Brain-Tissue Accumulation of Fluorescent Age Pigments in Four Poeciliid Fishes (Cyprinodontiformes) and the Estimation of “Biological Age”

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ABSTRACT: The concept of an intrinsic “biological age” or “physiological age” is very important in comparative ecological and evolutionary studies, but its implementation has been problematic. Although many authors have discussed the importance of intrinsic measures of age over the past 75 years, only Reiss (1989) has proposed an operational definition in terms of cumulative metabolism; however, Reiss’ measure cannot be easily applied to assessing the age of individual organisms. Measurement of the level of accumulation of fluorescent age pigments (particularly lipofuscins) in post-mitotic tissues, which seems to occur at a rate proportional to cumulative metabolism, in principle provides a mechanism for estimating the biological age of individual organisms. This study has shown that brain-tissue FAP levels vary in direct proportion to chronological age and body size within four species of poeciliid fishes, and has documented interspecific differences in rate of accumulation of fluorescent age pigments, differences that seem to be a function of the degrees of relatedness. Rather than proposing that FAP level by itself be used as a measure of biological age, however, I propose that it be used in conjunction with other estimates, such as chronological age and body size, to derive a composite “age factor.”

KEY WORDS: Body size, Gambusia holbrooki, Latent variable, Lipofuscin, Metabolic age, Path analysis, Poecilia reticulata, Xiphophorus helleri, Xiphophorus maculatus

INTRODUCTION

Growth is usually described and measured in relation to chronological age. However, chronological time has only an approximate relationship to the “biological age” of an organism, as indicated by the observations that rates of growth and aging are often asymptotic with respect to chronological age, are temperature-dependent, and are modulated by a host of environmental factors and metabolic and genetic states (Loeb and Northrup 1917, Spiegel 1972, Gerking and Raush 1979, Taylor 1981). Although sidereal time (the passage of calendar time in days or years) provides a convenient common scale for assessing ages of different individuals, a biologically more relevant measure would be physiological or
metabolic age, an intrinsic function of the "rate of living" of the organism (Du Noüy 1937). Like many other kinds of biological phenomena (Peters 1983), physiological age might in fact have a much more direct relationship with body size than with chronological time because body size reflects the cumulative product of net metabolism (Frazier 1985, Reiss 1989; but see Masoro, et al., 1991).

The assessment of the relative ages of organisms is obviously important in many kinds of ecological studies, particularly those involving age-structured populations (Fuiman 1998). It is less obvious that age assessments are important in comparative evolutionary studies, but the recent resurgence of interest in heterochrony (evolutionary change in the timing of developmental events) as a potential mechanism underlying morphological evolution has encouraged a reconsideration of the proper time scales for comparing developmental patterns in different species (Creighton and Strauss 1986, Strauss 1987, Gould 1988, McKinney 1988, Parichy, et al., 1992, Reilly, et al., 1997). While information about chronological age is clearly important in such research, we do not yet know enough about the relationships among chronological age, body size, and physiological age to be able to assess the proper metric on which to "map" developmental events (McMahon 1980, Lindstedt and Calder 1981, Martin and Palumbi 1993, Fuiman, et al., 1998). In studies of natural populations, the size- and temperature-dependence of developmental rates may make meaningless the direct comparison of developmental timing in different species. This is especially true in comparative studies in which poikilothermic organisms are raised under laboratory conditions (e.g., Strauss 1990, 1992) that may be suboptimal in different ways for different species and thus may differentially affect developmental patterns. As Reiss (1989) notes, "without a metric for developmental time, the extent and meaning of evolutionary changes in developmental timing simply cannot be assessed." An independent measure of physiological age would provide an intrinsic standard for comparative developmental studies (Dettlaf and Dettlaf 1961, Boddington 1978, Dettlaf 1986, Martin and Palumbi 1993).

