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Fluorescent age pigment accumulation as a determinant of chronological age in aquatic organisms

Hill, Kevin Thomas, Ph.D.

University of Hawaii, 1992
FLUORESCENT AGE PIGMENT ACCUMULATION AS A DETERMINANT OF CHRONOLOGICAL AGE IN AQUATIC ORGANISMS

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DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

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BY

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ABSTRACT

Age pigments have been intensively studied by gerontologists hoping to define the biochemical processes involved in aging. More recently, age pigment accumulation has been studied by field biologists hoping to estimate chronological age of animals based on quantities of pigments measured in postmitotic tissues. The present research was conducted to evaluate methods used for extraction and measurement of fluorescent age pigments (FAP), describe patterns of FAP accumulation in two teleost fish species and develop multivariate age prediction models based on FAP content and other somatic variables, and determine the effects of some environmental and physiological variables on FAP variability. Evaluation of procedures for handling specimens and extracts revealed increases in FAP-like fluorophores in vitro in Oreochromis mossambicus brain, heart, and muscle tissues and their extracts with increased storage temperature (-20°C and above) and time, particularly in the chloroform/methonal solvent system. Ultrasonication of tissue homogenates greatly enhanced this effect and generated other fluorophores in solution. Fluorescence assay temperature also affected expression of FAP. Some or all of these effects of handling procedures may have produced variability of results reported from previous FAP studies.

Nonpolar FAP extracted from O. mossambicus brain accumulated in an increasingly rapid manner with age. Nonpolar and polar FAP from Puntius conchonius brain leveled off with increasing age. FAP in P. conchonius heart increased linearly with age. Multiple regression analyses demonstrated the age-predictive value of FAP data for both species under controlled rearing conditions. FAP content and otolith dimension data consistently provided slightly stronger predictive models than somatic data alone. Temperature and body weight affected FAP content in P. conchonius brain and heart tissues. Brain FAP was inversely related to body weight, especially at the lower temperatures. Heart FAP was inversely related to body weight at 19°C, but positively
related at higher temperatures. Increasing temperature decreased brain FAP, but increased heart FAP. Different levels of saturation of cellular lipid constituents at different temperatures may affect the potential for FAP formation via lipid peroxidation. FAP differences detected between non-sibling fish indicated genetic variation in the ability to control FAP genesis.
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CHAPTER 1
INTRODUCTION

Overview

Managers of living aquatic resources are becoming increasingly concerned about the welfare of fishery stocks under continued exploitation. An understanding of the sustainable yield of a stock is essential for drafting and implementing effective fisheries management policies. Determination of age and growth rates is perhaps the most fundamental component of this type of assessment (Ricker 1979). Age data, in conjunction with length and weight measurements, can give information on life history parameters (natality, age at recruitment and sexual maturity, longevity, and mortality) necessary to evaluate the overall condition of the stock.

Traditional methods of age determination in fish have included such indirect measures as length-frequency analysis or the quantification of rhythmic events in hard tissues (Bagenal 1974). However, results from many length-frequency studies have been inconclusive because: 1) there is difficulty in obtaining adequate, representative numbers of specimens in a full range of life stages; 2) sampling introduces gear bias; 3) modes for older fish are difficult to distinguish due to asymptotic growth and variability within cohorts; and 4) cohorts are ill-defined in fish species with protracted recruitment periods (Neal & Maris, 1985).

Use of rhythmic events registered in hard tissues (e.g., bands in scales, otoliths, fin spines, vertebrae) has been the most productive technique for chronological age estimation in many fish species. These conventional techniques are often inadequate when applied to tropical fishes (Brothers 1987). Even with the discovery of daily growth increments in the otoliths of juvenile fishes (Pannella 1980), Prince & Lee (1991) have shown that the correspondence between days and increments can break down in older
individuals. These problems have led researchers to the realization that alternative, innovative methods of age determination are desirable to obtain the information required to manage fish populations.

The quantitative assessment of cellular metabolites accumulated during the process of physiological aging has been suggested as an alternative method for accurate estimation of chronological age. One class of metabolites, commonly termed 'fluorescent age pigments' (FAP), occurs in all animals studied to date, and is considered by many to be the most clearly discernible evidence of aging in the cell (Sohal & Wolfe, 1986). Not degradable by normal enzymatic processes, FAP polymers accumulate in cells over time, and their quantity may provide an index of the relative age of the cell, tissue, and ultimately the whole animal. Thus far, the study of FAP has centered mostly on gerontological research, but interest is growing among field biologists in the possible application of these methodologies to study rates of aging, estimate chronological age, and predict longevity in wild stocks. Early attempts to determine the age of animals in the field using this approach have met with limited success because of high variability in FAP levels and inadequate sampling. A satisfactory relationship between known chronological age and FAP content has yet to be validated.

**Cellular genesis of FAP**

Events leading to the production of FAP are generally well known. The term FAP is used to describe a heterogeneous family of compounds (pre-ceroids, ceroids, and lipofuscins) which accumulate in the cell as a result of both normal metabolic activity and general molecular 'wear and tear'. First described by Hannover (1842), age pigments can be observed as yellowish-brown, polymorphic granules in the cytoplasm. Chemical analysis has revealed that they are highly polymerized collections of lipids, protein fragments, and acid-hydrolysis resistant residues (Björkerud 1964). They are believed to
collect as the result of numerous intracellular digestions by lysosomes of peroxidized lipids and damaged proteins and organelles (Tsuchida et al. 1987).

Free radical production during cellular metabolism is considered the starting point for age pigment formation (see reviews by Harman 1984, Porta 1991). Superoxide radicals produced in the respiratory chain are converted to hydrogen peroxide in the presence of the superoxide dismutase (Fig. 1-1). Under homeostatic conditions, hydrogen peroxide is subsequently converted to oxygen and water by catalase or glutathione peroxidase. It is inevitable that some hydrogen peroxide will, in the presence of divalent metals, dissociate into a hydroxide ion and a hydroxyl radical (Fig. 1-1). Hydroxyl radicals are highly reactive, and there are no known protective mechanisms against their attack on biomolecules.

Hydroxyl radicals may damage many types of biomolecules, but the unsaturated fatty acids and membrane phospholipids are probably the most important to consider in terms of FAP formation. In the example illustrated in Fig. 1-2, attack on linolenic acid by a hydroxyl radical results in the formation of a lipid radical, which is oxidized in subsequent steps to form an endoperoxide. Endoperoxides dissociate into two smaller lipid radicals and malondialdehyde, a powerful bifunctional crosslinking agent in the cytoplasm. Free radical-mediated lipid peroxidation is further propagated by the two new lipid radicals unless controlled by antioxidants such as alpha-tocopherol (vitamin E).

Malondialdehyde, a by-product of lipid peroxidation, can crosslink with the amine groups of any two biological compounds to form autofluorescent condensation products such as amino-imino propene Schiff base fluorophores (Tappel, 1975), 1,4-Dihydropyridine-3,5-dicarbaldehydes (Kikugawa and Ido 1984), and polymalondialdehyde (Gutteridge et al. 1977) (Fig. 1-3). Biomolecules crosslinked through these reactions are inactivated, and the polymers are eventually engulfed by autophagic lysosomes for intracellular digestion. Crosslinked regions are highly resistant
to degradation through normal enzymatic processes, and gradually accumulate in number and size over time (Sohal and Wolfe 1986, Goebel 1987) as age pigment (pre-ceroid, ceroid, and lipofuscin) granules (Fig. 1-4).

Many factors are thought to affect the rate of FAP genesis. These include metabolic rate (Sohal & Donato 1979), temperature (McArthur & Sohal 1982), degree of fatty acid and membrane phospholipid saturation (Sohal et al. 1984), production of specific proteolytic enzymes (Katz & Shanker 1989), disease state (Hammer and Karakiri 1987, Koppang 1987, Hall et al. 1989), and in particular the efficiency of antioxidant defense mechanisms which intercept damaging free radicals (Armstrong 1984, Brizzee et al. 1984). Cell age may affect the ability to prevent free radical damage through changes in the expression of antioxidant enzymes (Sohal et al. 1983); FAP may accumulate at a faster rate in the tissues of senescent or diseased animals (Timiras 1974, Donato & Sohal 1978, Armstrong 1984, Nandy 1985, Porta 1987).

Quantification of FAP

Age pigments have been observed at both the histological and biochemical levels. Traditional histological methods have included direct quantification of age pigment granules in stained tissues by light microscopy (Strehler 1964), epifluorescence microscopy (Brizzee & Jirge 1981), and transmission electron microscopy (TEM) (Agius & Agbede 1984). Proponents of TEM methods have attempted morphometric quantification through digitization of micrographs. Unfortunately, TEM methods are time consuming and expensive. More significantly, morphometric quantification is subject to analytical biases associated with the selection of specific areas in the tissue and cell for counting granule numbers and for calculating percent volume of the cell occupied by the granules.

An alternative method, introduced by Tappel and coworkers (Chio & Tappel 1969, Chio et al. 1969, Fletcher et al. 1973, Tappel 1975), involves the measurement of
autofluorescent amino-imino propenes associated with age pigment polymers in whole tissues. This method has gained widespread use in gerontological research on a wide variety of animals, tissues, and model systems. It involves the homogenization, extraction and fluorometric quantification of the soluble FAP present in the tissue. Early studies used only the chloroform or lipid-soluble portion of these compounds, but it is now known that some FAP's are soluble in more polar solvents as well (Tsuchida et al. 1987). Although the overall chemical composition of FAP products is diverse, the presence of the autofluorescent crosslinks is universal. Thus, indirect measurement of soluble FAP by fluorometry produces an index of the amount of all types of FAP present in the tissue and provides a higher degree of overall replicability than light microscopic and electron microscopic methods.

Eldred et al. (1982), Eldred (1987), and Porta (1987) have argued that Tappel’s fluorometric technique does not measure the fluorescence of whole lipofuscin granules, but instead it measures direct products of lipid peroxidation not necessarily present in the granules. This view has been based on comparison of the fluorescent properties of solvent extracts with the properties of fluorescent granules visible by light microscopy. The possibility exists that FAP measured fluorometrically represent more immediate effects of lipid peroxidation associated with damaged membrane phospholipids (Tappel 1975, Vladimirov 1986), damaged mitochondria (Dillard & Tappel 1971, Tangeras 1983), or cross-linked proteins or oligonucleotides present in the cytosol, not yet incorporated into large polymers in the lysosome (Kikugawa et al. 1989). Regardless of the source of the measured fluorescence, Tappel’s method has been successfully applied to a large number of tissue systems over the past twenty years.

**Age pigments in aquatic animals**

Although age pigments have been described in a wide variety of animals, little work has focused on aquatic animals such as fish and crustaceans. Some fishes are
potentially excellent experimental subjects for gerontological research (Comfort 1960, 1961, 1963; Woodhead 1979, 1980, 1985) because they have relatively short lifespans, are easy to breed and maintain in captivity, and their metabolic rates can be manipulated.

Most researchers who have studied FAP in fishes have used histological procedures. Aboim (1946) reported age pigments in the interrenal cells of the elasmobranch Raja clavata. Other studies have noted the presence of age pigment granules in interrenal cells of Prionurus microlepidotus, Pterois lunulata, and Paralichthys olivaceus (Oguri & Hibiya 1957), and more recently in the same tissues of Lophius litulon and Triakis scyllia (Oguri 1986). Oguri (1986) commented on the abundant occurrence of age pigment granules in fish specimens of large size and "advanced age". Additional studies have focused on the accumulation of lipofuscin, haemosiderin, and melanin pigments in macrophages, relating pigment content to fish health and age (Grove 1968; Agius 1980, 1981; Agius & Agbede 1984; Wolke et al. 1981; Brown & George 1985). Brown & George (1985) found that the number of macrophage age pigment aggregates was more dependent on age than on the fish's health (as indicated by incidence of parasitism and condition index). Aloj Totaro and coworkers have examined effects of environmental pollutants on accumulation of lipofuscin granules in spinal ganglia of the electric ray, Torpedo marmoratus, finding higher levels in specimens from copper polluted areas (Aloj Totaro et al. 1985; Aloj Totaro & Pisanti 1987). Unfortunately, no consideration was given to the chronological age of their samples.

Fewer investigators have measured FAP in fish fluorometrically. Luo & Hultin (1986) found that chloroform soluble FAP increased with age (estimated with unvalidated scale annuli) in the skeletal muscle of winter flounder (Pseudopleuronectes americanus). Hammer & Madhusudhana-Rao (1987) found that presence or absence of dietary vitamin E affected the final quantity of FAP in Oreochromis aurea brain, but had no effect on levels in four other tissue types. Hammer & Karakiri (1988) measured FAP content in
field-captured *Limanda limanda* and found that individuals infected with lymphocystis had significantly higher levels of the pigment. Finally, Hammer (1988) tested effects of temperature on FAP levels in larval *Esox lucius*, finding higher rates of accumulation in larvae reared at suboptimal temperatures. Hill & Radtke (1988) measured FAP in *Dascyllus albisella* brains, finding a positive relationship between FAP content and specimen size and age (estimated from unvalidated annuli in sagittae). Similar studies have been conducted on Dover sole and trout (Vernet et al. 1988) as well as larval grunion, seabass, and halibut (Mullin & Brooks 1988) with variable results.

Ettershank (1983, 1984a, 1984b, 1985) was the first to apply FAP techniques to crustaceans. He attempted to resolve age cohorts in Antarctic krill (*Euphausia superba*), which were previously thought to live three years based on length frequency distributions. Ettershank found modal progressions of FAP content, assumed to be annual, which led him to the conclusion that krill live up to six or seven years. Nicol (1987) applied Ettershank's methods to field caught specimens of the euphausid, *Meganycytophanes norvegica*, and found a linear increase in FAP content with body weight; however, fluorescence could not be compared to chronological age. More significantly, Nicol pointed out a major flaw in Ettershank's use of formalin as a specimen preservative by demonstrating enhanced fluorescence of samples after storage in this powerful cross-linking agent. Subsequent investigators have committed similar errors in the use of formalin to preserve spider crab larvae (*Hyas araneus*; Hirche and Anger 1987) and krill (*E. superba*; Berman et al. 1989). Crossland et al. (1988) found an increase in FAP in lobster (*Panulirus cygnus*) and scampi (*Metanephrops andamanicus*) eye and tail muscle tissues with increasing size, however chronological age of their specimens was unknown. Sheehy and Ettershank (1989) extracted FAP from whole bodies of *Daphnia carinata*, finding an increase in absolute FAP content with increasing age, but a decrease in the weight-specific content with increasing age. The decrease in
weight-specific FAP can be attributed to the dilution of FAP by continued tissue growth. Most recently, Nicol et al. (1991) maintained populations of E. superba in the laboratory and measured significant increases in absolute and weight-specific FAP after one year of captivity.

Patterns of FAP accumulation have been examined in only two molluscan species. Nicol (1987) extracted FAP from mantle tissue of squid, Illex illecebrus, finding increases in FAP content with increasing size. Chronological age of his specimens was not known. Clarke et al. (1990) examined FAP accumulation in field specimens of the trochid gastropod Monodonta lineata whose chronological age had been estimated from shell annuli. Whole organ FAP content in brain and digestive gland increased with estimated age, but in a variable manner. Weight-specific FAP values decreased with age, a trend attributed to tissue growth.

Research problem

Little is yet understood about the relationship between FAP content and organismal age and growth in aquatic poikilotherms. Even though the previously mentioned studies seem promising, results are preliminary at best. Each fluorescence study has used some variation of a technique originally developed by Bligh & Dyer (1959) for whole lipid extraction and later modified by Fletcher et al. (1973) for the measurement of FAP. However, few of these variations have been replicable based on information in the published reports of the studies. Many aspects of the methodologies have simply been assumed to work properly, with no potentially detrimental effects to the final expression of fluorescence taken into account. In addition, the manner in which fluorescence results have been reported has been obscure at best, with no standardization or valid comparison possible between studies.
As a result of the above limitations, it has yet to be demonstrated that FAP can be used as an effective tool for predicting age of animals from the field. Only three investigators (Vernet et al. 1988, Hammer 1988, and Mullin & Brooks 1988) have examined FAP in fish of known age. Vernet et al. (1988) measured FAP content in three year classes of laboratory reared *Oncorhyncus mykiss* (of mixed brood stock origin), with highly variable results. Mullin & Brooks (1988) measured FAP in three larval fish species (*California grunion, Leuresthes tenuis*; white seabass, *Atractoscion nobilis*; and *California halibut, Paralichthys californicus*) up to 30 d in age, finding negative correlations between age and FAP content. Hammer's (1988) use of methanol to preserve larval pike specimens probably altered the final expression of FAP fluorescence in a fashion similar to Ettershank's krill samples (Nicol 1987). Even if a stable relationship between age and FAP were verified, its usefulness for aging field specimens would depend on a demonstration that information on FAP content can improve on the accuracy of age estimates presently available for fish through other means.

