ACCUMULATION OF AGE PIGMENTS (LIPOFUSCIN) IN TWO COLD-WATER FISHES

In fisheries management, age structured models are the preferred method for meeting the key objectives of estimating optimal yields and determining the effect of fishing on stock structure (Gulland 1978). However, few species of commercial marine fishes exist in which age can be determined with certainty (Boehlert 1985). The concentration of age pigments (lipofuscin) (Ettershank 1984) in fish tissues (Agius and Agbede 1984) may be a measure of fish age that could be used to validate other ageing techniques and might also improve estimates of age of long-lived species where other techniques are difficult to apply.

Lipofuscin originates in biological membranes through lipid peroxidation (Tappel 1975). Lipofuscin accumulation has been documented for a wide variety of animals, from mammals to the bread-mold *Neurospora* (Ettershank et al. 1983 and references therein). The rate of accumulation has been shown to be constant during the lifetime of laboratory-raised mice (Reichel 1968; Miquel et al. 1978), dogs (Munnell and Getty 1968), fleshflies (Ettershank et al. 1983); man (Strehler et al. 1959); and also wild populations of mice (Dapson et al. 1980). On the other hand, the rate of lipofuscin accumulation has been shown to vary with level of activity and lifespan (Sohal and Donato 1978). It is expected that the rate in which lipofuscin accumulates with age in a natural population of fishes, or stock, with its free genetic interchange and likely common habitat, would be fairly uniform (but see Smith 1987). In addition, if measurements are made in nonmitotic and constantly metabolizing tissues such as brain or myocardium, the variation in concentration due to environmental effects is least likely.

In this paper we present the results of a preliminary study designed to assess the usefulness of extracted lipofuscin as a method of ageing fishes. Two species of cold-water fishes are included: the rainbow trout, *Salmo gairdneri*, reared in captivity, and of known age; and the Dover sole, *Microstomus pacificus*, a long-lived fish, in which age is not known with certainty. We present spectral characteristics of the extracted lipofuscin from several tissues and the change in concentration of lipofuscin with fish age.

Materials and Methods

Specimens of Dover sole were collected on December 1986 off Point Conception. Ages were estimated by J. Butler, E. Lynn, and M. Drawbridge of the Southwest Fisheries Center using otolith sections. Ages ranged from 2.7 to 44.7 years (average of three readings). Specimens of the species *Salmo gairdneri* were collected in July 1986 at Hot Creek Hatchery. Ages ranged from 3 months to 3 years.

Samples were frozen after collection and kept at −80°C until analysis. Studies by Nicol (1987) showed that this form of preservation yielded the lowest fluorescence when compared with ethanol and formalin-preserved samples. There was no indication that the level of fluorescence changed as a function of time of preservation or by interaction with the extracted lipofuscin, as in the case for formalin. To excise the brain, the top of the skull was opened and the four brain lobes were removed as a unit, without the optic nerve. To excise the heart, we separated the muscle from connective tissue, blood vessels, and fat deposits.

Three methods were compared for maximum lipofuscin extraction. The first two methods (Tappel 1975; MacArthur and Sohal 1982) were specifically developed for lipofuscin extraction and employed chloroform:methanol (2:1) as the extractive solvent; they differ in the optimal volume-to-weight ratio (30:1 and 20:1, respectively), temperature of extraction, and number of times the chloroform phase is washed with water. The third method was developed for lipid extraction in fishes (Bligh and Dyer 1959) and uses chloroform:methanol:water (1:2:0.8) as the extractive solvent. Subsamples ($n = 3$) of cerebellum of *Stenella* sp. collected at the eastern tropical Pacific and kept at −30°C were defrosted, dried with lint-free paper, weighed, and extracted following the three methods as described originally.