Theories of cytological aging suggest that organisms age continuously, from fertilization to death, as the result of degradative cellular processes such as mechanical and thermal alterations, development of immobilizing protein cross-linkages, protein oxidation by free radicals affecting membranes, DNA, and collagen, and other degenerating biochemical interactions (Strehler 1977, Johnson, Jr., et al., 1986, Floyd 1991, Kowald and Kirkwood 1994, von Zglinicki, et al., 1995, Rubin, et al., 1996; but see Harrison 1992). Fluorescent age pigments (FAPs) are thought to be reliable correlates of cellular aging and thus have been widely studied at the molecular and cellular levels (Whiteford and Getty 1966, Aloj Totaro, et al., 1987, Sheehy 1990, Belchier, et al., 1998). FAPs comprise several different kinds of insoluble autofluorescent pigments (lipofuscins, ceroids, and possibly others), ranging in emission frequency from yellow to blue, that are generated under normal conditions in cells as a result of polymerization reactions between oxidized lipids and proteins (lipid peroxidation) (Hammer and Braun 1988, Eldred and Katz 1989, Hill and Womersley 1991, Wolf 1993). Because peroxidation products are not degraded by intracellular enzymatic processes, their rate of accumulation ostensibly is related directly to metabolic rate, at least in non-dividing (post-mitotic) cells such as those of nervous and myocardial tissues (Jolly, et al., 1995). The rate of FAP accumulation has been shown to be approximately constant over time in natural and laboratory-raised populations of animals such as crayfish and lobsters (Ettershank 1983, Wahle, et al., 1996, O'Donovan and Tully 1997, Sheehy, et al., 1998), mice (Reichel 1968, Reichel, et al., 1968, Miquel, et al., 1978, Dapson, et al., 1980, Moore, et al., 1995), dogs (Munnel and Getty 1968), and man (Strehler, et al., 1959, Terman and Brunk 1998). It has also been demonstrated that varying temperature or activity rate in insects can directly affect the rate of FAP accumulation, apparently in proportion to metabolic rate (Sohal and Donato 1979, Sohal 1981, Ettershank, et al., 1983, Sohal and Donato 1979). A general correlation between chronological age and FAP accumulation has been found in a number of different species of teleost fishes, although Hill & Womersley (1991)
have expressed doubts about the results of many of these studies due to various methodological problems.

Fletcher, et al., (1973) described a spectrofluorometric procedure for the whole-tissue assay of blue-emitting fluorophores that are produced by peroxidation and assumed to be related to age pigments. Their procedure has been used to demonstrate a general relationship between chronological age and the accumulation of intracellular autofluorescent pigments in insects, crustaceans, fishes, and mammals (references in Hammer and Braum 1988, Hill and Radtke 1988, Hill and Womersley 1991). In fishes, the first study documenting FAP accumulation as a function of growth was apparently that by Woodhead, et al., (1983) of FAP concentrations in the kidneys of two poeciliids: the guppy Poecilia reticulata (used in this study) and the Amazon molly Poecilia formosa. FAP accumulation has more recently been measured in adults of a variety of fish species (Aloj-Totaro, et al., 1986, Hammer and Madhusudhana-Rao 1987, Hill and Radtke 1988, Vernet, et al., 1988, Hill and Womersley 1991) and in larvae raised at various incubation temperatures (Hamer 1988) and food concentrations (Mullin and Brooks 1988). Although the results of almost all of these investigations have demonstrated weak to strong correlations of FAP concentration with body size or age, the results have been highly variable. Hill & Womersley (1991) commented on a number of serious methodological problems with the extraction and assay procedures of many of these studies, such as the use of formalin-fixed or inadequately frozen tissues, of ultrasonification of tissue homogenates to disrupt biological membranes and release cellular contents, and of lengthy incubation times, all of which may artificially increase fluorescence levels. Based on their systematic studies of these and other aspects of FAP-assay procedures, Hill & Womersley (1991) were able to recommend protocols designed to minimize the post-sampling production of fluorophores.

The objective of this study was to use this spectrofluorometric procedure, suitably modified to avoid known artifactual effects, to examine the accumulation of FAPs in the brain tissues of four species of poeciliid fishes as a function of chronological age and of conventional and multivariate measures of body size. The results lead to several interesting conceptual problems related to the general problem of age estimation.

MATERIALS AND METHODS

Study organisms

The advantages of poeciliid fishes for studies of developmental variability were reviewed by Strauss (1992). The four poeciliid species used for this study were Gambusia holbrooki (the eastern mosquitofish, formerly considered to be a subspecies of G. affinis), Poecilia reticulata (the guppy), Xiphophorus Helleri (the green swordtail), and X. maculatus (the southern platyfish). Parental stocks for the present study were obtained from natural populations (P. reticulata from Trinidad, G. holbrooki from South Carolina) or from commercial sources (X. helleri and X. maculatus). Fish stocks were maintained in aerated glass aquaria with a standardized lighting (12h on :12h off) and temperature (25-26°C) regime and were fed daily to satiation with newly hatched brine shrimp augmented by a standard diet of dry prepared food (Tetramin®).