Further - and thus far not considered in age estimations using FAP - a number of environmental and physiological factors are likely to influence the rates of FAP accumulation in aquatic poikilotherms. Temperature, light, and diet are foremost among the exogenous factors known to affect ontogenetic changes in growth (Kinne 1960; Paloheimo & Dickie 1966; Brett 1979; Wurtsbaugh & Cech 1983; Cui & Wooton 1988). Some of these factors have been implicated in determining the rate of FAP formation in various poikilothermic (Sheldahl & Tappe1974; Ragland & Sohal 1975; Sohal & Donato 1979; Sohal et al. 1985) and homeothermic animals (Porta 1987) and in liposomal model systems (Petkau 1986). If these factors influence both growth and rates of FAP accumulation in animals, they are likely to induce variability in this rate at a given age as well as cumulative amounts of FAP over longer periods of time. This is significant if FAP quantities are to be used to predict the chronological ages of wild specimens.
**Study objectives**

In this dissertation, I study patterns of FAP accumulation and variation in a freshwater (*Puntius conchonius* Hamilton) and a euryhaline (*Oreochromis mossambicus* Peters) fish to evaluate the potential for accurate age estimation for management of fisheries stocks. Specific objectives are: 1) to reassess aspects of FAP assay methodologies and determine effects of these procedures on final FAP fluorescence expression; 2) to establish the relationship between FAP content and known chronological age in the selected species and to build age-predictive models using multivariate analysis; and 3) to examine effects of specific factors (body size, ambient temperature, food ration, and photoperiod) which may induce variability in the relationship between age and FAP content.

Completing the first objective is critical to accomplishing the other two objectives. In Chapter 2, various aspects of sample handling and extraction procedures are investigated for their effects on final expression of FAP fluorescence in tissue samples. This work includes evaluation of the effects of sample storage, extraction, and extract storage procedures for a variety of post-mitotic tissues of the selected fish species. Once procedures for extraction and measurement of the compounds have been tested and defined, the methods are applied to tissues of the study species to determine the basic relationship between chronological age and FAP content (Chapters 2 and 3). After the patterns of FAP accumulation have been established, I incorporate fish size, otolith size, organ size, and FAP data into various multiple linear regression models (Boehlert 1985) to examine the utility of FAP data for chronological age prediction (Chapter 3). In Chapter 4, experiments are conducted to determine the degree to which FAP content may vary in *P. conchonius* tissues under different environmental and physiological conditions.
Literature cited


Fig. 1-1. Modulation of superoxide radicals produced through normal cellular metabolism. Adapted from Zs.-Nagy (1987).
Superoxide Radical

$2O_2^-$

$2H^+$

Superoxide dismutase

$O_2$

$H_2O_2$

Catalase

Glutathione peroxidase

$\rightarrow H_2O + \frac{1}{2} O_2$

Fe (II)

Fenton Reaction

Fe (III)

OH$^-$

Hydroxyl Radical
Fig. 1-2. Free radical-mediated lipid peroxidation and malondialdehyde production.
Adapted from Petkau (1986).
Hydroxyl Radical Attack

OH•

Linolenic Acid

Lipid Radical

Diene Conjugate

Peroxy Radical

Endoperoxide

Malondialdehyde
Fig. 1-3. Fluorescent crosslinkage reactions of malondialdehyde. Adapted in part from Gutteridge (1986).
Malondialdehyde

\[ \text{OHC-CH}_2\text{-CHO} \]

- \( \text{R}_1\text{NH}_2 \)

1,4-Dihydropyridine-3,5-dicarbaaldehydes (390/450nm)

- \( +\text{R}_1\text{NH}_2 \)

- \( +\text{R}_2\text{NH}_2 \)

Polymalondialdehyde (370/480nm)

- \( +[\text{OHC-CH}]=_n \)

Amino-imino propene
Conjugated Schiff Base Fluorophores
(Ex 340-360nm, Em 410-470nm)

26
Fig. 1-4. Summary schematic of processes leading to fluorescent age pigment formation.
Unsaturated Fatty Acids & Phospholipids

Free Radical Attack

Peroxidation

Malondialdehyde & Other Carbonyl Compounds

Primary Amine Groups
- phospholipids
- proteins
- free amino acids
- nucleic acids

Crosslinkages

Cross-Linked Macromolecules Containing Conjugated Schiff Base Fluorophores
-(N-C=C-C=N)-

fusion with lysosomes

Fluorescent Age Pigments
(Pre-ceroid, Ceroid, Lipofuscin)
CHAPTER 2
CRITICAL ASPECTS OF FLUORESCENT AGE PIGMENT
METHODOLOGIES: MODIFICATION FOR ACCURATE ANALYSIS AND
AGE ASSESSMENTS IN AQUATIC ORGANISMS

Introduction

Age pigments, first described by Hannover (1842), are now recognized as being
one of the most clearly discernible markers of aging in cells (see review by Hammer and
Braum 1988). Age pigments form under normal and pathological conditions in cells as
the result of polymerization reactions between oxidized lipids and proteins (Björkerud
1964, Davies 1988, Katz 1990). The residues from these reactions have been variously
classified by their chemical and physical properties, solvent solubilities, fluorescent
properties, and stage of formation, being assigned names such as "lipofuscin" (Hueck
1912), "wear and tear pigment" (Hamperl 1934), and "ceroid" (Lillie et al. 1941).

Derived from a diverse array of sources, age pigments can now be characterized as
containing numerous autofluorescent components, ranging in emission color from blue
(Hydén and Lindström 1950, Barden 1980, Porta et al. 1988) to yellow (Eldred et al.
1982, Katz et al. 1984, Eldred and Katz 1988), many of which have been found to
accumulate with age (Shimasaki et al. 1980, Eldred et al. 1982, Sohal et al. 1983,
Csallany et al. 1984). Many attempts have been made to identify the fluorescent
components of age pigments, however, due to their complex chemical composition, this
topic is likely to remain controversial for some time. Possible sources of fluorescence
include conjugated Schiff bases (N,N’ disubstituted-1-amino-3-iminopropenes; Chio and
Tappel 1969, Tappel 1975), polymalondialdehyde (Gutteridge et al. 1977), 1,4-
dihydropyridine-3,5-dicarbaldehydes (Kikugawa and Ido 1984), and membrane bound
In 1973, Fletcher et al. introduced a relatively simple spectrofluorometric assay for the measurement of blue-emitting fluorophores, assumed to be related to age pigments, from whole tissue samples. Numerous investigators have since adopted Fletcher et al.'s method, demonstrating a relationship between organismal age and fluorescence of tissue extracts (Tappel et al. 1973, Miquel et al. 1978, Bridges and Sohal 1980, McArthur and Sohal 1982, Ettershank et al. 1983). The use of the terms "lipofuscin" or "lipofuscin-like" to describe solvent-extracted fluorophores is currently disputed (Eldred 1987, Eldred and Katz 1989) due to the differential properties of fluorescence in tissue extracts compared to histologically observed preparations. It should at this point be recognized that Fletcher et al.'s method is probably not specific to a single type or class of age pigment; rather it is a more general assay for the blue-emitting components of age-related fluorophores generated by lipid peroxidation processes in the cell. Thus, for the purposes of this study, we will adopt the term 'fluorescent age pigment', hereinafter referred to as 'FAP' (Sohal and Buchan 1981, Hammer and Braum 1988), to describe age-related fluorophores which have been measured spectrofluorometrically. Use of more specific terms such as 'lipofuscin' or 'ceroid' should be reserved for describing age pigments observed histologically.

Results from these preliminary studies have been cursory and inconclusive with regard to the application of this method toward age estimation for population analyses. For example, relatively few investigators have tested the method on animals of known chronological age. Conversely, those studies using sample tissue of known age have used sampling periods insufficient to determine long-term ontogenetic changes in FAP content. In addition, a survey of the methods thus far employed reveals a number of unsubstantiated modifications to the extraction procedure and a lack of standardization for reporting FAP data, thus negating the direct comparability of results between studies.

Using three marine invertebrate species, Nicol (1987) was the first to test specific aspects (i.e., sonication and use of tissue preservatives) of Ettershank’s (1983, 1984 a,b) protocol. Of greatest significance was his finding that tissue preservatives (formalin and ethanol) caused in vitro elevation of sample fluorescence. These results were corroborated by Mullin and Brooks (1988), who induced similar effects in formalin-preserved fish larvae. Both of these findings raised questions not only as to the source of fluorescence (native FAP vs formalin induced) measured in Ettershank’s (1983, 1984 a,b, 1985) and Berman et al.’s (1989) krill samples, but also in Hirche and Anger’s (1987) spider crab larvae, and Hammer’s (1988) pike larvae. Further, additional questions with regard to the stability of extractable FAP compounds under different storage and handling conditions remained unanswered, and in essence have largely been ignored.

The purpose of the present study was two-fold. The first objective was to evaluate various aspects (i.e., tissue storage and extract incubation temperature and time, sonication of tissue homogenates, assay temperature and the use of aqueous-methanol extracts) of the methodologies currently adopted for the spectrofluorometric quantification of FAP and to determine their effects on the expression of and variation in sample fluorescence. For this purpose we chose Oreochromis mossambicus as the model
animal, concentrating on postmitotic tissues only. The second objective involved the development of modified handling, extraction, and assay procedures to negate in-sample fluorescence variation and the application of these procedures to brain tissues of *O. mossambicus* to determine whether a distinct relationship between FAP content and chronological age exists.

**Materials and methods**

**Model animal/tissues**

Known-age specimens of *Oreochromis mossambicus*, stocked as fry, were reared for up to 886 d at the Kewalo Basin Research Facility of the National Marine Fisheries Service, Honolulu Laboratory. Fish were held in circular fiberglass holding tanks at ambient temperature with daily water changes, and fed *ad libitum* rations of trout chow twice daily. Subsampled specimens were transported live to the laboratory, sacrificed by ice bath immersion, weighed to the nearest 0.01g, and standard and fork lengths measured to the nearest 0.05 mm. Fish were bled from the caudal region before removing tissues. A preliminary survey of numerous postmitotic tissues indicated that brain, ventricle, and skeletal muscle extracts contained detectable levels of FAP fluorescence with the least amount of interfering compounds; therefore, these were chosen as model tissues for the remainder of the study. Dissected tissues were placed in Eppendorf vials and immediately frozen in liquid nitrogen until analysis.

**Tissue storage temperature**

Skeletal muscle tissues were placed at various short- and long-term storage temperatures to detect changes in sample fluorescence over time. White skeletal muscle was dissected from the left anterior epaxial region of three sibling fish, briefly minced with a Virtis motorized blade, and stored in Eppendorf vials either at room (23°C) or refrigerated (3°C) temperatures for 0.5, 1, 2, 4, 8, and 12 h, or frozen at -20 or -80°C for
5, 15, 30, 60, 90, and 120 d. Sample vials were frozen in liquid nitrogen at the end of each time point. Sample fluorescence was compared to that of absolutely fresh tissue. Tissues were analyzed using the modified extraction procedure, and assayed as described in later sections.

**Unmodified extraction procedures**

Extraction and assay procedures adopted for the first part of this study were based on protocols outlined by Ettershank (1983, 1984 a,b). These included: maceration of tissues in spectroscopic grade chloroform/methanol (CHCl₃/MeOH, 2:1 v/v), sonication, mixing with a magnesium chloride solution [100 mM in deionized glass distilled water (GDW)], and centrifugation for 20 min at RCF = 3000 x g. Fluorescence of the lower CHCl₃ layer was compared to a standard solution of quinine sulfate (0.1 mg/L 1N sulfuric acid). Further details on extraction procedures are provided, when appropriate, in the following subsections.

**Incubation temperature of sample extracts**

The stability of FAP products in solvent was examined in intermediate-stage CHCl₃/MeOH (2:1 v/v) and final CHCl₃ fractions of tissue extracts at room temperature (23°C) and -20°C for up to 8 h. Lyophilized brain, heart, and muscle tissues from siblings were pooled by pulverizing and mixing dried tissues in 15 ml glass centrifuge tubes with a teflon pestle. Stability of intermediate stage extracts was tested by extracting triplicate aliquots of 20 mg of dry tissue in 2 ml CHCl₃/MeOH, centrifuging the homogenate for 20 min at a relative centrifugal force (RCF) of 3000 x g and 0 to 5°C, and collecting and storing the CHCl₃/MeOH supernatant in amber glass vials at the defined incubation temperatures. Final stage CHCl₃ extracts were prepared by adding an equal volume of 100 mM magnesium chloride solution in GDW to the CHCl₃/MeOH homogenates, centrifuging as defined above, and transferring the CHCl₃ hypophase to amber glass vials for incubation.
Effects of ultrasonication on fluorescence yield

Ultrasonication of tissue homogenates (widely adopted for the disruption of biological membranes and thus increased extraction efficiency) was tested to determine the overall effects on fluorescence yield of CHCl3-extractable FAP compounds. Eighty mg aliquots of lyophilized and pooled brain, heart, or muscle tissues were glass homogenized in 8 ml CHCl3/MeOH (2:1), transferred to a 15 ml glass centrifuge tube, and sonicated with a Sonics & Materials VibraCell model VC300 microtip probe sonicator for up to 35 min. Sonication output was 25 W at a 50% duty cycle with sample tubes held in ice and water to absorb heat. 1 ml aliquots of CHCl3/MeOH homogenate were removed at 5 min intervals, vortex mixed with an equal volume of the 100 mM MgCl2 solution, centrifuged, and the CHCl3 layer assayed for FAP content.

Confirmation and removal of interfering compounds

Fletcher et al. (1973) mentioned the importance of removing interfering fluorescent compounds from CHCl3/MeOH tissue extracts. Retinol fluorescence (excitation 325 to 340 nm, emission 475 nm), present in the final CHCl3 extract, could be removed by a brief (30 s) exposure of the solvent to high intensity ultraviolet light. Flavin compounds fluoresce at 510 to 520 nm with excitation maxima at 280, 350, and 450 nm and are present in the CHCl3/MeOH homogenate, removable by a water wash step. In the present study, complete excitation and emission fluorescence spectra were obtained before all sample assays to detect the presence of fluorescent contaminants. In addition, the fluorescent properties of all aqueous/methanol phase extracts were checked for the presence of these contaminants.

Assay temperature

Temperature dependence of sample and standard fluorescence has been established (Guilbault 1973), however, most researchers have conducted assays at ambient temperature. The importance of assay temperature control was tested on
Oreochromis mossambicus brain, heart, and muscle extracts by measuring sample luminescence at temperatures ranging from 0 to 42°C at 3°C intervals. Temperature of the sample solvent was maintained using a thermostatically controlled turret microcell holder connected to a Brinkmann Lauda Model RM6 refrigerated circulating water bath. Resulting changes in the calculated relative fluorescence intensity values were noted.

Modified extraction and assay procedures

Based on the results from the above experimental analyses a modified procedure for the extraction and assay of FAP was implemented. Tissues were prepared for extraction by immersion in liquid nitrogen and lyophilization to obtain the most accurate sample weight. Dry tissues were weighed on a Sartorius Model 4503 MP6 electronic microbalance to the nearest microgram, placed in glass tubes, and returned to the lyophiliizer until extraction. Tissues were macerated at room temperature for 4 min in a Potter-Elvehjem all-glass tissue homogenizer using at least 1 ml of CHCl3 per 10 mg dry tissue. Approximately one-third of the solvent typically evaporated during homogenization. Because of this the initial meniscus was marked and the homogenate adjusted back to its original volume after treatment. Homogenates were then transferred to a glass centrifuge tube and vortex mixed for 2 min with an equal volume of a solution containing 100 mM MgCl2 in GDW and methanol at a ratio of 3:1. Samples were centrifuged at 0-5°C for 20 min at RCF = 3000 x g. The lower CHCl3 layer was immediately removed and assayed for FAP content.

Chloroform extracts were placed in matched quartz silicate microcuvettes with a 4 mm pathlength, sealed with a teflon stopper, and assayed at 18°C in a Perkin Elmer model LS-5 fluorescence spectrophotometer equipped with a Xenon light source and standard S-5 photomultiplier tube. Excitation and emission slits were both set at 10 nm for standard assays, and luminescence readings were at a fixed scale setting of 1.000. Excitation and emission maxima were determined using PreScan mode, and the maximum luminescence.
at these settings was recorded. Fluorescence spectra reported herein were not corrected for the spectral emission characteristics of the light source, nor the spectral efficiencies of the monochromators, optics, and the photomultiplier tube. Quinine sulfate at a concentration of 0.1 mg/l 1N sulfuric acid (H$_2$SO$_4$) was used as a fluorescence standard, and the sample readings were expressed as a percentage of the standard luminescence at an excitation setting of 345 nm and emission setting of 450 nm. Sample FAP concentrations are expressed either as relative fluorescence intensity (RFI), where:

$$RFI = \frac{\text{sample luminescence}}{\text{standard luminescence}} \times \frac{\text{solvent volume (ml)}}{\text{sample dry weight (mg)}} \times 100$$

or as whole organ %fluorescence (%FL), where:

$$\text{Whole Organ } %FL = \frac{\text{sample luminescence}}{\text{standard luminescence}} \times \text{solvent volume (ml)} \times 100$$

Sample luminescence spectral scans were plotted for all samples with a Perkin Elmer model R100A chart recorder. Spectral scans presented herein were produced by recording luminescence at 1 nm intervals with the complementary excitation/emission wavelengths at a fixed setting.