Tissues from Dover sole were extracted basically following the MacArthur and Sohal (1982) technique, with two additional steps to ensure a complete washing out of flavoproteins and photooxidation of retinol when present (Fletcher et al. 1973). Tissue was freeze dried prior to analysis. Ten mL of chloroform:methanol (2:1, v/v) was added to a homogenizer containing the sample for a final solvent:sample ratio of about 120:1 (vol/dry weight). The sample was ground with a teflon pestle attached to an electric drill and later submerged in a 40°C water bath for 1 minute. A 2 mL subsample was taken from the homogenate. Three mL of deionized water was added, the sample was shaken, and the emulsion centrifuged 10 minutes at 1,912 *g* and 0°C. After centrifugation, the hyperphase was decanted and a second rinse performed. After decanting the hyperphase a second time, 1 mL of the hypophase (chloroform containing lipofuscin) was sampled and transferred to a polypropylene tube. Three mL of chloroform were added to the sample (for a total of 4 mL) which was then exposed to UV irradiation (254 nm) for 1–2 minutes to photooxidize retinol. This last step was routinely performed for liver tissue.

Samples were then transferred to glass tubes, sealed, and kept in the refrigerator until analysis. Sample fluorescence was measured at the emission peak (430–440 nm) in a quartz cuvette with a Perkin Elmer Fluorescence Spectrophotometer[1] Model MFP-44A. The sample was excited at the peak of fluorescence excitation (~360 nm). The intensity of the fluorescent emission (at 430 nm) was normalized to the intensity of the quinine solution standard (1 mg L^{-1} in 1N sulphuric acid) and expressed in fluorescence units.

Lipofuscin in Dover sole is expressed as 1) total lipofuscin content per organ and 2) weight-specific lipofuscin concentration, calculated by dividing the total lipofuscin content by the dry weight of the entire organ.

Lipofuscin from rainbow trout tissues was extracted following the Bligh and Dyer (1959) technique, without modifications. Whole tissues were ground in water with a tissue homogenizer to give a final concentration of 100 mg of tissue (wet weight) in 0.8 mL of homogenate. A sample of 0.8 mL was taken and solvents were added to give a final ratio of 1:2:0.8 (chloroform:methanol:water) with a solvent to sample ratio of 20:1 (vol/w). The sample was then filtered through a 2.4 cm glass fiber filter (Whatman GF/C), the tissue re-extracted with 1 mL of chloroform and refiltered. The extract was then washed with 3 mL of water, shaken, and centrifuged for 10 minutes at 1,912 *g* and 0°C. One mL of the chloroform hypophase was subsampled and fluorescence estimated as described above.

Lipofuscin in rainbow trout is expressed as 1) total lipofuscin content per organ and 2) weight-specific lipofuscin concentration (total lipofuscin content of the organ divided by its wet weight).

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

Results

Efficiency of Extraction

Lipofuscin was successfully extracted and quantified from three different fish tissues: brain, heart, and liver. Wavelengths of fluorescence excitation and emission maxima of extracted lipofuscin in chloroform are presented in Figure 1. All maxima are within the range cited by Sheldahl and Tappel (1973); fluorescence excitation maxima were between 340 and 370 nm and fluorescence emission maxima between 420 and 470 nm.

Retinol (wavelengths of maximum fluorescence excitation at 325–340 nm and maximum fluorescence emission at 475 nm) was photooxidized by exposing the chloroform extract to UV irradiation (254 nm). As expected, retinol was found in liver and sometimes it was present in brain and heart tissues. For example, wavelengths of fluorescence excitation and emission maxima in liver of Dover sole shifted from 352 to 365 nm and from 470 to 440 nm, respectively, after UV irradiation (see Figure 1C). Mullin and Brooks (1988) also found that this UV irradiation is effective in oxidizing retinol although they did not find significant retinol interference in fish tissue. It seems liver tissues may require UV irradiation to oxidize retinol while brain and heart tissue should be checked for retinol presence before irradiation.

The extractive efficiency of the three methods tested are compared in Table 1. All methods extracted similar fluorescent compounds from the brain of *Stenella* sp., as the fluorescence excitation and emission spectra were similar. The MacArthur and Sohal (1982) method extracted significantly more fluorescent pigment, than did either the Tappel (1975) or the Bligh and Dyer (1959) method which extracted 66% and 46% of maximum extraction, respectively). Thus all three methods are useful for quantitative estimation of extractable lipofuscin in fish tissue but

TABLE 1.—Comparison of the three methods of lipofuscin extraction in brain tissue. Fluorescence excitation at 360 nm and fluorescence emission at 440 nm. Results are presented as fluorescence units per mg of wet tissue, where the fluorescence emission signal is normalized to the intensity of emission of a standard quinine sulfate solution (1 mg L^{-1} in 1N sulphuric acid). The three methods were significantly different from each other, $P < 0.05$, Newman-Keuls range test (Zar 1974). ANOVA: F ratio = 21.81, 2 df, $P < 0.05$.

