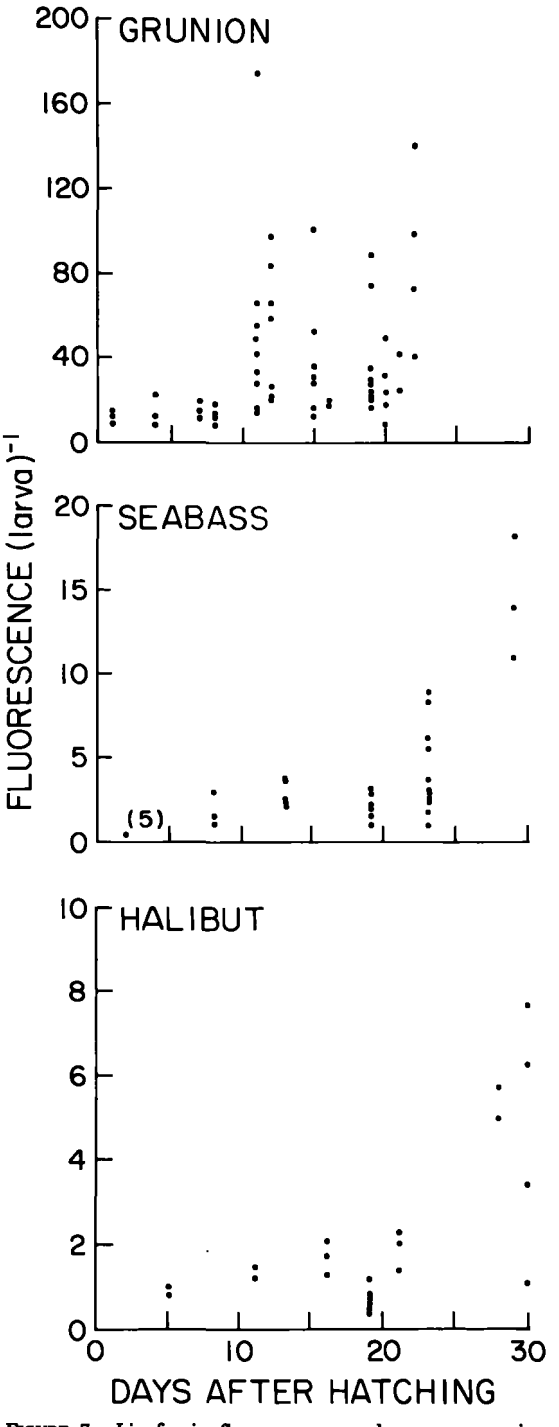


FIGURE 7.—Lipofuscin fluorescence per larva vs. age since hatching for laboratory-raised larval grunion (top), white seabass (center), and California halibut (bottom).

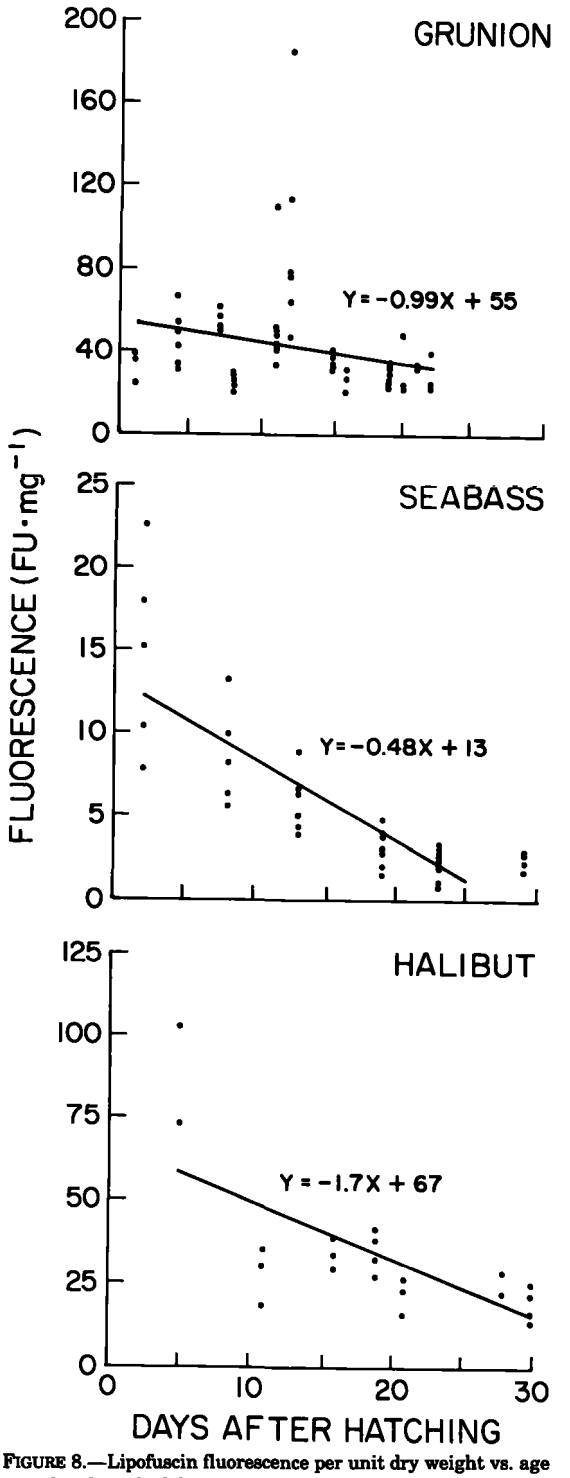


FIGURE 8.—Lipofuscin fluorescence per unit dry weight vs. age since hatching for laboratory-reared larval grunion (top), white seabass (center), and California halibut (bottom). Linear fits are for comparative purposes only; relations for seabass and halibut look curvilinear.

and is common in culture of larval fishes. Such variability may be reduced in nature, where runts may be subject to intense predation.

Lipofuscin analysis may be useful only when applied to postmitotic tissue, such as nervous tissue in mature fish, or to whole organisms whose mitotic growth has essentially ceased, such as adult copepods or insects (since the methods appear useful in arthropods—Ettershank et al. 1983; Sohal and Donato 1978). In these organisms, the vagaries of growth are reduced, and the accumulation of lipofuscin during starvation or exercise might show that lipofuscin concentration is interpretable as a measure of physiological age, habitat quality, and net growth efficiency.

Lipofuscin is known to be a polytypic substance, probably variable in composition among different organisms. We have assumed that a constant proportion of the same substance is extracted. This may not be true (Vernet et al. 1988), and considerable work remains to be done on the basic biochemistry of the component substance(s). Though the extraction and fluorometric measurement is tantalizingly simple, it may well be that the microscopical method used to quantify "ceroid bodies" is the best approach. Fluorescent techniques used in histochemical research (Brizzee and Jirge 1981), combined with automatic imaging procedures, might decrease the tedium of staining and visual microscopy.

Acknowledgments

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