**Results**

**Tissue storage temperature**

Chloroform extracts from fresh (t=0) pooled *Oreochromis mossambicus* muscle had an average RFI value of 0.4412 (±0.0275) at excitation maxima of 350 to 355 nm and emission maxima of 425 to 430 nm. Muscle samples stored at elevated (>0°C) temperatures exhibited linear increases in fluorescence after a matter of hours with no apparent shifts in excitation/emission maxima. Muscle samples held at room temperature (23°C) had elevated levels of fluorescence after only 1 h. A mean increase (ΔRFI) of 9.3% was evident after only 2 h of incubation, and this increased to as much as 87%
ΔRFI at the end of 12 h (Fig. 2-1 A) (%ΔRFI = -1.754 + 6.303 (Hours), r² = 0.96). Although refrigeration at 3°C slowed the rate of fluorophore accumulation, a mean increase of 9.1% ΔRFI was measured after 8 h incubation (%ΔRFI = 0.093 + 0.722 (Hours), r² = 0.56). Tissues stored for longer periods at -20°C also exhibited linear increases in fluorescence, with a mean ΔRFI of 9.7% after 15 d (Fig. 2-1 B) (%ΔRFI = -0.087 + 0.638 (Days), r² = 0.98). No significant changes in fluorescence were observed in muscle samples stored at -80°C after 120 d (%ΔRFI = 0.621 + 0.0029 (Days), r² = 0.003).

*Incubation temperature of sample extracts*

Fluorescence of CHCl3/MeOH- and CHCl3-soluble extracts of *Oreochromis mossambicus* brain, heart, and muscle increased over time at both room (23°C) and reduced (-20°C) temperatures (Figs. 2-2 and 2-3). Increases in CHCl3/MeOH extract fluorescence were accompanied by shifts in the excitation/emission maxima from approximately 355/430 to 380/455 nm, while fluorescence maxima of CHCl3 extracts remained unchanged. The change in CHCl3/MeOH extract fluorescence wavelengths occurred by 3 h at 23°C and by 4 h at -20°C. Chloroform/methanol extracts incubated at 23°C exhibited the most rapid rises in fluorescence, with heart extracts increasing by an average of 147% ΔRFI after 3 h and as much as 880% ΔRFI at the end of 8 h (Fig. 2-2 A). Final water-washed CHCl3 extracts of all three tissues increased in fluorescence on the average of 10 to 16% ΔRFI after only 2 h incubation at 23°C with heart extracts again increasing more than brain or skeletal muscle (Fig. 2-3 A). Incubation at lowered temperature (-20°C) slowed in vitro changes in sample fluorescence. Even so, increases were observed within 2 to 4 h of incubation (Figs. 2-2 B and 2-3 B). Brain CHCl3 extracts were the least stable at -20°C, with an average ΔRFI of 7.2% after 4 h and up to 18.5% after 8 h.
Effects of ultrasonication

Sonication of CHCl₃/MeOH homogenates at 25 W of output caused dramatic increases in fluorescence for all three tissues, with mean ΔRFI values of 200, 119, and 83% for brain, heart, and muscle, respectively, after only 5 min sonication (Fig. 2-4 A). Continued sonication for up to 30 min caused further increases in fluorescence, after which levels decreased or stabilized. Of the tissues studied, brain homogenates had the greatest change with an average ΔRFI of 902% after 30 min (Fig. 2-4 A). Following a sonication period of 5 min, the uncorrected fluorescence spectra of all tissue homogenates also revealed shifts in excitation/emission maxima similar to those described for CHCl₃/MeOH extracts in the previous subsection. Results are presented for brain tissue only (Fig. 2-4 B).

Fluorescent contaminants

Uncorrected fluorescence spectra from some brain, heart, and skeletal muscle extracts showed the presence of a second emission peak in the 510 to 520 nm range in the CHCl₃/MeOH of the intermediate extraction stage as well as in the aqueous phase final wash. Excitation scans of these samples at an emission setting of 520 nm revealed excitation maxima at 286, 351, and 447 nm (Fig. 2-5 B), confirming the presence of fluorescent flavin contaminants in the CHCl₃/MeOH and aqueous phase solvents. The second 510 to 520 nm emission peak was not obvious in all CHCl₃/MeOH and aqueous extracts (Fig. 2-5 A). However, excitation scans of all samples at emission =520 nm confirmed the presence of the three excitation maxima (Fig. 2-5 B). Flavin contaminants were not present at detectable levels in the final CHCl₃ sample solvent, and a second water wash of the CHCl₃ layer contained no flavin or FAP fluorescence. Thus, a single water wash was sufficient to remove all flavin contaminants. There was no evidence of retinol contaminants in any of the three chosen tissues.
**Assay temperature**

Absolute luminescence values of both standards and samples decreased with increasing assay temperature (Fig. 2-6 A). The relationship between assay temperature (T in °C) and brain extract luminescence (BL, as raw luminescence values) was linear, where:

\[ BL = 0.61109 - 0.01021 \times (T), \quad r^2 = 0.969 \]

and the relationship between assay temperature (T) and quinine sulfate luminescence (QL) was best described as:

\[ QL = 1.6532 - 0.00467 \times (T), \quad r^2 = 0.886 \]

The effect of temperature on fluorescence data is better observed when RFI values are considered (Fig. 2-6 B). A three-fold decrease in RFI occurred over the temperature range of 0 to 42°C, and inflections were apparent in this relationship (Fig. 2-6 B) within the working range of room temperatures (20 to 24°C).

**FAP accumulation with age**

The extraction and assay of whole brain FAP content (% FL) using our modified procedures resulted in a positive correlation with chronological age (Fig. 2-7 A). This increase was evident both in immature fish and in mature male and female specimens, and for the portion of the lifespan examined, it was well described by second order polynomial regressions (Fig. 2-7 A) (Immature + males: %FL = 7.407 - 4.586*10^{-2} (Days) + 2.33*10^{-4} (Days)^2, r^2 = 0.926, P< 0.0001, n = 101; Immature + females: %FL = 6.347 - 1.41*10^{-2}(Days) + 1.074*10^{-4} (Days)^2, r^2 = 0.936, P< 0.0001, n = 99). FAP content (% FL) accumulated gradually during the first 370 d, after which it increased rapidly and resulted in a marked difference between males and females.

Relative fluorescence intensity values decreased rapidly between 32 and 92 d, remained low until approximately 330 d, and gradually increased thereafter, also differentiated by sex (Fig. 2-7 B). After about the first 80 d, relationships between age...
and RFI were well described using second order polynomial equations (Fig. 2-7 B)
(Immature + males: \( RFI = -0.366 + 2.033 \times 10^{-3} \) (Days) + \( 3.843 \times 10^{-6} \) (Days)\(^2\), \( r^2 = 0.921, P< 0.0001, n = 101 \); Immature + females: \( RFI = 0.866 - 2.678 \times 10^{-3} \) (Days) + \( 5.872 \times 10^{-6} \) (Days)\(^2\), \( r^2 = 0.935, P< 0.0001, n = 99 \)).

Brain growth with age was most rapid initially, slowing after 300 d, and mirrored the relationship between age and RFI. Relationships between age and brain dry weight (BW in mg) were well described by logarithmic equations (Fig. 2-7 C) (Immature + males: \( BW = -36.488 + 23.333 \times \log_{10}(\text{Days}) \), \( r^2 = 0.943, P< 0.0001, n = 123 \); Immature + females: \( BW = -32.101 + 21.067 \times \log_{10}(\text{Days}) \), \( r^2 = 0.914, P< 0.0001, n = 121 \)).

**Discussion**

It is apparent from the results of this study that the temperatures at which tissues and extracts are handled and the time frame involved markedly affect expression of FAP fluorescence and can introduce considerable error unless controlled. Similarly, application of ultrasonication to tissue homogenates drastically alters the properties and levels of sample fluorescence. These observed changes in tissue and extract fluorescence suggest the *in vitro* formation of FAP-like substances caused by lipid peroxidation (Luo and Hultin 1986), the production of cross-linking agents (e.g., malondialdehyde and formaldehyde), and the subsequent genesis of fluorophores in the sample solvent.

The effects of storage, handling, and assay temperatures on the accurate analysis of FAP are not surprising. The chemical instability of fish tissues under different freezing conditions (as related to food quality) has been well documented. Changes occurring during freezing include "browning" reactions between free amino acids and sugars (Jones 1959), denaturation of proteins (Sawant and Magar 1961), dissociation of trimethylamine to dimethylamine and formaldehyde (Amano and Yamada 1964,
Tokunaga 1964, Castell et al. 1971), loss of membrane phospholipid through autoxidation (Hardy et al. 1979), and increases in muscle fluorescence (Davis 1982). Davis (1982) used fluorometric methods to document effects of freezing (-5 to -30°C) on muscle tissues. In a companion paper, increases were attributed to formaldehyde production and browning reactions between fructose, ribose, and free amino groups (Davis and Reece 1982). The results reported for skeletal muscle of Oreochromis mossambicus in the present work indicate similar changes affecting levels of apparent FAP content, and demonstrate the necessity for proper freezing temperatures from the moment the animal is sacrificed (i.e., immediate placement and storage in liquid nitrogen, or at least at temperatures of -80°C or lower).

Because temperature would seem to be a critical factor affecting the native FAP content of tissues, the increases observed with time in this research suggest that they are probably due to the in vitro formation of other chemical complexes. We suspect that these complexes are similar to those described in studies seeking to determine the chemical nature of FAP fluorophores (Chio and Tappel 1969). Their formation is presumably initiated by lipid peroxidation, which has been well defined for muscle microsome and sarcoplasmic reticulum (SR) model systems of fish. Lipid peroxidation, measured by malondialdehyde (MDA) production, has been demonstrated for muscle microsomal fractions from red hake (McDonald et al. 1979) and herring (Slabyj and Hultin 1982). MDA production was dependent upon the levels of peroxidizing cofactors (NADH, ATP, and Fe^{3+}) and incubation temperature, and occurred under all conditions over short periods of time (0 to 60 min) in both studies (McDonald et al. 1979, Slabyj and Hultin 1982). Related studies using SR model systems have found similar cofactor effects (Luo and Hultin 1986) as well as inhibition of peroxidation by phospholipase A2 (Shewfelt and Hultin 1983). Luo and Hultin (1986) incubated SR from winter flounder, documenting increases in both MDA and fluorescence after only 1 and 3 h, respectively.
Once formation of MDA has occurred, a cascade of chemical reactions has been proposed that eventually results in the genesis of blue and blue-green emitting fluorophores.

Chio and Tappel (1969) first described the cross-linking reaction between MDA and amino acids to form N,N'-disubstituted 1-amino-3-iminopropenes which fluoresce at wavelengths between 400 and 550 nm. Similar cross-linking reactions have now been established between MDA and the primary amine groups of phospholipids (Dillard and Tappel 1971, Bidlack and Tappel 1973, Trombly and Tappel 1975), proteins (Shimasaki et al. 1982, Fukuzawa et al. 1985, Kikugawa and Beppu 1987), and nucleic acids (Nair et al. 1986). Lipid-soluble extracts used for FAP studies represent complex mixtures of phospholipids, fatty acids, non-polar amino acids, proteins, and many other biological compounds which are highly susceptible to peroxidation and polymerization reactions. Our results support the conclusion that temperature and time are important in the accelerated formation of fluorescent complexes. To eliminate further synthesis of fluorescent adducts, tissue extracts should be handled expeditiously within a critical window of time (from beginning of extraction through fluorescence assay) and assayed at controlled temperature.

The fluorescence maxima shifts observed in CHCl₃/MeOH extracts (whether or not ultrasonication was applied to the homogenate) suggest the formation of compounds similar to 1,4-dihydropyridine-3,5-dicarbaldehydes first isolated by Kikugawa and Ido (1984) through the reaction of primary amines with the hydrolysate of tetramethoxypropane; however, this process has yet to be substantiated. Regardless of the source of change, CHCl₃/MeOH tissue homogenates are highly unstable under normal extraction conditions, and ultrasonication of homogenates greatly enhanced the rates of fluorophore production. This may be due either to generation of free radicals in sample solution through cavitation or to the localized generation of heat within.
homogenates. Our results indicate that ultrasonication should be avoided because of the rapid alteration of sample fluorescence properties.

Overall, our data raise questions as to the nature of fluorescence described in previous investigations on FAP in aquatic animals (Table 1). A survey of the methods adopted in these studies emphasizes the fact that few used fresh tissues or stored tissues at temperatures adequate for stabilization of cellular constituents, most did not mention the time elapsed between freezing and analysis, and none took extract incubation time and temperature into full consideration. Additionally, ultrasonication was applied to tissue homogenates in at least half of these studies (Table 1), and whereas the majority employed sonication for less than 5 min, most applied a power output four times greater than that found to generate additional fluorophores under our experimental procedures. Conversely, Nicol (1987) found no significant differences between sonified and non-sonified Meganyctiphanes norvegica individuals. This was probably because the samples were preserved in formalin and had already increased in fluorescence prior to extraction. Moreover, several of the studies cited in Table 1 (i.e., Hammer and Madhusudhana-Rao 1987, Hirche and Anger 1987, Hammer 1988, Hammer and Karakiri 1988) used the polar extract phase to quantify FAP but failed to verify the absence of flavin contaminants - which we found to comprise as much as half of the total sample fluorescence. Sheehy and Ettershank (1989) also reported considerable quantities of flavin-like fluorescence in CHCl₃/MeOH and polar phase extracts from whole Daphnia carinata. Ettershank (1983, 1984 a,b, 1985) was the only investigator to control temperature during fluorometric analysis; the others conducted assays at room temperature.

When all these critical aspects of FAP analysis are considered, it is not surprising that the data from previous investigations have been too inconsistent for direct comparison. More importantly, the main goal of most previously published research has been to establish a relationship between FAP content and chronological age; however,
because of the high degree of variability and the fact that the techniques were applied to animals of known age in only a few studies (Table 1), the relationships reported are open to question. In all probability, they reflect fluorescence modified by analytical artifacts rather than native tissue fluorescence. Of those studies on fishes that have taken chronological age into account, the most complete is probably that of Vernet et al. (1988), who measured FAP content in adult Oncorhynchus mykiss of known age (3 mo to 3 yr). However, they sampled only 21 individuals derived from different broodstock and did not take into account extract incubation or assay temperature and time.

In contrast to these previous investigations, our study not only took into account sources of variability in most aspects of the extraction and assay procedure, but also provided a chronological range of samples (from 33 to 886 d), with more stringent sampling procedures. The present research is the first to describe a clear relationship between blue-emitting FAP accumulation and chronological age in an aquatic organism. However, other sources of variability have been subsequently exposed that require further research, in order that the technique can be validated for accurate assessment of age. For example, initial decreases in the weight-specific FAP concentrations in juvenile Oreochromis mossambicus appear to correspond to a period of rapid brain growth, suggesting that although FAP accumulates in this tissue, growth of the whole organ exceeds the rate of accumulation at this developmental stage. Siakoto et al. (1977) found similar patterns of accumulation of human brain lipofuscin which was extracted and purified by differential centrifugation, and speculated that the large pool of lipofuscin found in young individuals may be related to the lysosomes derived from the remodeling of cellular structures during early developmental stages. The marked differences in FAP accumulation between male and female O. mossambicus have yet to be explained, but will have direct bearing on the applicability of FAP analysis for accurate age assessment of wild stocks.
An additional problem must be overcome before spectrofluorometric assays for FAP can be applied successfully to crustaceans. Many crustacean species contain considerable quantities of carotenoid compounds such as astaxanthin and canthaxanthin (Thommen and Wackernagel 1964, Herring 1968a,b, Goodwin 1984), that have light absorptive properties within the emission range of FAP compounds (Zeller et al. 1959, Cooper et al. 1975) and are highly soluble in nonpolar solvents such as chloroform. Thus, these carotenoid compounds may cause internal quenching of FAP fluorescence. Preliminary investigations of FAP quantification in *Penaeus vannamei* (Hill, Moss, and Womersley unpublished data) and *Heterocarpus laevigatus* (Hill, Shiota, and Womersley unpublished data) have revealed large quantities of orange colored pigments in nonpolar extract fractions which have UV/visual absorption maxima similar to those reported for astaxanthin and canthaxanthin. Increases in orange pigment concentrations were accompanied by decreases in FAP concentrations. If the observations indicate quenching phenomena, they may explain the negative correlations between age and weight-specific FAP fluorescence described by Hirche and Anger (1987) for *Hyas araneus* and by Sheehy and Ettershank (1989) for *Daphnia carinata*.

Clearly, the fluorometric assay for FAP holds a great deal of promise. However, further investigation is necessary to determine the source in variations in FAP content between sexes and the effects of environmental factors (e.g., temperature, light, food ration) on tissue fluorescence. These and other problems must be resolved before measurement of FAP can become a standard method for aging aquatic animals.
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Table 2-1. Previous studies of spectrofluorometrically measured fluorescent age pigment accumulation in aquatic animals. Tissues: b = brain; ceph. = cephalothorax; h = heart; l = liver; m = muscle; s = spleen. Storage: FA, preserved in formalin; FA/S, preserved in formalin then transferred to storage solution; refrig., refrigerated. Solvent: C, chloroform; M, methanol. Sonic.: ultrasonication. rt: assayed at room temperature. -: no information available based on the described methods. Abbreviations for species names are: E. superba = Euphausia superba, P. americanus = Pseudopleuronectes americanus, O. aureus = Oreochromis aureus, H. araneus = Hyas araneus, C. hyperboreus = Calanus hyperboreus, M. norvegica = Meganystiphanes norvegica, I. illecebrosus = Ilyanassa illecebrosus, E. lucius = Esox lucius, L. limanda = Limanda limanda, D. albidella = Dasyatis albidella, L. tenuis = Leuresthes tenuis, A. nobilis = Atractoscion nobilis, P. californicus = Paralichthys californicus, O. mykiss = Oncorhynchus mykiss, M. pacificus = Microstomus pacificus, D. carinata = Daphnia carinata.