	Fluorescence units			Fluorescence maxima	
	Average 10–3	SD 10–3	n	Excitation (nm)	Emission (nm)
MacArthur and Sohal (1982)	4.30	0.39	3	362	445
Tappel (1975)	2.83	0.37	3	362	440
Bligh and Dyer (1959)	1.98	0.45	3	360	445

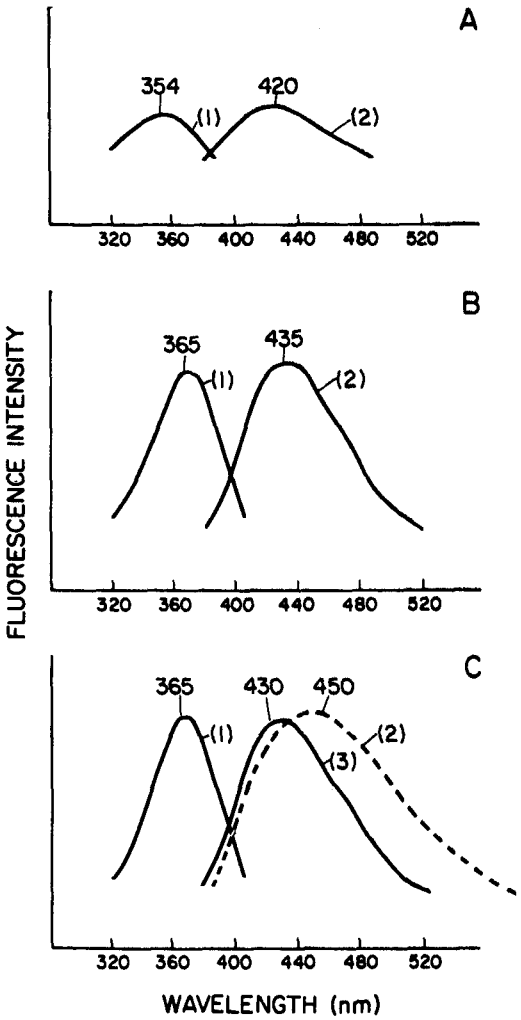

FIGURE 1.—Spectral characteristics of lipofuscin extracted from Dover sole, *Microstomus pacificus*, tissues (uncorrected spectra). In chloroform. (A) Brain: (1) Lipofuscin fluorescence excitation spectrum (emission wavelength 440 nm); (2) lipofuscin fluorescence emission spectrum (excitation wavelength 365 nm). (B) Heart: (1) Lipofuscin excitation spectrum (emission wavelength 430 nm); (2) lipofuscin fluorescence emission spectrum (excitation wavelength 365 nm). (C) Liver: (1) Lipofuscin fluorescence excitation spectrum (emission wavelength 430 nm); (2) lipofuscin emission spectrum (excitation wavelength 365 nm) before UV radiation; (3) Same as (2) after UV radiation (254 nm).

comparisons of amount of concentrations between tissues or species cannot be made if different extraction methods are used.

Lipofuscin Concentration

The total concentration of extracted lipofuscin in Dover sole brain tissue was positively correlated with fish length (Fig. 2A) ($Y = -15.4 + 0.516\,X$, $r^2 = 0.43$; $b \neq 0$, $P < 0.01$, $t = 5.1$, df = 34). Furthermore, total extracted lipofuscin content in the brain was positively correlated with age in organisms estimated by annular increments in otoliths to be from 2 to 15 years old (Fig. 3A) ($Y = -2.5 + 1.0\,X$, $r^2 = 0.75$; $b \neq 0$, $P < 0.01$, $t = 5.5$, df = 10). On the other hand, in older Dover sole (estimated age from 20 to 45 years; $n = 26$) the total extracted lipofuscin in the brain did not increase with their estimated age. The concentration of lipofuscin in the brain (extracted lipofuscin concentration per unit of g dry weight) followed a similar pattern (Fig. 3B). The concentration in the brain increased linearly with age for fish 2–15 years old ($Y = 30.39 + 14.85\,X$, $r^2 = 0.79$, $b \neq 0$, $P < 0.01$, $t = 6.66$, df = 10) but fishes older than 15 years did not show an increase in pigment concentration with age.