For each species, a cohort of fry was produced from a single parental mating of a batch-reared male with a virgin female. Time of impregnation was recorded to within one day. Each cohort was divided into two groups that were randomly assigned to different aquaria to control for “tank effects” on growth. Individuals from sib cohorts were sequentially sampled over time at regular intervals to obtain sufficiently resolved size and age series. Three cohorts from different parents were sampled for each species. Total sample sizes were N = 42 for G. holbrooki, 61 for P. reticulata, 38 for X. helleri, and 33 for X. maculatus. Females of these species release live young at about 30 days after fertilization. Ages at sampling varied from 30-120 days post-fertilization. By the age of 120 days, most individuals have become reproductively mature but sexual differences in body size are just beginning to become evident; therefore sexual dimorphism is not a factor in the patterns reported here. Interspecific differences in body form are also minor at this size.


**Autofluorescence**

Individuals were sacrificed by overdose of tricaine methanesulfonate (MS-222) and were immediately photographed and weighed (as described below) and frozen at -80° until dissected. For larger individuals (greater than about 9 mm standard length), brain tissues were sampled from frozen carcasses by excising the top of the skull with a scalpel and removing the four brain lobes as a unit. For smaller individuals, the head was removed and the non-cranial portions were excised, leaving the brain and eyes along with some residual cartilage and muscle tissue. Tissues were blotted, stored in glass tubes, and re-frozen at -80° until being assayed for fluorescence.

Hill and Womersley (1991) have shown the duration and temperature of tissue storage to be critical in FAP studies. Times at sacrifice, sampling, and assay were recorded for individual samples. Durations between sacrifice and sampling ranged from 1-32 hrs (mean 3.2 hrs), and between sampling and assay from 1-70 hrs (mean 22.7 hrs).

Brain tissues were prepared for spectrofluoroscopy using the procedures of Fletcher, et al., (1973) and Ettershank (1983), following many of the modifications of Hill & Womersley (1991), particularly those involving temperature control. A sample of 10 mg (wet weight) of frozen tissue in 5 ml chloroform (spectroscopic-grade CHCl₃) was homogenized in a Potter-Elvehjem jacketed tissue grinder at 0° and rinsed into a glass centrifuge tube with sufficient chloroform to return the solvent volume to 5 ml after evaporation. An equal volume of a chilled magnesium-chloride/methanol solution (100 mM MgCl₂/MEOH, 3:1 v/v) was added and the sample was shaken on a vortex mixer for 2 min, centrifuged at 3000 g for 20 min at -4°, and transferred to an ice/water bath. The lower chloroform layer was extracted immediately into a sealed vial and stored in the ice/water bath until assayed.

Prior to assay, samples were transferred to a room-temperature water bath (approximately 20°) for 10 min. Sample fluorescence was measured with a Perkin Elmer Fluorescence Spectrophotometer (MFP-44A) at wavelengths of 365 nm excitation and 422 nm emission in Teflon-covered quartz cuvettes at room temperature. The intensity of the fluorescence emission was standardized with respect to freshly mixed quinine sulfate (1 mg/l in 1 N H₂SO₄) measured at 350 nm excitation and 445 nm emission. Sample fluorescence results are expressed as standardized whole-tissue fluorescence (as a percentage) by multiplying the standardized sample luminescence by the solvent volume (2 ml).

In order to estimate the precision of the method, brain tissues of three large adult female swordtails (*X. helleri;* 56.8, 58.7, and 51.2 mm standard length) were divided into three replicate subsamples each, with the durations between sacrifice and sampling being 1, 12, and 24 hrs, respectively. Three separate assays were done, each utilizing one subsample from each fish, with the durations between sampling and assay being 4, 24, and 36 hrs, respectively.

**Morphometric data**

All specimens were photographed, with metric scale, in lateral and dorsal projection for morphometric study using an Olympus SZH dissecting microscope fitted with a photographic tube and camera. They were then blotted until superficially dry and weighed to the nearest 0.1 g to provide an estimate of "wet weight."

Standard length (SL), the conventional measure of body size in fishes, is measured from the tip of the "snout" (the premaxilla at the anteroventral symphysis) to the base of the caudal fin (posterior edge of the hypural plate). However, because standard length might not sufficiently represent overall body size in species having different body forms (Bookstein, et al., 1985), a multivariate measure of general size was used for comparative purposes.