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Table 2-1. (Continued) Previous studies of spectrofluorometrically measured fluorescent age pigment accumulation in aquatic animals. Tissues: b = brain; ceph. = cephalothorax; h = heart; l = liver; m = muscle; s = spleen. Storage: FA, preserved in formalin; FA/S, preserved in formalin then transferred to storage solution; refrig., refrigerated. Solvent: C, chloroform; M, methanol. Sonic.: ultrasonication. rt: assayed at room temperature. -: no information available based on the described methods. Abbreviations for species names are: E. superba = Euphausia superba, P. americanus = Pseudopleuronectes americanus, O. aureus = Oreochromis aureus, H. araneus = Hyaenus araneus, C. hyperboreus = Calanus hyperboreus, M. norvegica = Meganyctiphanes norvegica, I. illecebrosus = Illex illecebrosus, E. lucius = Esocidae lucius, L. limanda = Limanda limanda, D. albisella = Dascyllus albisella, L. tenuis = Leuresthes tenuis, A. nobilis = Atractosteon nobilis, P. californicus = Paralichthys californicus, O. mykiss = Oncorhynchus mykiss, M. pacificus = Microstomus pacificus, D. carinata = Daphnia carinata.

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Fig. 2-1. *Oreochromis mossambicus*: Effect of storage temperature on relative fluorescence intensity of skeletal muscle. (A) short-term storage of tissues at 23 and 3°C; (B) long-term storage of tissues at -20 and -80°C. Uncorrected excitation and emission maxima were 350 to 355 nm and 425 to 430 nm, respectively. Maxima did not shift as intensity increased. Data presented are means ± standard deviations for triplicate analyses.
Fig. 2-2. *Oreochromis mossambicus*: Effect of incubation temperature on relative fluorescence intensities of unfractionated chloroform/methanol-soluble extracts of brain, heart, and muscle tissues. Extracts incubated at 23°C (A) and -20°C (B). Initial uncorrected excitation and emission maxima were 355 and 430 nm, respectively, shifting to 380 and 455 nm after 2 and 3 h of incubation at either temperature. Data presented are means ± standard deviations for triplicate analyses.
Fig. 2-3. *Oreochromis mossambicus*: Effect of incubation temperature on relative fluorescence intensities of final chloroform-soluble extracts of brain, heart, and muscle tissues. Extracts incubated at 23°C (A) and -20°C (B). Uncorrected excitation and emission maxima were 350 to 355 nm and 425 to 430 nm, respectively. Maxima did not shift as intensity increased. Data presented are means ± standard deviations for triplicate analyses.
Brain • Heart • Muscle

Incubation Time (hours)

%Δ Relative Fluorescence Intensity

A

23°C

B

-20°C

0 1 2 3 4 5 6 7 8

61
Fig. 2-4. *Oreochromis mossambicus*. Effects of ultrasonication at 25 W of output on the expression of sample fluorescence. (A) change in relative fluorescence intensities of sonified homogenates of brain, heart, and muscle, (B) uncorrected fluorescence spectra of brain extracts before sonication (excitation/emission maxima = 355/440 nm, respectively) and after 5 min sonication (excitation/emission maxima = 383/453 nm, respectively). Data in (A) are means ± standard deviations for triplicate analyses.
**A**

- Plot of % Relative Fluorescence Intensity vs. Sonication Time (minutes)
- Different tissues: Brain (○), Heart (●), Muscle (▲)

**B**

- Plot of Luminescence vs. Excitation/Emission Wavelength (nm)
- Peaks at 383, 453, 355, and 440 nm
- EX and EM labels indicate excitation and emission wavelengths respectively.
Fig. 2-5. *Oreochromis mossambicus*. Uncorrected fluorescence spectra of aqueous/methanol phase extracts of brain tissue. (A) excitation spectrum at emission = 455 nm and emission spectrum at excitation = 338 nm; (B) excitation spectrum of same sample at emission = 520 nm. Excitation peaks at 286, 351, and 447 nm indicate contaminating flavin compounds.
Fig. 2-6. *Oreochromis mossambicus*. (A) Relationship between assay temperature and luminescence of quinine sulfate standard (0.1 mg/l 1N H$_2$SO$_4$) and chloroform extracts from brain, and (B) resulting relative fluorescence intensity values from same sample data.
Fig. 2-7. *Oreochromis mossambicus*. Fluorescent age pigment (FAP) accumulation and brain weight as functions of chronological age. (A) Total FAP content of an entire brain; (B) weight-specific FAP (relative fluorescence intensity) in brain; (C) brain dry weight.
CHAPTER 3

FLUORESCENT AGE PIGMENT ACCUMULATION AND THE USE OF MULTIPLE REGRESSION MODELS FOR AGE ESTIMATION IN TWO FISH SPECIES

Introduction

For the past decade, aquatic biologists have attempted to establish whether a relationship exists between chronological age and the accumulation of soluble fluorescent age pigments (FAP) extracted from postmitotic tissues, with the ultimate goal of applying this method to age prediction in population studies. To date, results from FAP studies have been published for crustacean (Ettershank, 1983, 1984a,b, 1985; Hirsch & Anger, 1987; Nicol, 1987; Crossland et al., 1988; Berman et al., 1989; Sheehy & Ettershank, 1989; Nicol et al., 1991), mollusk (Nicol, 1987; Clarke et al., 1990), and fish (Luo & Hultin, 1986; Hammer & Madhusudhana-Rao, 1987; Hammer, 1988; Hammer & Karakiri, 1988; Hill & Radtke, 1988; Mullin & Brooks, 1988; Vernet et al., 1988; Hill & Womersley, 1991) species. Results from some of these preliminary studies have been variable, leading to skepticism about the applicability of FAP assays for age estimation; however, variability of results from some of these early studies has been explained in part by the application of methodological procedures leading to inaccuracies in the FAP assay. Nicol's (1987) discovery that formalin fixation of samples artificially induced FAP-like fluorescence in the tissue essentially invalidated the results of numerous studies (Ettershank 1983, 1984a,b, 1985; Hirsch & Anger, 1987; Hammer, 1988; Berman et al., 1989). Furthermore, Hill & Womersley (1991; see Chapter 2) demonstrated that other factors related to sample storage, extraction technique and handling time may have affected variability of the FAP assay in numerous other studies.

An additional problem that has led some investigators to question the FAP assay has been the presumption that weight-specific concentrations of FAP (commonly referred
to as "relative fluorescence intensity", or RFI) should show some positive correlation with age. On the contrary, most studies have shown that RFI values are either negatively correlated with age (Mullin & Brooks, 1988; Vernet et al., 1988; Crossland et al., 1988; Sheehy & Ettershank, 1989; Clarke et al., 1990), or decrease initially, increasing later with advancing age (Clarke et al., 1990; Hill & Womersley, 1991). Only one study reports consistent age-related increases in weight-specific FAP concentrations in an aquatic species (Luo & Hultin, 1986). Earlier reports describe such a trend in other vertebrates (e.g. - Tappel et al., 1973; Shimasaki et al., 1977; Miquel et al., 1978; Shimasaki et al., 1980). As many investigators have pointed out, any relationship between tissue FAP concentration (RFI) and age will be inherently confused as long as the rate of FAP genesis differs from the rate of accumulation of tissue mass (Hammer & Madhusudhana-Rao, 1987; Vernet et al., 1988; Hill & Womersley, 1991; Sheehy & Roberts, 1991). Thus, RFI values are only useful for comparing FAP from areas within the same tissue, FAP in different tissues from the same organism, FAP between different animals of identical age, or for examining trends in FAP accumulation in animals whose somatic or organ growth has approached some asymptote.

Questions have also been raised as to the validity of the FAP technique originally developed by Fletcher et al. (1973). This controversy has centered mainly on discrepancies in the autofluorescence properties of histologically observed age pigment granules (lipofuscins and ceroids) having emission maxima in the 500 to 630 nm wavelength range, and soluble age-related fluorophores (FAP) which emit light in the blue (400-490 nm) range. Eldred et al. (1982) suggested that this problem was related to differential sensitivity of fluorescence instrumentation which biased fluorescence readings in the blue-green range unless corrected; however, Eldred & Katz (1989) later demonstrated that this difference remained even after instrumental biases were corrected. Numerous attempts have been made to characterize age pigment fluorescence by
reproducing crosslinking reactions involved in the pathology of aging, and all have resulted in model fluorophores with autofluorescence in the 400-490 nm range (Chio & Tappel, 1969; Malshet et al., 1974; Gutteridge et al., 1977; Kikugawa & Ido, 1984; Fukuzawa et al., 1985; Kikugawa et al., 1989; Yin & Brunk, 1991b). Despite this controversy, a number of studies have clearly demonstrated that soluble FAP is present in the tissues of aquatic animals and that it accumulates in a manner that correlates with chronological age (Luo & Hultin, 1986; Mullin & Brooks, 1988; Vernet et al., 1988; Hill & Womersley, 1991; Nicol et al., 1991). Subsequently, Yin & Brunk (1991a) provided data to support all FAP studies, and thus the FAP assay, by demonstrating that model FAP fluorophores undergo a metachromatic shift in fluorescence emission from the orange and yellow range at high concentration to the blue range at diluted concentrations. This 'inner-filter' effect goes a long way toward explaining the differences between fluorescence spectral properties of intact age pigment granules that are dense, light-absorbing bodies (Katz et al., 1984; Docchio et al., 1991) and those that have been isolated and solvent-extracted (Hendley et al., 1963; Siakotos & Koppang, 1973).

The question of primary importance to most field biologists interested in the FAP method is whether FAP content, which is a function of physiological aging processes, can be used to predict chronological age. The present study was designed to address this issue by examining age-related patterns of FAP accumulation in laboratory-reared populations of a freshwater (Puntius conchonius Hamilton) and a euryhaline (Oreochromis mossambicus Peters) teleost. The problem of age prediction was approached by developing multiple linear regression models which include information available from somatic growth, otolith morphometrics, and FAP content, to establish whether additional information available from the FAP method can improve the ability to predict chronological age.
Materials and methods

Specimen culture and preparation

Known-age specimens of *O. mossambicus* were reared for up to 886 d and sampled under conditions described previously (Hill & Womersley, 1991). *P. conchonius* fry were hatched at the Kewalo Basin Research Facility of the National Marine Fisheries Service, Honolulu, and reared for up to 538 d in cylindrical fiberglass tanks. Fish were reared at room temperature (22 to 24°C), with an approximate 12 h photoperiod, bidaily water changes, and fed ground trout chow twice daily. Specimens remaining after 538 d were subsequently transferred to wet room facilities at the Department of Zoology, University of Hawaii, and held under similar conditions until sacrifice at 1517 d.

Sampled *P. conchonius* individuals were transported live to the laboratory, anesthetized by ice-bath immersion, weighed to the nearest 0.01 g, and standard length measured to the nearest 0.05 mm using calipers. Specimens were labeled, wrapped in aluminum foil, frozen in liquid nitrogen, and either stored in liquid nitrogen or at -80°C until dissection. Specimens were dissected while still in semi-frozen condition. Skull tops were removed and the region surrounding the brain thoroughly rinsed with Ringer's saline solution to remove blood and lipid deposits. Brain tissues removed included the cerebral, inferior, optic, and cerebellar lobes with the medulla being severed posterior to the tenth cranial nerve. Cranial nerves were severed at their point of attachment to the brain. Hearts were excised and ventricle myocardial tissues separated and saved. Brain and myocardial tissues were briefly rinsed in Ringer's solution, placed in Eppendorf vials and immediately frozen in liquid nitrogen until analysis.

Sagittal otoliths from both species were removed from the otic cavity, cleaned of extraneous tissue, rinsed in distilled water, soaked in 95% ethanol, and stored dry. *O. mossambicus* otoliths were weighed to the nearest 0.001 mg using a Sartorius Model 4503 MP6 electronic microbalance, and otoliths from *P. conchonius* were weighed to the
nearest 0.0001 mg using a Cahn Model C-32 electronic microbalance. Otolith lengths and widths for both species were measured to the nearest 0.01 mm using a stereo dissecting microscope with an ocular micrometer.

**FAP extraction and spectrofluorometric assay**

Soluble FAP materials from brain and myocardial tissues were quantified following the modified extraction and assay protocols outlined by Hill & Womersley (1991). Tissues were lyophilized and their dry weight measured to the nearest 0.1 μg on a Cahn Model C-32 electronic microbalance. Tissues were macerated at room temperature for 4 min in a Potter-Elvehjem glass tissue homogenizer using at least 1 ml of spectroscopic grade chloroform (CHCl₃) per 10 mg dry tissue. The initial meniscus was marked and the volume reconstituted after homogenization. Homogenates were transferred to glass centrifuge tubes and vortex-mixed for 2 min with an equal volume of a solution containing 100 mM magnesium chloride in glass distilled water and spectroscopic grade methanol at a ratio of 3:1. Samples were centrifuged at 0-5°C for 10 min at RCF = 3000 x g. Nonpolar and polar fractions were immediately removed and assayed for FAP content. Sample extractions were performed singly and rapidly to prevent in vitro production of FAP-like fluorophores in the sample solvent (Hill & Womersley, 1991).

Extract solvents were placed in quartz-silicate microcuvettes with a 4 mm pathlength and sealed with a teflon stopper to prevent evaporation. Temperature of the sample solvent was maintained at 18°C using a thermostatically controlled turret microcell holder connected to a Brinkmann Lauda Model RM6 refrigerated circulating water bath. A Perkin Elmer LS-5B luminescence spectrometer, Acer computer, and Perkin Elmer CFS PC-control software (version 3.0) were used for the fluorometric assay. The LS-5B luminescence spectrometer was equipped with a Xenon light source and a Hamamatsu Type R928 red-sensitive photomultiplier tube to reduce possible bias in the 500-700 nm region (Eldred et al., 1982). Excitation and emission slits were set at 5 and 20 nm,
respectively. Fluorescence spectra reported herein were corrected for the spectral emission characteristics of the source lamp, the spectral efficiencies of the excitation and emission monochromators, optics, and the photomultiplier tube using the method of Rhys-Williams et al. (1983) and the emission correction routine in the CFS PC-control software. All sample readings were expressed as a percentage of Quinine sulfate (0.1 mg/l 1N sulfuric acid) standard luminescence at an excitation setting of 345 nm and an emission setting of 450 nm. Sample FAP concentrations were expressed as whole-organ %fluorescence (%FL), where:

\[
\text{%FL} = \frac{\text{sample luminescence}}{\text{standard luminescence}} \times \text{solvent volume (ml)} \times 100
\]

Only brain tissues were available for *O. mossambicus*, and only their chloroform-soluble (nonpolar) FAP was quantified due to high levels of interfering flavin compounds in the methanol/water (polar) fraction (Hill & Womersley, 1991).

Preliminary assays of *P. conchonius* brain and heart tissues revealed large quantities of FAP in the polar extract fraction. Excitation scans (250 to 500 nm) at an emission setting of 520 nm (Fletcher et al., 1973) revealed low quantities of flavin compounds in the polar fraction which, in most cases, contributed less than 5% of the total luminescence at the FAP maxima settings. A simple algorithm was applied to polar fraction FAP data to subtract the luminescence contributed by flavin compounds. Flavin luminescence contribution at the FAP emission maxima was approximated and corrected by; 1) assuming that the flavin luminescence spectrum is symmetrical in form, 2) measuring luminescence at a point equidistant from the wavelength difference between the FAP emission maximum and the flavin emission maximum (520 nm), and 3) subtraction of this luminescence from the FAP raw luminescence value. For example, if the FAP emission maximum was at 425 nm, then luminescence was also measured at 615 nm (= 520-425+520 nm) and subtracted from the FAP raw luminescence at 425 nm.

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Multiple regression analysis

Multiple regression models for fish, otolith, and FAP measurements were fitted to the following form:

\[ \log_{10} (\text{Age}) = a + b_1X_1 + b_2X_2 + b_3X_3 + \ldots + b_nX_n \]

where age (days) is known, \( a \) is the intercept, \( b_i = \) regression coefficients, \( X_i = \log_{10} \) independent variables. Regressions were fitted in a stepwise manner with the inclusion level for all variables set at \( P = 0.05 \). Independent variables included fish size [fish weight (W) and standard length (SL)], otolith size [otolith weight (OWt), otolith length (OL), and otolith width (OW)], brain and heart dry weight (BW and HW) and FAP content (%FL). All variables were log transformed to meet the assumptions of normality and homogeneous variances. Four model types were developed in order to compare the usefulness of information available from combinations of fish size, otolith size, and FAP data. These models were developed for males and females separately, with data from immature specimens included in both.