The total extracted lipofuscin content extracted from rainbow trout brains increased with age (Fig. 4A) ($Y = 0.14 + 0.18\,X$, $r^2 = 0.37$; $b \neq 0$, $P < 0.01$, $t = 3.75$, df = 23), as did the content of the heart (Fig. 4C) ($Y = -0.54 + 0.82\,X$, $r^2 = 0.67$; $b \neq 0$, $P < 0.01$, $t = 7.36$, df = 23), and liver (Fig. 4E) ($Y = -2.86 + 5.81\,X$, $r^2 = 0.62$; $b \neq 0$, $P < 0.01$, $t = 7.54$, df = 34) from 3 months to 3 years. The concentration of extracted lipofuscin per unit wet weight of heart tissue did not change with age (Fig. 4D) whereas that of brain and liver tissue decreased with age (Fig. 4B, F).

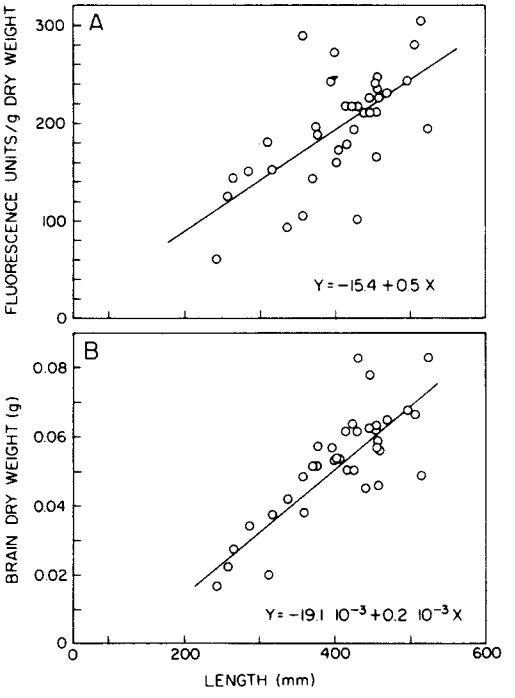

FIGURE 2.—Dover sole, *Microstomus pacificus*, brain: (A) Extracted lipofuscin per unit of dry weight as a function of fish length (mm) (n = 36); (B) brain dry weight (g) as a function of fish length (mm). Fluorescence units: the intensity of fluorescence normalized to a standard solution of quinine sulfate (1 mg L^{-1} in 1N sulphuric acid).

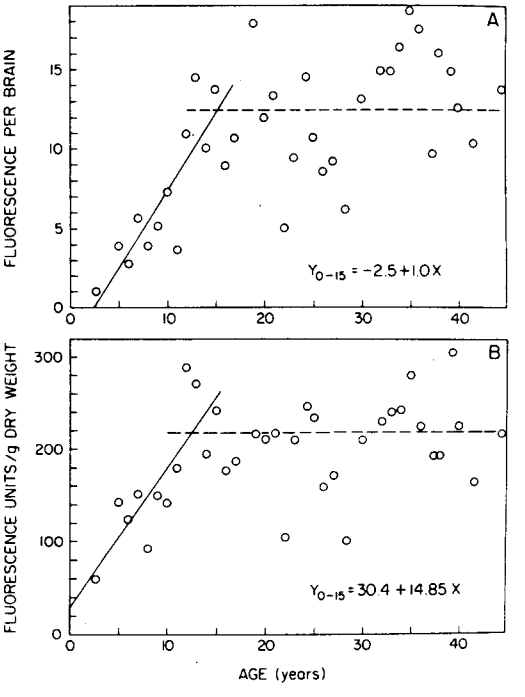

FIGURE 3.—Lipofuscin in Dover sole, *Microstomus pacificus*, brain tissue as a function of age ($n = 36$). (A) Total lipofuscin content per brain; (B) weight-specific (g dry weight) lipofuscin in brain.