Morphometric data were obtained from lateral and dorsal photographs by digitizing point coordinates of lateral- and dorsal-view anatomical landmarks corresponding to structures judged to be homologous from form to form. (Landmarks from morphological structures visible in both projections were conservatively given different labels in case they did not agree precisely in position; however, correspondences are noted in the descriptions.) The following anatomical landmarks, digitized from lateral (1-19) and dorsal (20-25) projections, are diagrammed in Fig. 1: (1) Chin, the dentary bone at the
Figure 1: External anatomical landmarks (hollow circles) and associated interpoint distances used in the assessment of general body size. Descriptions of the anatomical landmarks are provided in the Appendix. (A) Lateral view, anterior to the left. (B) Dorsal view.

anterior symphysis; (2) snout, the premaxilla at the anterior symphysis; (3) posterior midsaggital edge of the supraoccipital crest (comparable to #24); (4) anterior edge of orbit (comparable to #21, 21'); (5) posterior edge of orbit (comparable to #22, 22'); (6) postero-dorsal edge of opercular hinge; (7) posterior edge of maxilla (comparable to #20, 20'); (8) midsaggital isthmus; (9) ventral base of pectoral fin; (10) dorsal base of pectoral fin (comparable to #23, 23'); (11) base of most anterior dorsal-fin ray; (12) base of most posterior dorsal-fin ray; (13) midsaggital base of anterodorsal procurent caudal ray; (14) base of most anterior pelvic-fin ray; (15) base of most anterior anal-fin ray; (16) base of most posterior anal-fin ray; (17) midsaggital base of anterodorsal procurent caudal ray; (18) posterior edge of the hypural plate; (19) posterior edge of the central caudal-fin ray; (20, 20') posterior edges of maxillae (comparable to #7); (21, 21') anterior edges of orbit (comparable to #4); (22, 22') posterior edges of orbit (comparable to #5); (23, 23') dorsal bases of pectoral fins (comparable to #10); (24) posterior midsaggital edge of the supraoccipital crest (comparable to #3); (25, 25') posterior edges of pectoral-fin margins.

Forty-three metric distances, standardized with respect to digitized scale bars, were computed among selected pairs of points (Strauss 1992). In poeciliids such homologous interpoint distances can be measured reliably on individuals as small as 4 mm SL. General body size is then represented by the first principal component of the covariance matrix of log-transformed distances for each species (Jolicoeur 1963, Reyment 1991:100-105). This measure is consistent with other studies (Strauss 1990, 1992) of morphometric variation in poeciliids in relation to developmental timing. The units of the general-size axis are arbitrary, depending on the scaling of the principal-component coefficients, but are comparable among species for a constant number and selection of characters.

Statistical analytic methods

The functional (predictive) relationships of FAP accumulation on size and age within and among species were assessed by simple and multiple regression and by analysis of covariance. Multivariate patterns of covariation in FAP level, general body size, and chronological age were analyzed by principal components analysis of the correlation matrix.

Path-model coefficients were fitted from the correlation structure using the structural-equation modeling program LISREL (Hayduk 1987, Jöreskog
and Sorbom 1988), based on the standard assumptions of linear modeling (collinear responses, additivity, multivariate normality), which have been discussed in detail by Karlin, et al., (1983), Cloninger, et al., (1983), Wright (1983), and Breckler (1990). All other statistical analyses, including the mapping of adjusted FAP means onto the hypothetical phylogenetic tree (McArdle and Rodrigo 1994), were carried out in Matlab (Mathworks 1993) using functions written by the author.

RESULTS

Covariation of size, weight, and age

Because of the large ranges in body size, general-size factors accounted for more than 99% of total morphometric variation within all species, thus providing comparable measures of body size among species that are robust against differences in body form. As expected, the correlations of general size with both body weight and standard length are quite high (>0.9, Table 1) and are comparable to those reported for three of these species by Strauss (1990). This suggests that the greater resolution provided by the general-size measure over univariate measures such as body weight or length might not compensate for the much greater effort involved in its estimation. However, the multivariate measure is likely to be more valuable when comparisons are made among species differing substantially in body form, as in the present case. Multivariate allometric coefficients for a subset of the interlandmark distance measures are provided in Figure 2. The 95% confidence intervals about 1 (isometry) for the four samples are [0.982, 1.018] for G. holbrooki, [0.987, 1.013] for P. reticulata, [0.978, 1.022] for X. helleri, and [0.974, 1.026] for X. maculatus; coefficients outside of these intervals indicate statistically significant negative (<1) or positive (>1) allometry. Because growth patterns are allometric and differ significantly among the species, size differences are confounded with shape differences and a multivariate measure of general size is justified to distinguish them.