Results

Somatic and otolith growth

Somatic growth of *O. mossambicus* differed by sex, with males reaching a mean standard length (SL) of 246.3 ± 15.3 mm and a mean weight (W) of 462.6 ± 101.9 g at an age of 886 d (Fig. 3-1a,b). The largest male weighed 576.97 g with a SL of 264 mm. Females reached a mean 185 ± 10.8 mm SL and 212.1 ± 44.3 g W by 886 d, with the largest specimen being 253.85 g at a SL of 194 mm. Quadratic growth models determined by regression and some associated statistics are provided in Fig. 3-1. Growth in SL was asymptotic in nature up to 886 d (Fig. 3-1a) for both males and females, whereas the growth rate of W continued to increase with age (Fig. 3-1b).
O. mossambicus males had larger otoliths than females (Fig. 3-1c). The best quadratic models describing the relationship of otolith weight (OWt) to age for both males and females are shown in Fig. 3-1c. The rate of growth in otolith weight did not decrease significantly during this experiment. At 886 d, males had a mean OWt of 49.149 ± 1.299 mg and females had a mean OWt of 33.955 ± 3.661 mg.

Somatic growth in P. conchonius differed little between sexes for the first 538 d of age (Fig. 3-2a,b). Logarithmic models described the relationship between SL and age reasonably well up to 538 d. Growth was asymptotic in nature for both males and females (Fig. 3-2a). Growth in W, also modeled using logarithmic equations (Fig. 3-2b), was most rapid between 60 and 300 d, leveling off between 300 and 538 d. Individuals held until 1517 d continued to grow, with females attaining a mean W of 16.59 ± 3.56 g (max. = 18.86 g) and males having mean W of 7.43 ± 1.93 g (max. = 9.05 g). Otolith growth (OWt) in P. conchonius was well described by logarithmic regressions for both males and females and did not vary greatly between the sexes (Fig. 3-2c).

Organ growth and FAP accumulation with age

Spectral characteristics of nonpolar FAP extracted from O. mossambicus brain tissues have been previously reported (Hill & Womersley, 1991). Brain growth and whole-brain FAP (%FL) has also been previously described (Hill & Womersley, 1991). Regression models describing brain growth and nonpolar FAP accumulation (%FL) with age are presented again for comparative purposes (Fig. 3-3). In brief, whole brain nonpolar FAP (%FL) accumulated gradually during the first 370 d, after which it accumulated more rapidly, with males (mean %FL = 140.6 ± 37.3) having almost twice as much FAP as females (mean %FL of 74.3 ± 8.51) by 886 d (Fig. 3-3b).

Characteristics of corrected emission (EM) spectra of FAP extracted from P. conchonius brain and heart tissues are presented in Fig. 3-4. Brain tissues had far greater
proportions of polar to nonpolar FAP than did heart tissues. Polar FAP extracted from both tissue types had similar corrected emission maxima, which ranged from 415 to 420 nm when excited (EX) at 340 nm wavelength. There was no obvious evidence of fluorescence from interfering compounds (e.g., retinols and flavins), however, excitation scans of polar extracts at EM = 520 revealed minor levels of flavin fluorescence, and steps were taken to account for this contribution to the total fluorescence at the EX and EM maxima of the FAP (see Methods section).

Brain growth and FAP accumulation with age in *P. conchonius* were expressed with logarithmic models (Fig. 3-5). Brain growth was most rapid during the first year of life, and was similar for males and females (Fig. 3-5a). At 1517 d, males had a mean brain dry weight (BW) of $9.730 \pm 1.259$ mg, and females had a mean BW of $8.236 \pm 2.050$ mg.

Soluble brain FAP in *P. conchonius* was detected in both the nonpolar and polar solvent fractions; levels were up to 100-fold greater in the polar fraction (Fig. 3-5b,c). Nonpolar and polar FAP were detected in immature fish sampled at 60 d and gradually increased in quantity with age in both sexes. The relationship of brain nonpolar FAP to age, modeled up to 538 d by logarithmic regression, was variable for both males ($r^2 = 0.615, P < 0.0001$) and females ($r^2 = 0.682, P < 0.0001$) at each age, and the rate of accumulation declined with increasing age (Fig. 3-5b). The highest mean values of nonpolar FAP were present in the oldest individuals sampled. Brain polar FAP was also present in large quantity in brain tissues at 60 d, and they slowly increased with time. Regression models were highly variable for both males ($r^2 = 0.312, P < 0.0001$) and females ($r^2 = 0.405, P < 0.0001$).

Relationships of heart weight and FAP content to age in *P. conchonius* were modeled using logarithmic regression (Fig. 3-6). Heart weight increased continuously but became more variable with increasing age (Fig. 3-6a).
FAP extracted from the ventricle myocardium was also more soluble in polar than nonpolar solvents (Fig. 3-6b,c), but the difference was not as great as was found for brain tissues. Both nonpolar and polar FAP were detectable in heart tissues from the youngest individuals (60d) which had an average ventricle dry weight of 0.147 mg. Nonpolar heart FAP increased linearly with increasing age (Fig. 3-6b), but with considerable variability in the data (male $r^2 = 0.605$, $P < 0.0001$; female $r^2 = 0.517$, $P < 0.0001$). Similar trends were apparent for polar heart FAP (Fig. 3-6c), which was approximately four times as abundant as nonpolar FAP extracted from the same tissues.

**Multiple regression analyses**

*Oreochromis mossambicus*

Multiple linear regression models using various combinations of independent variables from fish size, FAP, and otolith morphometric data provided strong predictions of age for *O. mossambicus* (Table 3-1). Four model types were developed in order to compare the relative usefulness of information available from fish size, FAP, and otolith data. For the first type considered, data included fish size (SL and W) and otolith morphometrics; for the second type, fish size and organ weight and FAP data were considered; for the third type, all variables except FAP were considered; for the fourth type, all these independent variables were considered.

Stepwise regression analysis of fish size and otolith data (organ size and FAP data excluded) demonstrated that fish weight and the three otolith measurements (OWt, OL, and OW) were the most important variables in predicting age for both males and females (Table 3-1). All of the correlations were highly significant, and the included variables explained 97.99% of the variation in age for males and 97.20% for females, as measured by the adjusted $r^2$ values.

Stepwise regression analysis of fish size, organ size and FAP data (otolith data excluded) demonstrated that fish weight, brain dry weight, and nonpolar FAP (%FL)
were the best predictors of age for both sexes (Table 3-1). Again, coefficients for each variable were highly significant, and coefficients of determination were only slightly higher for both males ($r^2 = 0.9820$) and females ($r^2 = 0.9830$) than in the model that excluded brain and FAP information. Thus, in the absence of information on otolith size, a model including FAP content provided more information on age than did body size alone.

When all independent variables except FAP were considered, it was determined that fish weight, brain weight, otolith weight and otolith length were the best predictor variables for males ($r^2 = 0.9815$), and that brain weight, otolith weight and otolith length were best for females ($r^2 = 0.9798$). The fit of these models was slightly poorer than those including somatic and FAP data, and slightly better than those including only somatic and otolith data.

When information from all independent variables was included in the stepwise multiple regression analysis, it was determined that brain weight, nonpolar FAP (%FL), and otolith weight were the best variables for predicting age of males, and that these same variables along with otolith length were best for age prediction in females. Coefficients of determination were marginally highest for these models, explaining 98.43% of the variation in age for males and 98.60% for females.

**Puntius conchonius**

The four multiple regression model types developed for *P. conchonius* were similar to those described for *O. mossambicus*, with the addition of information on heart weight as well as polar and nonpolar FAP (%FL) data from both brain and heart. As for *O. mossambicus*, fish size, otolith morphometrics, organ weight and FAP data all proved useful to various degrees for the prediction of chronological age (Table 3-2).

Through stepwise regression analysis of fish size and otolith data (minus organ and FAP data) it was determined that fish weight and otolith weight were the most important
variables for age prediction in male P. conchonius, as were fish standard length, otolith weight, and otolith width for females (Table 3-2). These variables explained 95.35% of the variation of age for males, and 97.12% for females, and the coefficients of all the variables were significant (Table 3-2).

Stepwise multiple regression of fish size, organ weight, and FAP data revealed that brain weight and heart nonpolar and polar FAP (%FL) were the best predictors of age in males ($r^2 = 0.9406$) and that fish weight, brain weight, and brain and heart nonpolar FAP (%FL) were the best variables for females ($r^2 = 0.9402$) (Table 3-2). Coefficients of determination were slightly less than for the fish size/otolith size model, however information on FAP content strengthened the prediction of age over that of somatic variables alone.

When all independent variables except FAP were considered, brain weight, heart weight, and otolith weight were the best predictor variables for males ($r^2 = 0.9604$), and fish standard length, otolith weight, and otolith width were best for females ($r^2 = 0.9712$). These models provided equal or better age prediction than those including somatic and otolith data or somatic and FAP data.

As was the case for O. mossambicus, the strongest models for age prediction in P. conchonius were the ones including some information from each of the data types (Table 3-2). Stepwise regression analysis of all independent variables determined that brain weight, heart nonpolar and polar FAP, and otolith weight were the most important variables for age prediction in males ($r^2 = 0.9633$). For females, the best variables for age prediction included fish standard length, heart polar FAP, otolith weight, and otolith width ($r^2 = 0.9725$).
Discussion

Results from this study provide additional evidence that FAP accumulates in brain and heart tissues of fish over time and, more importantly, that information on soluble-FAP content can, at the very least, strengthen the prediction of chronological age. This result is particularly relevant to researchers wishing to apply an age pigment technique, either histological or biochemical, as an alternative or supplement to standard methods of age determination. Patterns of FAP accumulation reported in this research clearly show that rates of FAP genesis can be both species- and tissue-specific. Nonpolar FAP fractions extracted from brain tissue of *Q. mossambicus* and *P. conchonius* increased with age, but the rate of FAP genesis apparently began to slow fairly early in life in *P. conchonius*. The rate of accumulation was increasingly rapid to the maximum age sampled in *Q. mossambicus*. FAP accumulation differed in pattern between brain and heart tissues of *P. conchonius*, with the rate of accumulation of heart FAP remaining more constant with age. Within a given tissue type, considerable similarity was evident in the pattern of accumulation of nonpolar and polar FAP in *P. conchonius*, despite vast differences in the relative solubilities. Results of this nature emphasize the importance of examining more than one type of tissue when initiating studies on FAP accumulation for age prediction.

The decline in rate of accumulation of FAP observed in brains of *P. conchonius* with age appears inconsistent with the assumption that age pigments accumulate in a linear fashion throughout an animals' lifespan (Strehler et al., 1959); however, the assumption of linear age pigment accumulation may not be entirely valid if ontogenetic changes in metabolic rate are involved. While information on such changes is presently unavailable for *P. conchonius*, studies on other fish species have demonstrated age-related declines in weight-specific oxygen consumption, a trend related to the decreased ratio of area to volume at greater age (De Silva et al., 1986; Oikawa et al., 1991). In one of the few
studies on FAP accumulation in aquatic species, Vernet et al. (1988) also reported a leveling-off in the apparent rate of formation of FAP in brain tissue of Dover sole (Microstomus pacificus). Miquel et al. (1978) reported a similar decline in FAP extracted from mouse testes.

A surprising result of the present study was the extreme ratio (ca. 100:1) of polar to nonpolar FAP extracted from P. conchonius brain. This is much higher than the ratios reported in brain tissues of either Oreochromis aureus (Hammer & Madhusudhana Rao, 1987) or O. mossambicus (Hill & Womersley, 1991). Polar FAP was also higher in P. conchonius heart extracts, but only by a ratio of ca. 4:1. We were only able to provide these data for P. conchonius because of relatively low levels of flavins in tissue samples. Such was not the case for O. mossambicus, where high levels of contaminating flavin compounds are present in brain tissue (Hill & Womersley, 1991). Clearly, flavin contamination prevents useful measurement of polar FAP in some cases, but where it is not masked by such interference, the polar fraction should not be ignored.

The extraction of age-related FAP which is more polar in solubility is not new (Desai et al., 1975; Taubold et al., 1976; Klass 1977; Davis et al., 1982), but the subject involves some controversy. For example, Sheehy & Roberts (1991), working exclusively with fluorescent compounds extracted from insect tissues, asserted that most of the nonpolar fluorescence measured in other studies is in fact due to polar pteridine contamination and not to the presence of FAP. This does not appear to be the case for P. conchonius, however, because the ratios of polar:nonpolar fluorescence were so vastly different between tissue types. If nonpolar fluorescence were merely an artifact of polar contaminants, consistent ratios between fraction quantities would be expected. Sheehy & Roberts (1991) also assumed that all lipofuscin compounds are lipid in nature and that FAP should therefore be soluble only in nonpolar solvents; however, this is not the case. Detailed studies on the biochemical composition of isolated lipofuscin and ceroid granules...
have revealed that the relative protein and lipid composition of these residues can vary greatly, with protein fragments comprising up to 70% of the total mass (Porta, 1991). Furthermore, most investigators using malondialdehyde (a breakdown product of lipid peroxidation) to synthesize crosslinked products with FAP-like fluorescence, have done so in polar solvents using amino acids and proteins as substrates (Chio & Tappel, 1969; Shimasaki et al., 1982; Kikugawa & Ido, 1984). Thus, it is not surprising that much of the FAP extracted from *P. conchonius* brain and heart, and the tissues of other animals, (excluding insects) is polar in solubility.

The application of multiple linear regression analysis for chronological age prediction is a recent development in fisheries biology (Boehlert 1985). This statistical methodology appears to be effective for examining the utility of FAP for age assessment. Previous studies have included variables obtained from measurement of otoliths (Boehlert, 1985) or a combination of somatic and otolith data (Radtke et al., 1989; Radtke 1990; Beckman et al., 1991). Our multiple regression analyses show improvement in the prediction of chronological age in both *O. mossambicus* and *P. conchonius* when FAP data are added. This is the first time that such a utility has been demonstrated. FAP variables entered all fitted models in the analysis when included in the initial variable list, however the fits (as measured by $r^2$) were never more than slightly better than models which included only size, otolith, and organ weight variables.

The strongest model for *O. mossambicus* males and females was that which incorporated all variables, with brain dry weight, brain nonpolar FAP (%FL), and otolith weight (plus otolith length for females) being most important. When otolith information was arbitrarily excluded from male and female models, brain dry weight and FAP (%FL) remained in the model with the addition of fish weight, to produce an age prediction model with only slightly smaller coefficients of determination. The model with the smallest coefficients of determination was the one that included only somatic and otolith
data. In the absence of FAP information, fish weight and otolith weight and dimensions were the most important age prediction variables, with fish standard length consistently rejected from the model. Thus, for O. mossambicus, it would appear that FAP information combined with fish and brain weight can provide a more reliable prediction of age than otolith and somatic data alone, but that brain FAP and otolith weight data are the best overall predictor variables.

The best age prediction models developed for P. conchonius also included FAP data. The most important variables for age prediction in males were heart nonpolar and polar FAP, brain weight, and otolith weight. Fish standard length, heart polar FAP, and otolith weight and width were best for predicting female age. However, unlike O. mossambicus, the model including only somatic and FAP data did not provide as strong a prediction of age as the model including only somatic and otolith data. Nevertheless, the model excluding otolith variables presented strong evidence that FAP information can provide a more reliable prediction of age than somatic data alone. This was certainly the case for P. conchonius males in which only brain weight, and heart nonpolar and polar FAP data were included in the best predictive model.

From the above, it is clear that whole-organ FAP data can increase the accuracy of age prediction in multivariate analyses of carefully standardized specimens. The use of two teleost species, with calcified structures traditionally used for aging, to demonstrate the importance of FAP was an important aspect of the experimental design. This allowed us to assess by comparison the importance of FAP in relation to calcified structures. Thus, it was of particular interest when a combination of otolith and FAP information provided the best predictive model. This held true even when the patterns of FAP accumulation were highly variable, as was the case for P. conchonius brains and hearts.

The retention of variables such as fish weight, otolith weight, organ weight, and some FAP variables in the multiple regression models was probably due to their more
consistent patterns of increase with age as compared to some other variables. For example, fish standard length was consistently excluded from multiple regression models for *O. mossambicus*, whereas fish weight and otolith weight were, in most cases, included in the models. Weight increases with age were consistent and fairly rapid for *O. mossambicus* males and females, with sizes-at-age comparable to those reported by Hodgkiss & Man (1977) for this species. The continuous pattern of growth in somatic and otolith weight should not be surprising. *O. mossambicus* in this study were sampled through less than half of their lifespan (Bruton & Allanson, 1974), and animals had only reached 20% of their potential maximum weight (2953 g; Jubb, 1967).

Many animals of interest do not contain calcified skeletal structures suitable for aging. Thus, it is encouraging that results of our models that excluded otolith data demonstrated that FAP data can at least supplement, if not replace, somatic data for predicting age. This analytical tool is especially pertinent to the problem of aging commercially important fisheries species (e.g.- hagfish, lobster, shrimp, and squid) for which tagging and size-based methods are currently the only alternatives.

With regard to field populations, further questions obviously must be addressed before FAP assays or histological lipofuscin methods (Sheehy 1990b) can be used as tools for age estimation. Age-related pigments (FAP and lipofuscin) are thought to form as products of physiological aging processes related to metabolic rate. The rates of these processes can vary greatly in poikilothermic animals, and little is known of the possible effects of temperature or other environmental variables on the aging process and thus on the rate of FAP genesis. Hammer (1988) tested the effect of temperature on FAP accumulation in pike (*Esox lucius*) larvae, but these results have been questioned due to the use of formalin for specimen preservation. Sheehy's (1990a) histological study of age pigment accumulation in crayfish (*Cherax cuspidatus*) brain demonstrated a direct effect of temperature on lipofuscin volume fraction in the olfactory lobe region. Studies
of temperature effects on "FAP" accumulation have been conducted on insects (Ragland & Sohal, 1975; Sohal et al., 1981; McArthur & Sohal, 1982), but the fluorescence extracted from insects is now suspected to be pteridine in origin (Lehane & Mail, 1985; Sheehy & Roberts, 1991).