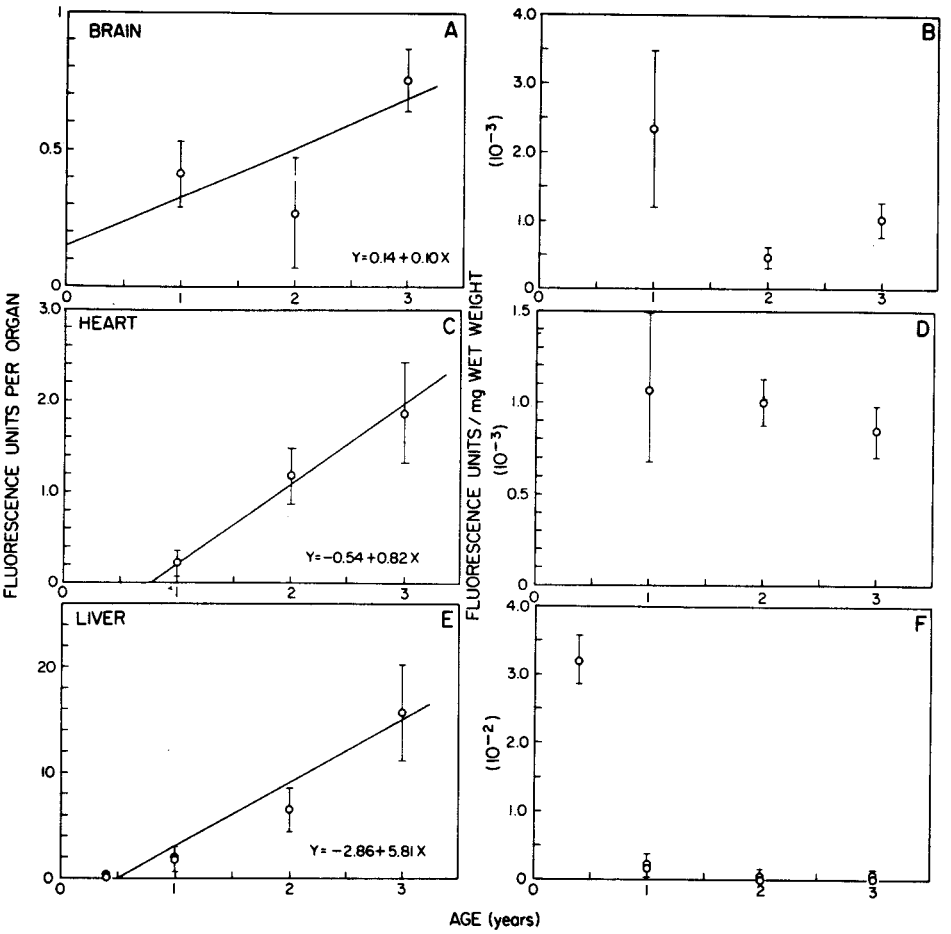

FIGURE 4.—Lipofuscin in rainbow trout, *Salmo gairdneri*, as a function of age (n = 21). (A) Total lipofuscin in brain; (B) weight-specific (mg wet weight) lipofuscin in brain; (C) total lipofuscin in heart; (D) weight-specific (mg wet weight) lipofuscin in heart; (E) total lipofuscin in liver; (F) weight-specific (mg wet weight) lipofuscin in liver.

Discussion

Our results indicate that lipofuscin accumulated in the brain of Dover sole with time (Fig. 2A). The concentration of lipofuscin increased over a wide range of fish lengths and with estimated age of 15 years but did not increase with older fish. Several explanations exist for the lack of change in concentration in older fish: 1) older fish were incorrectly aged; 2) growth of brain tissue masked the actual rate of accumulation; and 3) the rate of accumulation changed during the lifespan of this species due to changes in metabolic activity. Either one or several of these factors may cause the lack of lipofuscin accumulation in older fish. We consider each of these issues below.