Application of FAP content as an aging tool for field populations will ultimately be contingent on obtaining baseline information on age, growth, and accumulation patterns, together with concurrent data on environmental and physiological variables that affect the rate of FAP accumulation. The animals used for the present study were reared under strictly controlled conditions in the laboratory. The resulting data represent a near optimum which is probably unattainable with wild specimens. Sources of FAP variability must be determined and taken into account before multiple regression models can be used for age prediction in wild stocks. A combination of mark-recapture and laboratory growth studies will probably be required over relevant portions of the animal's lifespan.
Literature cited


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Table 3-1. - Coefficients and associated statistics from multiple regressions of multivariate linear models of age (log₁₀) for *Oreochromis mossambicus*. Models were fitted in a stepwise manner using combinations of the independent variables including fish size and otolith data, fish size, organ size, FAP data, and all variables combined. Data from immature specimens were included in both male and female analyses. All variables were log₁₀ transformed for analyses. Intercepts, coefficients and their standard errors (SE) are for log transformed data.

<table>
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<tr>
<th>Independent Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
<th>Model Adj. r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n = 92)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fish size model)</td>
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<td></td>
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<tr>
<td>Intercept (a) = 1.729</td>
<td></td>
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<td></td>
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<tr>
<td>Fish weight</td>
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<tr>
<td>Intercept (a) = 2.153</td>
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<td></td>
<td></td>
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<tr>
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<tr>
<td>Intercept (a) = 2.062</td>
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Table 3-1. (Continued) - Coefficients and associated statistics from multiple regressions of multivariate linear models of age (log10) for Oreochromis mossambicus. Models were fitted in a stepwise manner using combinations of the independent variables including fish size and otolith data, fish size, organ size, FAP data, and all variables combined. Data from immature specimens were included in both male and female analyses. All variables were log10 transformed for analyses. Intercepts, coefficients and their standard errors (SE) are for log transformed data.

<table>
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<tr>
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Table 3-2. Coefficients and associated statistics from multiple regressions of multivariate linear models of age (log10) for *Puntius conchonius*. Models were fitted in a stepwise manner using combinations of the independent variables including fish size and otolith data, fish size, organ size, FAP data, and all variables combined. Data from immature specimens were included in both male and female analyses. Animals sampled at 1517 d were excluded from the analysis. All variables were log10 transformed for analyses. Intercepts, coefficients and their standard errors (SE) are for log transformed data.

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Table 3-2. (Continued) - Coefficients and associated statistics from multiple regressions of multivariate linear models of age ($\log_{10}$) for *Puntius conchonius*. Models were fitted in a stepwise manner using combinations of the independent variables including fish size and otolith data, fish size, organ size, FAP data, and all variables combined. Data from immature specimens were included in both male and female analyses. Animals sampled at 1517 d were excluded from the analysis. All variables were log$_{10}$ transformed for analyses. Intercepts, coefficients and their standard errors (SE) are for log transformed data.

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Figure 3-1. *Oreochromis mossambicus*. Body size and otolith weight as functions of chronological age (Days). Quadratic growth regressions for immature + males (n = 101) and immature + females (n = 99) all have P < 0.0001. (A) Standard length (SL) as a function of age for immature + males: $SL = 3.3813 + 0.51878(Days) - 2.8061*10^{-4}(Days^2), r^2 = 0.976$; and for immature + females: $SL = 8.0858 + 0.45049(Days) - 2.8413*10^{-4}(Days^2), r^2 = 0.983$. (B) Fish wet weight (W) as a function of age for immature + males: $W = -15.692 + 0.21892(Days) + 3.8805*10^{-4}(Days^2), r^2 = 0.929$; and for immature + females: $W = -17.042 + 0.26895(Days) + 3.2033*10^{-5}(Days^2), r^2 = 0.940$. (C) Otolith weight (OWt) as a function of age for immature + males: $OWt = -2.5115 + 4.997*10^{-2}(Days) + 9.6809*10^{-6}(Days^2), r^2 = 0.950$; and for immature + females: $OWt = -2.1908 + 4.7062*10^{-2}(Days) - 5.3785*10^{-6}(Days^2), r^2 = 0.973$. 

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Figure 3-2. *Puntius conchonius*. Body size and otolith weight as functions of chronological age (Days). Logarithmic growth regressions for immature + males (n = 90) and immature + females (n = 89) all have P < 0.0001. Animals sampled at 1517 d were excluded from the regression analyses. (A) Standard length (SL) as a function of age for immature + males: SL = -23.418 + 25.536 * log₁₀(Days), r² = 0.905; and for immature + females: SL = -26.240 + 26.997 * log₁₀(Days), r² = 0.906. (B) Fish wet weight (W) as a function of age for immature + males: W = -5.6956 + 3.3363 * log₁₀(Days), r² = 0.785; and for immature + females: W = -7.0110 + 4.0120 * log₁₀(Days), r² = 0.828. (C) Otolith weight (OWt) as a function of age for immature + males: OWt = -0.70798 + 0.41255 * log₁₀(Days), r² = 0.898; and for immature + females: OWt = -0.75479 + 0.43729 * log₁₀(Days), r² = 0.908.
Figure 3-3. *Oreochromis mossambicus.* Brain weight and fluorescent age pigment (FAP) level as functions of chronological age (Days). Logarithmic and quadratic regressions for immature + males (n = 101) and immature + females (n = 99) all have P < 0.0001. (A) Brain dry weight (BW) as a function of age for immature + males: BW = -36.488 + 23.333 * log_{10}(Days), r^2 = 0.943; and for immature + females: BW = -32.101 + 21.067 * log_{10}(Days), r^2 = 0.914. (B) Brain nonpolar FAP (%FL) as a function of age for immature + males: %FL = 7.4078 - 4.5872*10^{-2}(Days) + 2.3313*10^{-4}(Days)^2, r^2 = 0.926; and for immature + females: %FL = 6.3477 - 1.4104*10^{-2}(Days) + 1.0736*10^{-4}(Days)^2, r^2 = 0.936.
Figure 3-4. *Puntius conchonius*. Corrected emission spectra for soluble fluorescent age pigments (FAP) extracted from brain and heart tissues. (A) Brain polar and nonpolar FAP corrected emission maxima were 415 and 420 nm when excited at 340 and 345 nm, respectively. (B) Heart polar and nonpolar FAP corrected emission maxima were 415 and 425 nm when excited at 340 and 345 nm, respectively.
Brain FAP

Heart FAP

Emission wavelength (nm)

Luminescence (arbitrary units)
Figure 3-5. *Puntius conchonius*. Brain weight and fluorescent age pigment (FAP) level as functions of chronological age (Days). Logarithmic regressions for immature + males (n = 90) and immature + females (n = 89) all have P < 0.0001. Animals sampled at 1517 d were excluded from the regression analyses. (A) Brain dry weight (BW) as a function of age for immature + males: BW = -10.337 + 6.2202 * log_{10}(Days), r^2 = 0.919; and for immature + females: BW = -9.3086 + 5.6154 * log_{10}(Days), r^2 = 0.926. (B) Brain nonpolar FAP (%FL) as a function of age for immature + males: %FL = -4.5132 + 3.8772 * log_{10}(Days), r^2 = 0.615; and for immature + females: %FL = -5.6471 + 4.3583 * log_{10}(Days), r^2 = 0.682. (C) Brain-polar FAP (%FL) as a function of age for immature + males: %FL = -234.80 + 200.43 * log_{10}(Days), r^2 = 0.312; and for immature + females: %FL = -348.42 + 258.05 * log_{10}(Days), r^2 = 0.405.
Figure 3-6. *Puntius conchonius*. Heart weight and fluorescent age pigment (FAP) level as functions of chronological age (Days). Linear and logarithmic regressions for immature + males (n = 90) and immature + females (n = 89) all have P < 0.0001. Animals sampled at 1517 d were excluded from the regression analyses. (A) Heart dry weight (HW) as a function of age for immature + males: HW = -1.6957 + 1.0180 * log10(Days), r² = 0.574; and for immature + females: HW = -1.2374 + 0.77254 * log10(Days), r² = 0.566. (B) Heart nonpolar FAP (%FL) as a function of age for immature + males: %FL = -1.0720*10⁻² + 9.2545*10⁻³ (Days), r² = 0.605; and for immature + females: %FL = 0.16355 + 7.8694*10⁻³ (Days), r² = 0.517. (C) Heart-polar FAP (%FL) as a function of age for immature + males: %FL = 4.0984 + 1.9406*10⁻² (Days), r² = 0.313; and for immature + females: %FL = 4.1528 + 1.6479*10⁻² (Days), r² = 0.368.
A

Heart dry weight (mg)

- Immature
- Male
- Female

B

Heart nonpolar FAP (%FL)

C

Heart polar FAP (%FL)

Age (days)

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CHAPTER 4

INTERACTIVE EFFECTS OF SOME ENVIRONMENTAL AND PHYSIOLOGICAL VARIABLES ON FLUORESCENT AGE PIGMENT ACCUMULATION IN BRAIN AND HEART TISSUES OF AN AQUATIC POIKILOTHERM

Introduction

The accumulation of age pigment complexes related to physiological aging is considered one of the most consistent events in all senescing, nonmitotic cells (see reviews by Sohal and Wolfe 1986, Tsuchida et al. 1987, Hammer and Braum 1988, Porta 1991). Age pigments have been variously classified as pre-ceroid, ceroid, lipofuscin, and fluorescent age pigment, depending upon their physical and chemical properties determined by either histologic or biochemical methods. The age-related accumulation of these pigments has been of interest to gerontologists who hope to define biochemical aspects of aging and ultimately slow or arrest this process. More recently, age pigment studies have been initiated by field biologists wishing to develop predictive models, based on pigment content, for the estimation of chronological age for population studies (Ettershank 1983, 1984 a,b, 1985; Brown and George 1985, Hirche and Anger 1987, Hill and Radtke 1988, Hammer 1988, Mullin and Brooks 1988, Vernet et al. 1988, Berman et al. 1989, Clarke et al. 1990, Sheehy 1990a, Hill and Womersley 1991, Nicol et al. 1991, Hill and Womersley, in review).

The majority of these field studies have examined soluble fluorescent age pigments (FAP) using the extraction methods and spectrofluorometric assay described by Fletcher et al. (1973). This method of age pigment quantification has recently been criticized because of discrepancies between fluorescent emission properties of solvent-extracted pigments (blue emission at 400 to 490 nm; Siakotos and Koppang 1973, Tappel 1975) and those observed in situ using fluorescence microscopy (green to yellow...
emission at 500 to 630 nm; Eldred et al. 1982, Eldred and Katz 1989). This issue has been partly resolved by Yin and Brunk (1991), who provided strong evidence for concentration-dependent metachromatic shifts in fluorophore emission from yellow at high density to blue under diluted conditions. In addition to this controversy, Nicol (1987) and Hill and Womersley (1991) defined flaws in the sample handling procedures that most likely affected variability of FAP results in many of the early field studies. In spite of these problems, FAP has been shown to accumulate over time in nonmitotic tissues and organs of numerous aquatic poikilotherms (Luo and Hultin 1986, Mullin and Brooks 1988, Vernet et al. 1988, Hill and Womersley 1991, Nicol et al. 1991, Hill and Womersley, in review) as well as mammalian species traditionally studied in gerontological research (Tappel et al. 1973, Shimasaki et al. 1977, Miquel et al. 1978, Shimasaki et al. 1980).

It is of primary importance to both gerontologists and field fish biologists that the factors that lead to age pigment genesis and variability be defined. Biochemical events involved with age pigment formation are considered to be stochastic in nature, determined in part by rates of cellular metabolism and the generation of superoxide and hydroxyl radicals in organelles and the cytoplasm (Harman 1984). Free radicals, in the presence of metals and divalent oxygen, can initiate a cascade of lipid peroxidation events leading to malondialdehyde and other carbonyl cross-linking reactants (Gutteridge 1987). Amine-containing biomolecules (amino and nucleic acids, proteins, phospholipids, etc.) that have been cross-linked become deactivated and eventually digested in the lysosomal recycling system (Harman 1989). Cross-linked portions that are nondigestable remain in the cytoplasm as age pigment residues and have autofluorescent properties. Thus, there are numerous critical factors that may determine the rate of age pigment genesis at the cellular level, including: 1) the rate of free radical production through oxidative metabolism (Fridovich 1976, Armstrong 1984, Freeman 1984), 2) the efficiency of
antioxidant mechanisms responsible for free radical control (Sohal et al. 1990, Cutler 1991), 3) the susceptibility of cellular lipid components to free radical-mediated oxidation (Packer et al. 1967, Tappel 1975, Mead 1976), 4) presence of catalytic factors such as oxygen (Sohal and Brunk 1989, Sohal et al. 1989) and metals (Aloj Totaro et al. 1985, Gutteridge 1985), and 5) the efficiency of lysosomal digestive enzymes (Davies 1988, Marzabadi et al. 1991).

Relatively few studies have examined the effects of metabolism on age pigment formation. Rearing *Drosophila melanogaster* at different temperatures, Sheldahl and Tappel (1974) first demonstrated a direct effect on the quantity of fluorescent products extracted from the flies. Other studies on FAP in insects followed, testing the effects of oxygen consumption (*D. melanogaster*, Miquel et al. 1976), physical activity level (*Musca domestica*, Sohal and Donato 1979, Sohal and Buchan 1981), and ambient temperature (*Oncopeltus fasciatus*, McArthur and Sohal 1982). Questions have now been raised as to whether the fluorescent products extracted in these studies represent FAP or pteridine metabolites that are ubiquitous in insects (Ziegler and Harmsen 1969, Lehane et al. 1986, Sheehy and Roberts 1991). Nonetheless, Sohal and Donato (1979) and Sohal (1981) confirmed the positive effect of activity level on lipofuscin accumulation in *M. domestica* using electron microscopy. Activity level has also been found to affect rates of age pigment accumulation in rats (Basson et al. 1982) and hamsters (Papafrangos and Lyman 1982). Most recently, Sheehy (1990b) reared crayfish (*Cherax cusicatus*) at three temperatures, finding greater quantities of lipofuscin in the olfactory lobe cell mass of animals reared at higher temperature.

The following investigation was conducted to address additional questions related to metabolic and growth-related effects on FAP genesis in nonmitotic tissues, using a vertebrate aquatic poikilotherm as the study organism. Our primary objectives were: 1) to determine the degree to which FAP content may vary within and between sibling and
non-sibling fish of identical age, and 2) to determine whether body weight, rearing temperature, ration level, photoperiod, or a combination of these factors, can influence FAP genesis, ultimately affecting the ability to predict chronological age.

Materials and methods

Model animal

We chose the tropical freshwater fish *Puntius conchonius* (Cyprinidae), the rosy barb, as our study organism. *P. conchonius* is small, easy to breed, and tolerant to a wide range of physiological temperatures (15 to 33° C), lending itself well to physiological studies requiring large numbers of individuals in limited laboratory space. Clutches used for the following experiments were obtained by breeding adult pairs in 19 l all-glass aquaria for 8 to 12 h at 25° C. Adults were removed immediately following the spawning event. Larvae hatched within 3 to 4 d after fertilization, and yolk sack absorption was complete after 3 to 4 d, at which time the larvae were fed a diet of 50-150 μm diameter Hatchfry Encapsulon (Argent Chemical Laboratories) for 4 d. Fry were subsequently fed a combination of larval *Artemia salina* and crushed flake food until transfer to experimental aquaria at approximately 45 d of age.

Aquarium system

The experimental system consisted of 18 118 l all-glass closed-system aquaria. Water quality was maintained using air-driven undergravel filters with 25% of the water exchanged biweekly. Dissolved oxygen and pH were monitored weekly and temperature was monitored daily. Aquaria were cooled to 19° C using a thermostatically controlled refrigerated water bath which circulated chilled water through coiled glass tubing in each aquarium. All 25° and 31° C aquaria were held at temperature using 100 W thermostatic heaters. In addition, all 25° C aquaria were equipped with glass coils cooled by tap water (22 to 23°C) to assure proper temperature on days when room temperature exceeded 25°
C. Photoperiod was controlled by enclosing each aquarium in heavy gauge black plastic sheeting to exclude external light. All aquaria were covered with full hoods equipped with 15 W fluorescent bulbs controlled by timer boxes.

**Experimental design**

Two independent experiments were conducted to assess the interactive effects of temperature, ration level, photoperiod, clutch, and individual size on FAP accumulation in brain and heart tissues of *P. conchonius*. In the first, temperature and ration level were varied while photoperiod was held constant. In the second experiment, temperature and photoperiod were varied while ration level was held constant. Possible interactions between photoperiod and ration level were not addressed in this study.

**Temperature/ration experiment:** This experiment was designed to test for interactive effects of temperature, ration level, clutch, and individual body size on variability of FAP. At an age of 45 d, three uniparental clutches of fry (referred to as C1, C2, and C3) were randomly divided into experimental aquaria at a stocking density of 24 fish per aquarium. Temperature levels were 19°C ± 0.2°C, 25°C ± 0.2°C, and 31°C ± 0.2°C. Within each temperature, fish were fed commercial flake food (Wardley's Basic Flake, The Wardley Corporation) either *ad libitum* twice daily (high ration, or Hi), or *ad libitum* once on alternating days (low ration, or Lo). This three-temperature, two-ration design was repeated for each of the three clutches, which were acquired from different parental sources. All aquaria were held at a constant 12 h light: 12 h dark photoperiod. Individuals were sacrificed for FAP measurement after being held under experimental conditions for 8 mo, at an age of 280 to 290 d. The effects of temperature and body size on metabolic rate were measured on individuals from each of the six treatments in this experiment. Methods for oxygen consumption measurement are described in a later subsection.
**Temperature/photoperiod experiment:** Interactive effects of temperature, photoperiod, clutch, and body size on FAP content were tested using a design similar to the temperature/ration experiment. Individuals were divided into aquaria at 45 d with 24 fish per aquarium. Treatments included three temperature levels (19, 25, and 31°C) and photoperiod was held at two levels within each temperature; 18 h light : 6 h dark (referred to as 18L), or 6 h light : 18 h dark (referred to as 6L). All treatments were fed high ration levels. Three uniparental clutches, referred to as C4, C5, and C6 to avoid confusion with the previous experiment, were reared under each of the six treatment conditions. Individuals were held under experimental conditions for 8 mo, and sacrificed at 280 to 290 d of age.

**Oxygen consumption**

Laboratory determinations of oxygen consumption were made on three randomly selected *P. conchonius* from each of the 6 temperature/ration treatments (C3 only) approximately one month prior to the end of the experiment. Respiration was determined by measuring changes in oxygen tension in the water medium using a polarographic electrode. The respiration chamber consisted of a one liter specimen flask connected in series to another one liter flask containing oxygen and temperature electrodes and a mixing bar driven by a magnetic stirrer. Oxygen tension was measured directly as mg O\textsubscript{2} per liter using an Omega Model PHH-71 dissolved oxygen/temperature probe. Water medium was recirculated through the two flasks in series using a motorized pump. The two flasks were held in a water bath whose temperature was maintained using a submerged glass coil connected to a thermostatically-controlled Lauda Model RM5 water circulator. Temperature was held at either 19, 25, or 31°C ± 0.2°C throughout each observation.

Triplicate oxygen consumption measurements were made for each individual, with measurements lasting between 20 and 40 min, depending upon the rate of
consumption. Oxygen measurements were only taken within the range of 80 to 100% of air saturation (Belman and Childress, 1973), and the medium was resaturated between observations using an air stone. New water medium, sterilized by UV irradiation, was used for each specimen, and background oxygen depletion due to microbial respiration was negligible. Observations were made between 0900 and 1500 hrs to account for daily changes in metabolic rhythms. Specimens were starved for at least 16 h prior to the observations to minimize specific dynamic action (Saunder, 1963). Fish wet weight and chamber water volume were measured at the end of the three replicate observations.

**Specimen/tissue preparation**

*Puntius conchonius* individuals were anesthetized by ice-bath immersion, and weighed to the nearest 0.01 g. Upon removal of cranial bones, the brain cavity was thoroughly rinsed with Ringer's saline solution to remove blood and lipid deposits. Brain tissues removed for FAP assay included the cerebral, inferior, optic, and cerebellar lobes, with the medulla being severed posterior to the tenth cranial nerve. Cranial nerves were severed at their point of attachment to the brain. Hearts were excised and ventricle myocardial tissues separated and saved for FAP analysis. Brain and myocardial tissues were briefly rinsed in Ringer's solution, placed in Eppendorf vials, and immediately frozen in liquid nitrogen until analysis.

**FAP extraction and assay**

Soluble FAP materials in tissues were quantified following the modified protocols outlined by Hill and Womersley (1991). Tissues were lyophilized and their dry weights measured to the nearest 0.0001 mg on a Cahn Model C-32 electronic microbalance. Tissues were macerated at room temperature for 4 min in a Potter-Elvehjem glass tissue homogenizer using at least 1 ml of spectroscopic grade chloroform (CHCl3) per 10 mg dry tissue. The initial meniscus was marked and the volume reconstituted after homogenization. Homogenates were transferred to glass centrifuge tubes and vortex
mixed for 2 min with an equal volume of a solution containing 100 mM magnesium chloride (MgCl) in glass distilled water and spectroscopic grade methanol at a ratio of 3:1. Samples were centrifuged at 0–5°C for 10 min at RCF = 3000 x g. Nonpolar and polar fractions were immediately removed and assayed for FAP content. Sample extractions were performed singly and rapidly to prevent in vitro production of FAP-like fluorophores in the sample solvent (Hill and Womersley, 1991).

Extract solvents were placed in quartz-silicate microcuvettes with a 4 mm pathlength and sealed with a teflon stopper to prevent evaporation. Temperature of the sample solvent was maintained at 18°C using a thermostatically controlled turret microcell holder connected to a Brinkmann Lauda Model RM6 refrigerated circulating water bath. A Perkin Elmer LS-5B luminescence spectrometer, Acer computer, and Perkin Elmer CFS PC-control software (version 3.0) were used for the fluorometric assay. The LS-5B luminescence spectrometer was equipped with a Xenon light source and a Hamamatsu Type R928 red-sensitive photomultiplier tube to reduce possible bias in the 500-700 nm region (Eldred et al., 1982). Excitation and emission slits were set at 5 and 20 nm, respectively. Fluorescence spectra reported herein were corrected for the spectral emission characteristics of the source lamp, the spectral efficiencies of the excitation and emission monochromators, optics, and the photomultiplier tube using the method of Rhys-Williams et al. (1983) and the emission correction routine in the CFS PC-control software. All sample readings were expressed as a percentage of Quinine sulfate (0.1 mg/l 1N sulfuric acid) standard luminescence at an excitation setting of 345 nm and an emission setting of 450 nm. Sample FAP concentrations were expressed as whole-organ %fluorescence (%FL), where:

\[
%FL = \frac{\text{sample luminescence}}{\text{standard luminescence}} \times \text{solvent volume (ml)} \times 100
\]
Statistical analysis

Unpaired two-tailed t-tests were applied to data within treatments to test for differences in FAP content between sexes. Preliminary examination of the data revealed a body weight effect on FAP content, thus it was necessary to standardize this effect by applying Analysis of Covariance (ANCOVA) to the data. ANCOVA was used, whenever possible, to evaluate the effects and interactions of temperature, ration level or photoperiod, and clutch on FAP extracted from brain and heart tissues, with body weight regressed as a covariate. Temperature, ration, photoperiod, and clutch were treated as class variables, with clutch being random. All FAP data were log-transformed to homogenize the variances. Computations were made using GLM procedures of the SAS statistical software (SAS, 1985).

Results

Temperature/ration experiment

Both temperature and ration level had considerable effects on body weight in this experiment by 280 to 290 d of age (Fig. 4-1a). At low ration level, mean (± 95% confidence limits) fish weight ranged from 0.79 ± 0.18 g (C1-31°-Lo) to 1.33 ± 0.15 g (C3-19°-Lo). High ration fish ranged in weight from 1.72 ± 0.19 g (C1-19°-Hi) to 3.97 ± 0.73 g (C2-31-Hi). Within temperatures, weight was greater in fish fed high rations, and this effect was accentuated with increasing temperature (Fig. 4-1a). Mean weight ratios between high and low ration treatments were approximately 2:1 at 19°C, 3:1 at 25°C, and 4:1 at 31°C. Thus, under low ration conditions, temperature had little or no effect on final size, whereas temperature had a positive effect on weight at high ration levels.

Polar and nonpolar FAP extracted from P. conchonius brain tissues had excitation and emission characteristics similar to those previously reported for this species
(Hill and Womersley, 1992). There were no apparent treatment effects on excitation and emission maxima or the overall shape of the spectra. FAP extracted from brain was much more polar in solubility with ratios of polar:nonpolar FAP varying greatly (ca. 6:1 to 50:1) within and between clutches and treatments (Fig. 4-2).

Brain polar FAP content varied greatly between all experimental treatments with mean %FL values ranging from $52.1 \pm 12.5$ (C1-31°-Hi) to $541.7 \pm 98.1$ (C2-19°-Lo) (Fig. 4-2a). The most notable trend was the inverse relationship between rearing temperature and polar FAP content. This trend held for all three clutches and for both ration levels. There was a tendency for low ration treatments to have higher polar FAP values than high ration treatments at both 19°C and 31°C in all three clutches (Fig. 4-2a). The above trends were not apparent for nonpolar FAP extracted from the same brain tissues (Fig. 4-2b). Brain nonpolar FAP, present in much lower quantity than polar FAP, varied between clutches both within and between treatments. Within clutches, nonpolar FAP tended to be higher in both 25°C treatments.

An attempt was made to apply ANCOVA models to the above brain FAP data. The data failed to meet one of the most basic assumptions of ANCOVA, which is a parallel relationship between the dependent variable (in this case, brain FAP) and the covariate (fish weight) under the different treatment conditions. Scatter plots of brain polar FAP as a function of fish weight revealed a negative relationship between the two variables, and this was evident for most treatments (Fig. 4-3); however, the nature of this response varied under the different treatment conditions. For example, body weight appears to have a far greater effect on brain polar FAP at 19°C (Fig. 4-3a) than for animals of similar size at 31°C (Fig. 4-3c). Because of this, it was not possible to apply further statistical analyses to these data to determine possible interactive effects of temperature, ration level, or clutch on brain FAP. While ANCOVA was not feasible, it is
clear that brain polar FAP values are inversely correlated with temperature in fish of similar size.

FAP extracted from heart tissues also had excitation and emission properties similar to those previously reported for this species (Hill and Womersley, 1992). Rearing conditions in this experiment did not affect the shape of the luminescence spectra of either soluble fraction. The solubility of heart FAP in polar and nonpolar solvent fractions varied between clutches, but was approximately equal or slightly greater in the polar fraction.

Heart polar FAP varied between treatments and clutches, with %FL values ranging from 2.44 ± 0.34 (C2-19°-Lo) to 11.77 ± 2.08 (C2-31°-Hi) (Fig. 4-4a). Over all temperatures, except for C1-19° and C3-19°, FAP values were higher in fish fed high ration levels. Excluding C3-19°-Lo, temperature had no clear effect on heart polar FAP in low ration treatments. Temperature did, however, have a positive effect on polar FAP in high ration animals, especially between 25° and 31°C (Fig. 4-4a). Heart nonpolar FAP were variable between clutches both within and between treatments with no obvious consistent trends related to rearing temperature or ration level (Fig. 4-4b).

Attempts to apply ANCOVA to heart FAP data failed for reasons similar to those stated above for brain data. Plots of heart total FAP as a function of fish weight (Fig. 4-5) revealed that FAP was negatively correlated with fish weight at 19°C, and positively correlated at 25° and 31°C.

Metabolic rate, measured as oxygen consumption, varied with both fish weight and temperature. Within the three temperatures, the weight-specific rate of oxygen consumption was inversely related to fish weight, presumably due to the greater ratio of surface area to volume at smaller body sizes (Fig. 4-6). The effect of body weight on oxygen consumption was stronger at 19°C and 25°C than at 31°C. Temperature showed the expected positive effect on metabolic rate.
Temperature/photoperiod experiment

Fish weight was not greatly affected by temperature, photoperiod, or clutch in this experiment (Fig. 4-1b). Mean (± 95% confidence limits) fish weights ranged from 2.57 ± 0.28 g (C6-19°-6L) to 4.27 ± 0.70 g (C6-31°-18L). There were no apparent differences in weight between photoperiods within temperatures, and size differences between temperatures were not as obvious as those for high ration treatments in the temperature/ration experiment.

Brain polar FAP content was inversely related to rearing temperature for all three clutches, with mean %FL values ranging from 275.1 ± 84.6 (C4-19°-6L) to 50.5 ± 16.6 (C5-31°-6L) (Fig. 4-7a). The relation of polar FAP to temperature was similar to that reported for high ration animals in the temperature/ration experiment (Fig. 4-2a). Photoperiod did not appear to have a consistent influence on polar FAP content.

Nonpolar FAP, present in much lower quantities than the polar fraction, did not appear to be affected by either rearing temperature or photoperiod in C5 or C6, but was lower at 19° and 25°C in C4 (Fig. 4-7b).

Results of the ANCOVA model developed for brain polar FAP data are presented in Table 4-1. The ability to meet ANCOVA criteria was probably due to the smaller magnitude of size differences resulting from the experimental treatments. The ANCOVA test revealed that both temperature (F 2,219 = 96.54, P < 0.0001) and clutch (F 2,219 = 203.96, P < 0.0001), as well as their interactive term (F 4,219 = 147.96, P < 0.0001), were significant factors affecting polar FAP content in P. conchonius brain (Table 4-1). Neither fish weight or photoperiod alone significantly affected polar FAP. The interactive term of photoperiod x clutch was significant (F 2,219 = 7.92, P = 0.0005). Brain nonpolar FAP data failed to meet ANCOVA assumptions, and a model was not applied.

Heart polar FAP appeared to be positively correlated to rearing temperature, with mean %FL values ranging from 7.80 ± 1.99 (C6-19°-18L) to 12.33 ± 4.75 (C5-31°-18L).
Application of an ANCOVA model to the data revealed that fish weight ($F_{1,219} = 30.60, P < 0.0001$), temperature ($F_{2,219} = 20.05, P < 0.0001$), and clutch ($F_{2,219} = 12.29, P < 0.0001$) all significantly affected polar FAP content, and that photoperiod had no general effect ($F_{1,219} = 0.01, P = 0.9202$) (Table 4-2). Significant interaction terms included temperature x photoperiod, temperature x clutch, and temperature x photoperiod x clutch, indicating a highly mixed response of heart polar FAP to each these factors. Application of ANCOVA procedures to heart nonpolar FAP was not possible, however the most generalized trend was for %FL values to be lowest at 25°C (both photoperiods and all clutches), slightly higher at 19°C, and highest at 31°C (Fig. 4-8b).

**Discussion**

The results of the present study provide strong evidence for a relationship between temperature, body weight, metabolic rate, and FAP genesis in *P. conchonius*. Further, the differences observed between clutches suggest that this is not merely a physiological effect, but that genetic factors are integrally involved. This is the first study to demonstrate a temperature effect on FAP accumulation in a vertebrate organism, confirming the usefulness of fish and other higher order poikilotherms for aging research (Comfort 1963, Woodhead 1980).

Both temperature and body size showed differential effects on FAP accumulation in brain and heart tissues. The most striking pattern, repeated for all clutches in both experiments, was the inverse relationship between ambient temperature and brain polar FAP. This result is entirely contrary to patterns reported by Sheehy (1990b) for lipofuscin detected in the olfactory lobe cell mass of crayfish brains. Moreover, our brain polar FAP data are contradictory to the precept that metabolic rate, free radical production, and age pigment genesis should be positively correlated with temperature (Sohal 1984).
While McArthur and Sohal (1982) have cautioned against assuming that oxygen consumption is strictly temperature dependent, our own data clearly show that *P. conchonius* has higher rates of oxygen consumption at higher temperatures. In comparison, heart data from the same animals demonstrates a significant positive relationship of FAP to ambient temperature, a result that agrees with previously published age pigment research (Sheehy 1990b).

The fact that different patterns of FAP accumulation occur in *P. conchonius* brain and heart tissues have led us to suspect the involvement of other unknown physiological/biochemical factors in the genesis of FAP. These differences are better understood when the determinants of age pigment genesis are considered in terms of the adaptive physiology of the organism. It is well established that poikilothermic animals acclimate to colder ambient temperatures by increasing the degree of unsaturation of the free fatty acids and fatty acid moieties of phospholipids to maintain membrane permeability and fluidity (Hazel and Prosser 1974, Cossins et al. 1977, Cossins and MacDonald 1989, Hazel and Williams, 1990). Homeoviscous adaptation could theoretically predispose cold-adapted tissues to be more susceptible to free radical attack and peroxidative breakdown, leading to the increased production of cross-linking reactants in the cytoplasm. In the event that increased lipid radical generation is not compensated for by increases in non-enzymatic antioxidants (e.g., beta carotene, alpha tocopherol, ascorbic acid), higher rates of age pigment formation would result. Thus, temperature may affect age pigment genesis in two different manners: through increased metabolism and free radical production at higher temperatures, and by increased lipid peroxidation potential at lower temperatures.

Though speculative, the above theory may partially explain the differences in FAP accumulation observed in *P. conchonius* brain and heart tissues under the various experimental conditions. If FAP genesis is indeed dependent on some balance between
metabolism and lipid peroxidative potential, then it would be expected that brain (a lipid rich tissue) would be more susceptible to lipid peroxidation events than heart tissues at lower temperature. Consequently, if the potential for lipid peroxidation is great enough, it could mask any positive temperature effects related to metabolism. The converse may hold true for myocardial tissues, which possess a lower lipid content and whose metabolic activity is directly affected by temperature (Bowler and Tirri 1990).