We do not know the accuracy of the age determination in Dover sole but believe it is unlikely that 3 readers would confuse fish aged 30–40 years with those of 20 years. Although future research will shed more light on this controversial subject, we think that grossly inaccurate age determination is the least likely an explanation.

A key difference between fishes and other organisms in which lipofuscin accumulation has been clearly documented as a function of chronological age (mammals and invertebrates) is that

fishes have indeterminate growth. For example, Figure 3B indicates that the brain of Dover sole continues to grow long after this fish begins reproduction (450 mm). Thus, unlike mammals and invertebrates which have determinate growth, the total content of extractable lipofuscin in any fish organ cannot be used as a measure of age but rather concentration (content per unit of weight) must be used. Furthermore, even the concentration of lipofuscin in the brain, a slow growing tissue, is a function of both the rate of lipofuscin accumulation and the rate of tissue growth. It follows then, that if the rate of brain growth is such that it masks the actual rate of lipofuscin accumulation in older organisms, lipofuscin concentration will not increase with time.

A critical assumption underlying the use of lipofuscin as a determinant of age is that metabolic rate, and hence lipofuscin accumulation rate, remains fairly constant over the portion of the life history of interest. The rainbow trout were reared in a hatchery where no net change in the environment occurred from 1983 to 1985. Dover sole, on the other hand, gradually migrate into deeper, colder, and less oxygenated waters as they age. Presumably the metabolism of fish under these conditions would be lower as might be the rate of lipofuscin accumulation in the brain.

The literature on fishes provide no conclusive evidence that lipofuscin is an accurate index of age over the entire lifespan. Hill and Radtke (in press) reported that the extracted lipofuscin per unit dry weight in the brain of the tropical fish *Dascillus albisella* accumulates exponentially with age. The relationship is driven by a single point for an 11-year-old individual and it would probably be linear without that single point, if only fish 1–7 years old were considered. In hatchery-reared *Cyprinus carpio* the total extracted lipofuscin per unit of dry weight in the brain of fish of the same age (6 years) increased with weight over 8-fold range in weight (Griven et al.[2]). About 45% of the lipofuscin content could be explained by difference in weight among fish. Thus, in fish of the same age there was a strong size effect on lipofuscin concentration. These results are very similar to those found in this study. Aloj Totaro et al. (1985) found that lipofuscin increased over a range of 0–2 years. But the method was different as they measured lipofuscin granules present in the electric lobe of *Torpedo marmorata* brains rather than using extractable lipofuscin of the entire brain. Thus, all studies to date seem to indicate that accumulation occurs in the brains of fishes, but results are not conclusive owing to small sample sizes, limited age ranges, and failure to identify the effects of brain growth on rates of accumulation.

Additional research is required to evaluate lipofuscin as a method of age determination in fishes. The effect of brain growth on lipofuscin accumulation rates must be considered in such studies. A promising approach in this regard may be to estimate lipofuscin on a per cell basis instead of on a weight basis. This could be accomplished by either expressing extracted lipofuscin relative to DNA concentration or by histological techniques.

[2]Girven, R. J., R. W. Gauldie, Z. Czochanska, A. D. Woolhouse. Manuscr. in prep. A critical tests of the lipofuscin technique of age estimation in fish.

Acknowledgments

This work was funded by Sea Grant contract R/NP-1-15C and by NOAA contract 43ABNF6 1987. The authors would like to thank A. Dizon, M. M. Mullin, E. Brooks, and J. Butler for helpful discussions; J. Butler, E. Lynn, and M. Drawbridge for age determination by otoliths of Dover sole specimens; and M. Rowan of California Fish and Game for providing the rainbow trout samples.

Literature Cited

AGIUS, C., AND S. A. AGBEDE.
1984. An electron microscopical study on the genesis of lipofuscin, melanin, and haemosiderin in the haemopoietic tissues of fish. J. Fish Biol. 24:471–488.

ALOJ TOTARO, E., F. A. PISANTI, P. RUSSO, AND P. BRUNETTI.
1985. Evaluation of aging parameters in *Torpedo marmorata*. Ann. Soc. R. Zool. Belg. 115(2):203–209.