An additional factor important to the above considerations is the dependence of metabolic rate and FAP genesis on body size. Both brain and heart FAP contents varied with fish weight in this study. The nature of this relationship varied between tissues as well as between temperatures within tissue type. Respiration measurements also demonstrated that fish of smaller body size had higher rates of oxygen consumption per unit of body weight at any given temperature. Higher weight-specific metabolism in smaller specimens has been widely described in animals including fish (Wieser 1984, Schmidt-Nielson 1984, Taylor 1987). Using freshwater carp (Cyprinus carpio), Itazawa and Oikawa (1986) demonstrated a decrease in weight-specific metabolic rate with increasing size that was due to a relative increase in quantity of tissue of low metabolic activity (bone and white muscle). Based on this widely accepted relationship between size and metabolism, it could be predicted that smaller fish would accumulate higher quantities of FAP than larger fish of identical age, particularly smaller fish living at lower temperatures where the potential for lipid peroxidation is higher. This is indeed the case for both brain and heart tissues of P. conchonius. While no previous study has considered weight-specific metabolic rate as a factor affecting age pigment accumulation within a given species, comparisons have been made between species. Dogs, which have a fivefold greater weight-specific rate of oxygen consumption than humans, also accumulate myocardial lipofuscin five times faster than humans (Munnel and Getty 1968, Strehler et al. 1959).
Restriction of caloric intake has been shown to increase mean life span and maximum lifespan potential in numerous invertebrate and vertebrate animals (see reviews by Weindruch 1984, and Finch 1990). There are many potential mechanisms by which caloric restriction might slow the aging process, yet the precise manner in which it occurs is still unknown. The broadest interpretation of most caloric restriction studies is that reduced caloric intake acts to decrease oxidative metabolism, reducing free radical production and associated molecular damage (Weindruch 1984). In addition, diet restriction induces synthesis of catalase (Koizumi et al. 1987, Semsei et al. 1989, Rao et al. 1990), free glutathione (Lang et al. 1990), glutathione peroxidase (Rao et al. 1990) and superoxide dismutase (Semsei et al. 1989, Rao et al. 1990) in livers of diet-restricted rodents. Superoxide dismutase catalyzes conversion of the superoxide radical to hydrogen peroxide, while catalase and glutathione peroxidase are responsible for converting hydrogen peroxide into oxygen and water, preventing its dissociation into hydroxide and the highly-damaging hydroxyl radical.

We found no evidence that diet restriction lowers oxygen consumption in _P. conchonius_. On the contrary, fish fed low rations had an overall smaller body size and higher weight-specific rates of oxygen consumption. While fish held on low ration attained less weight than their high ration counterparts, they were by no means undernourished or starved. All low ration fish had well developed gonads and abundant visceral fat deposits by the end of the experiment. Thus, even though ANCOVA analysis of FAP data was not possible for the temperature/ration experiment, we feel that any FAP differences observed between dietary treatments were due mainly to the differential effects of body size and the lipid peroxidative potential of the respective tissues rather than malnourishment stress.

Photoperiod - frequently a determinant of growth and activity level in fishes - appears to have no significant effect on FAP accumulation in _P. conchonius_. However,
our experiments were conducted only through the first quarter of the potential lifespan of

*P. conchonius*; longer-term effects may occur. This is the first study to test for effects of

photoperiod on accumulation of age pigments, but it is not the first to question the role of

the level of physical activity. Physical activity level has been shown to directly affect age

pigment levels in flies (Sohal and Donato 1979, Sohal 1981) and rodents (Basson et al.


hibernation in Turkish hamsters, slowing the rate of lipofuscin accumulation in the heart

and brain and prolonging their lifespan.

The significant differences in FAP observed between *P. conchonius* non-siblings

of identical age suggest that genetic factors may be involved in their formation.

Differential expression of genes related to metabolism, production of antioxidant

enzymes, or lysosomal digestive enzymes could all potentially influence the rate of age

pigment production. This is highly speculative with regard to *P. conchonius*, but the

topic deserves further investigation.

With respect to aging research, the results of this study provide further support

for Strehler's (1959) suggestion that age pigments represent physiological events

associated with the aging process. We have provided evidence that FAP production is

dependent upon temperature, body size, and oxygen consumption, and that the manner in

which these factors influence FAP differs between tissue types. While the

interdependence of these relationships is not completely understood, our results provide

indirect evidence that homeothermic adaptation may affect the susceptibility of cellular

lipid components to peroxidative decomposition, a process which may itself lead to

increased FAP production. This possibility has not previously been considered in studies

on temperature and FAP accumulation in poikilotherms.

The results reported in the present work are also relevant to biologists hoping to

apply FAP methodologies to models for chronological age estimation in wild stocks of
aquatic poikilotherms. Hill and Womersley (in review) examined the time course of FAP accumulation in brain and heart tissues of *P. conchonius* ranging from 60 to 538 d of age. Brain and heart FAP accumulated with time, but brain FAP was highly variable at any given age, and less useful than heart FAP for purposes of age prediction. Hill and Womersley's findings were based on animals that had been reared in the laboratory under stable temperature and dietary regimes. In the present study, the degree of brain and heart FAP variation measured between temperatures, body sizes, and clutches in *P. conchonius* was induced in approximately half the time frame of Hill and Womersley's (in review) study and over a relatively short portion of the animals' potential lifespan (ca. 4 yr). Presumably, these variations would increase with time, and this significant variability must be taken into account in considering the use of any age-predictive model based on FAP content. The large effects of interactions between variables reported in the present study indicate that it will be difficult to account for individual effects of variables and accurately calibrate age against FAP content in wild specimens. Although many marine animals live under relatively stable thermal conditions, many other species experience ranges of temperature that, according to our results, would produce major variations in FAP content. The lifetime history of exposure to temperature or other variables that affect FAP content is usually unknown for a wild specimen at the time of aging. Even without temperature variability, size variability within age classes may pose a problem if the rate of FAP accumulation is dependent upon the weight-specific rate of oxygen consumption. Variability in FAP accumulation may occur as a direct metabolic result of variability in ration level; such an effect could not be separated from the effect of growth in our study. FAP variability may be accentuated in freshwater species in temperate regions, or in nearshore or migratory marine species that experience wide fluctuations in temperature, salinity, food availability, pollution (Aloj Totaro et al, 1985), or other environmental or physiological variables. All these factors must be given high priority if FAP
methodologies are to be used for chronologically aging natural populations of aquatic animals. Based on the amount of variability we have encountered, the number of variables involved, and the complexity of the interactions, successful estimation of age of wild specimens is likely to be very difficult.
Literature cited


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Table 4-1. Results of the ANCOVA model testing the effects of fish weight (as covariate), temperature (Temp), photoperiod (Phot), and clutch (Clutch) on polar FAP extracted from *Puntius conchonius* brain tissues. FAP data were log-transformed to stabilize variances. Model $r^2$ (0.895) significant at $P < 0.0001$.

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<td>0.0046</td>
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Table 4-2. Results of the ANCOVA model testing the effects of fish weight (as covariate), temperature (Temp), photoperiod (Phot), and clutch (Clutch) on polar FAP extracted from *Puntius conchonius* heart tissues. FAP data were log-transformed to stabilize variances. Model $r^2 (0.532)$ significant at $P < 0.0001$. 

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
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<tr>
<td>Heart polar FAP</td>
<td>Fish weight</td>
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<td>0.2373</td>
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<tr>
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<td>Phot</td>
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<td>0.01</td>
<td>0.9202</td>
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<tr>
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<td>Clutch</td>
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<td>12.29</td>
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</tr>
<tr>
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<td>Temp x Phot</td>
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<td>0.0422</td>
<td>3.56</td>
<td>0.0300</td>
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<tr>
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<td>0.0718</td>
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<td>Phot x Clutch</td>
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<td>0.0033</td>
<td>0.28</td>
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<tr>
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<td>Temp x Phot x Clutch</td>
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<td>0.0466</td>
<td>3.94</td>
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<td>2.5926</td>
<td>0.0118</td>
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Fig. 4-1. *Puntius conchonius*. Mean fish weight ± 95% confidence limits at 280 to 290 d of age as affected by experimental treatments: (A) temperature/ration experiment with low and high rations at each temperature (°C), and (B) temperature/photoperiod experiment with 6 h and 18 h light (6L and 18L) per 24 h period at each temperature (°C).
The graphs represent fish weight (g) across different treatments and clutches. The x-axis denotes the treatment temperature (19°, 25°, 31°) and light duration (6L, 18L). The y-axis shows the fish weight. Each graph compares the performance of different clutches at varying conditions, illustrating how temperature and light duration affect fish growth.
Fig. 4-2. *Puntius conchonius*. Effects of temperature (°C) and ration level (low and high) on brain FAP (%FL) soluble in (A) polar and (B) nonpolar solvent fractions. Bars represent means ± 95% confidence limits for Clutches 1, 2, and 3.
Fig. 4-3. *Puntius conchojus*. Brain polar FAP (%FL) as a function of fish weight for the temperature/ration experiment, Clutch 1 animals. Solid and open circles represent fish fed low and high ration levels, respectively.
Fig. 4-4. *Puntius conchonius*. Effects of temperature (°C) and ration level (low and high) on heart FAP (%FL) soluble in (A) polar and (B) nonpolar solvent fractions. Bars represent means ± 95% confidence limits for Clutches 1, 2, and 3.
Fig. 4-5. *Puntius conchonius*. Heart total FAP (%FL) as a function of fish weight for the temperature/ration experiment, Clutch 1 animals. Solid and open circles represent fish fed low and high ration levels, respectively.
Fig. 4-6. *Puntius conchonius*. Weight-specific rate of oxygen consumption as a function of fish weight and ambient temperature. Data are means ± 95% confidence intervals for triplicate determinations on individuals subsampled from Clutch 3, temperature/ration experiment.
Fig. 4-7. *Puntius conchonius*. Effects of temperature (°C) and photoperiod (6L and 18L) on brain FAP (%FL) soluble in (A) polar and (B) nonpolar solvent fractions. Bars represent means ± 95% confidence limits for Clutches 4, 5, and 6.
Treatment

Brain polar FAP (%FL)

Clutch 4
Clutch 5
Clutch 6

Brain nonpolar FAP (%FL)

19° 25° 31°
Fig. 4-8. *Puntius conchonius*. Effects of temperature (°C) and photoperiod (6L and 18L) on heart FAP (%FL) soluble in (A) polar and (B) nonpolar solvent fractions. Bars represent means ± 95% confidence limits for Clutches 4, 5, and 6.
CHAPTER 5
SUMMARY AND CONCLUSIONS

This dissertation has examined three important aspects of FAP complexes formed in postmitotic tissues of aquatic poikilotherms. It provides new insights relative to applications in fisheries biology and biomedical gerontology. The primary objective of this research was to critically evaluate the potential for using FAP methodologies for accurate estimation of age in fish (and ultimately in other aquatic animals such as mollusks and crustaceans). This goal was accomplished by 1) reassessing various aspects of methodologies used to quantify FAP in animal tissues; 2) establishing the nature of the relationship between measured levels of FAP and chronological age in two fish species, and 3) examining the effects of several environmental and physiological variables that can alter the rate of FAP accumulation and induce variability in the relationship to chronological age.

In the first part of this research (Chapter 2), it was found that the temperature and time involved in storage, incubation and analysis of tissues and their solvent extracts can critically affect FAP expression through the in vitro production of additional FAP-like fluorophores in tissues of *Oreochromis mossambicus*. Such production was drastically increased if sample homogenates were ultrasonicated. The finding that FAP-like fluorophores can form in dead tissues or sample solvent is not surprising in view of the biochemical processes leading to fluorophore formation. The complex and unstable biochemical composition of dead tissues and their extracts predisposes them to lipid peroxidation, malondialdehyde production, and fluorophore formation as described in Chapters 1 and 2. Ultrasonication, commonly applied to disintegrate cells, generates free radicals in solution and expedites these reactions. The findings described in Chapter 2 can have profound implications in terms of the sources of fluorescence described in all
previous FAP studies. In most FAP studies on aquatic animals (Table 2-1), some or all of these critical sources of error have been ignored. This has probably led to artifactual fluorescence and high variability in the results of some of these early studies, as well as erroneous conclusions as to the usefulness of the technique. My study did not attempt to address such problems for the hundreds of pathological and gerontological studies employing FAP methods over the past twenty years, but similar oversights have probably occurred.

An additional problem, common to most of the preliminary studies of FAP in aquatic species, has been the use of animals whose chronological age was estimated, but not validated by other methods. In some cases, age was not even estimated, and FAP content was only compared with body size. Several studies have attempted to examine FAP accumulation in whole animals without regard for differential rates of accumulation in different tissue types. The sampling frequency and time scale of many studies have been inadequate for accurately describing ontogenetic patterns of FAP accumulation in the study animals.

In the second phase of this dissertation research (Chapter 3), the above problems were addressed by examining patterns of FAP accumulation for extended periods of time in specimens of two fish species of known chronological age. Nonpolar FAP accumulated in brain tissues of Oreochromis mossambicus during the first 2.5 years of life, and it is assumed that accumulation would continue through the latter half of the lifespan as well. FAP also accumulated in brain and heart tissues of Puntius conchonius over the first 1.5 years, and the highest values in both tissues were found in isolated measurements of the oldest animals sampled (> 4 years old). Patterns of accumulation were different in the two tissue types over the first 1.5 years; brain tissues had greater and more variable quantities of FAP than heart tissues. Brain FAP tended to level off with increasing age, whereas heart FAP increased in a more linear pattern. Reasons for
these differences are not clear; it is assumed that the rates of accumulation are affected by ontogenetic changes in tissue metabolism as well as changes in the activity of the antioxidant enzymes responsible for the control of free radicals. Differences were also found in the relative solubilities of FAP in polar and nonpolar solvents within and between tissue types, emphasizing the importance of examining both fractions whenever possible.

This is the first study to incorporate FAP data into multiple regression models for prediction of chronological age. Of the several types of multiple regression models developed in Chapter 3, the best models for age prediction in both species were those that included all types of data (body size, otolith size, organ weight, and FAP). However, these best models provided only slightly stronger age prediction than similar models constructed without FAP data. This result raises the question of whether obtaining the additional information available from FAP measurement would be a cost effective addition to gathering the fish size, otolith size, and organ weight data; however, a major goal of FAP research is to use FAP to age animals lacking skeletal structures such as otoliths. When otolith data were excluded from the multiple regression models in this study, inclusion of FAP still provided a better age-predictive model than somatic data alone. This was especially evident for male P. conchonius, for which organ weight and FAP data were the only significant variables remaining in the final fitted regression model. The relationship between FAP content and chronological age was variable in this study, but FAP variables consistently remained in the final multiple regression models. The animals used for these multiple regression studies were reared under closely similar, strictly controlled conditions in the laboratory, so the resulting data represent a near optimum which is probably unattainable with field specimens.

The final goal of this dissertation was to determine the possible effects of some environmental (temperature, photoperiod) and physiological (body weight, weight-
specific metabolic rate, ration level, genome) variables on FAP accumulation in brain and heart tissues of *P. conchonius* (Chapter 4). These effects were tested in two experiments: one tested for effects of temperature, ration level, and clutch; the other tested for effects of temperature, photoperiod, and clutch. Temperature and body weight affected FAP content in brain and heart in both experiments, but in a different manner in each organ. Brain FAP was inversely related to body weight in both experiments, especially at the lowest rearing temperature (19°C). Heart FAP was inversely related to body weight at 19°C, but positively related to weight at higher temperatures. Increasing temperature decreased brain FAP in both experiments, but increased heart FAP content. Ration level appeared to affect FAP level by modulating body size, however statistical analyses and separation of possible direct effects were not possible. Photoperiod had no significant effect on FAP in either tissue. Significant differences in FAP were detected between non-sibling fish, implicating genomic variation in the ability to control FAP genesis. The mixed effects of body size, temperature, and weight-specific oxygen consumption for each tissue indicated involvement of factors in addition to metabolic rate in the formation of FAP products. Homeothermic adaptation via modification of cellular lipid constituents may predispose lipid-rich brain tissues to lipid peroxidation at lower temperatures. In contrast, heart tissues, which are lower in lipid content, are more susceptible to the positive effects of temperature on oxygen consumption, and thus have higher FAP values at higher temperatures. This is the first study to demonstrate these environmental and physiological effects on FAP accumulation in a vertebrate poikilotherm.

Biologists hoping to apply FAP methodologies for age estimation of field populations will be faced with a number of serious complications. The ability to apply FAP as an aging tool will ultimately be contingent on obtaining experimental baseline information on age, growth, and FAP accumulation patterns which can be related to animals collected from the natural populations. Measuring FAP fluorescence in a tissue
is a relatively simple procedure to perform, however interpretation of the data for age prediction is not nearly as straightforward a procedure as counting growth increments in skeletal parts. Age pigment content of a tissue, whether measured as extractable FAP or quantified using histological/morphometric methods, will always be dependent upon the physiological and environmental life history of the animal. Physiological aging processes are correlated only roughly with chronological age. Thus, investigators wishing to apply FAP methods to natural populations will be required to conduct baseline research defining the relationship between FAP accumulation and chronological age in combined laboratory growth and mark-recapture studies, through relevant portions of the animal's lifespan. Laboratory growth studies should take into account variation in FAP induced by temperature, body size, nutrition and genetic factors, which were shown to be important in this study. Other factors that may be important to consider (but not studied here) include disease history and exposure to salinity fluctuations and radiation, xenobiotics such as heavy metals, or other environmental extremes. Based on the amount of variability and the complexity of the interactions encountered in this study, successful estimation of age of wild specimens using FAP is likely to be difficult.