BLIGH, E. G., AND W. J. DYER.
1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.

BOEHLERT, G. W.
1985. Using objective criteria and multiple regression models for age determination in fishes. Fish. Bull, U.S. 83:103–107.

DAPSON, R. W., A. T. FELDMAN, AND G. PANE.
1980. Differential rates of ageing in natural populations of old-field mice (*Peromyscus polionotus*). J. Gerontol. 35:39–44.

ETTERSHANK, G.
1984. A new approach to the assessment of longevity in the Antarctic krill *Euphausia superba*. J. Crustacean Biol. 4:295–305.

ETTERSHANK, G., I. MACDONNELL, AND R. CROFT.
1983. The accumulation of the age pigment by the fleshfly

Sarcophaga bullatta Parker (Diptera: Sarcophagidae). Aust. J. Zool. 31:131–138.

FLETCHER, B. L., C. J. DILLARD, AND A. L. TAPPEL.
1973. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. Anal. Biochem. 52:1–9.

GULLAND, J. A.
1978. Analysis of data and development of models. *In* J. A. Gulland (editor), Fish population dynamics, Ch.4. Wiley.

HILL, K. T., AND R. L. RADTKE.
In press. Gerontological studies of the damselfish, *Dascyllus albisella*. Bull. Mar. Sci.

MACARTHUR, M. C., AND R. S. SOHAL.
1982. Relationship between metabolic rate, aging, lipid peroxidation, and fluorescent age pigment in milkweed bug, *Oncopeltus fasciatus* (Hemiptera). J. Gerontol. 37:268–274.

MIQUEL, J., P. LUNDGREN, AND J. E. JOHNSON, JR.
1978. Spectrofluorometric and electron microscopic study of lipofuscin accumulation in the testis of ageing mice. J. Gerontol. 33:5–19.

MULLIN, M. M., AND E. BROOKS.
1988. Extractable lipofuscin in larval marine fish. Fish. Bull., U.S. 86:407–415.

MUNNELL, J. F., AND R. GETTY.
1968. Rate of accumulation of cardiac lipofuscin in the ageing canine. J. Gerontol. 23:154–158.

NICOL, S.
1987. Some limitations on the use of the lipofuscin ageing technique. Mar. Biol. (Berl.) 93:609–614.

REICHEL, W.
1968. Lipofuscin pigment accumulation and distribution in five rat organs as a function of age. J. Gerontol. 23:145–153.

SHELDAHL, J. A., AND A. L. TAPPEL.
1973. Fluorescent products from aging *Drosophila melanogaster*: an indicator of free radical lipid peroxidation damage. Exp. Gerontol. 9:33–41.

SMITH, P. J.
1987. Homozygous excess in sand flounder, *Rhombosolea plebeia*, produced by assortive mating. Mar. Biol. (Berl.) 95:489–492.

SOHAL, R. S., AND H. DONATO.
1978. Effects of experimentally altered life spans on the accumulation of fluorescent age pigment in the housefly, *Musca domestica*. Exp. Gerontol. 13:335–341.

STREHLER, B. L., D. D. MARK, A. S. MILDVAN, AND M. V. GEE.
1959. Rate and magnitude of age pigment accumulation in the human myocardium. J. Gerontol. 14:430–439.

TAPPEL, A. L.
1975. Lipid peroxidation and fluorescent molecular damage to membranes. *In* B. F. Trump and A. V. Arstila (editors), Pathobiology of cell membranes, Vol. 1. Acad. Press, N.Y.

ZAR, J. H.
1974. Biostatistical analyses. Prentice-Hall, Englewood Cliffs, NJ, 620 p.

MARIA VERNET

Institute of Marine Resources
Scripps Institution of Oceanography
University of California San Diego
La Jolla, CA 92093-0202

JOHN R. HUNTER

Southwest Fisheries Center La Jolla Laboratory
National Marine Fisheries Service, NOAA
P.O. Box 271
La Jolla, CA 92037

RUSSELL D. VETTER

Marine Biology Research Division
Scripps Institution of Oceanography
University of California San Diego
La Jolla, CA 92093-0202