Body size and chronological age are imperfectly correlated in these species (Fig. 3, Table 1), ranging from a high of 0.93 between age and general size for G. holbrooki to a low of 0.72 between age and weight for X. helleri. Although in all species the multivariate size measure is significantly more highly correlated with age and FAP level than the original measured variables, it is such incongruence among variables that raises the issue of which measure of age or size is the “best” standard for comparing de-
TABLE 1

Correlations among FAP fluorescence level (FAP), chronological age, general body size, standard length (SL), and wet weight for four species of poeciliid fishes. Values in brackets are asymmetric lower and upper 95% confidence limits.

<table>
<thead>
<tr>
<th>Variables</th>
<th>G. holbrooki</th>
<th>P. reticulata</th>
<th>X. helleri</th>
<th>X. maculatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP - Age</td>
<td>0.78 [0.72, 0.83]</td>
<td>0.76 [0.69, 0.81]</td>
<td>0.53 [0.42, 0.62]</td>
<td>0.60 [0.50, 0.68]</td>
</tr>
<tr>
<td>FAP - Size</td>
<td>0.83 [0.78, 0.87]</td>
<td>0.85 [0.80, 0.88]</td>
<td>0.78 [0.72, 0.83]</td>
<td>0.78 [0.72, 0.83]</td>
</tr>
<tr>
<td>FAP - SL</td>
<td>0.75 [0.68, 0.80]</td>
<td>0.73 [0.66, 0.79]</td>
<td>0.70 [0.62, 0.76]</td>
<td>0.72 [0.64, 0.78]</td>
</tr>
<tr>
<td>FAP - Weight</td>
<td>0.73 [0.66, 0.79]</td>
<td>0.76 [0.69, 0.81]</td>
<td>0.64 [0.55, 0.71]</td>
<td>0.65 [0.56, 0.72]</td>
</tr>
<tr>
<td>Age - Size</td>
<td>0.93 [0.91, 0.95]</td>
<td>0.91 [0.88, 0.93]</td>
<td>0.78 [0.72, 0.83]</td>
<td>0.85 [0.80, 0.88]</td>
</tr>
<tr>
<td>Age - SL</td>
<td>0.82 [0.77, 0.86]</td>
<td>0.82 [0.77, 0.86]</td>
<td>0.67 [0.58, 0.74]</td>
<td>0.77 [0.70, 0.72]</td>
</tr>
<tr>
<td>Age - Weight</td>
<td>0.79 [0.73, 0.84]</td>
<td>0.75 [0.68, 0.80]</td>
<td>0.62 [0.52, 0.70]</td>
<td>0.72 [0.64, 0.78]</td>
</tr>
<tr>
<td>Size - SL</td>
<td>0.89 [0.86, 0.91]</td>
<td>0.84 [0.79, 0.88]</td>
<td>0.82 [0.77, 0.86]</td>
<td>0.84 [0.79, 0.88]</td>
</tr>
<tr>
<td>Size - Weight</td>
<td>0.83 [0.78, 0.87]</td>
<td>0.84 [0.79, 0.88]</td>
<td>0.84 [0.79, 0.88]</td>
<td>0.81 [0.75, 0.85]</td>
</tr>
<tr>
<td>SL - Weight</td>
<td>0.81 [0.75, 0.85]</td>
<td>0.83 [0.78, 0.87]</td>
<td>0.85 [0.80, 0.88]</td>
<td>0.83 [0.78, 0.87]</td>
</tr>
</tbody>
</table>

TABLE 2

Results of replications of the FAP-assay procedures using three individuals of Xiphophorus helleri. \( \bar{x} \) is the mean, and CV the coefficient of variation, among the four FAP measurements per specimen.

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>Sampling to assay (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacrifice to sampling</td>
<td>4</td>
</tr>
<tr>
<td>SL (hrs)</td>
<td></td>
</tr>
<tr>
<td>51.2</td>
<td>24</td>
</tr>
<tr>
<td>56.8</td>
<td>1</td>
</tr>
<tr>
<td>58.7</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 3: Three-dimensional scatterplots of standardized whole-tissue fluorescence level as a function of chronological age and body size for the four species of poeciliid fishes.
TABLE 3

Regressions of FAP levels on general body size and chronological age.

All regressions are significant at a probability level of \( p \leq 0.001 \). \( b_0 \) = intercept; \( b_1 \) = slope; \( se \) = standard error; \( R^2 \) = proportion of total variance in FAP level accounted for the independent variable.

<table>
<thead>
<tr>
<th></th>
<th>Size</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( b_0 \pm se )</td>
<td>( b_1 \pm se )</td>
</tr>
<tr>
<td>\text{G. holbrooki}</td>
<td>2.57 ± 1.32</td>
<td>1.99 ± 0.21</td>
</tr>
<tr>
<td>\text{P. reticulata}</td>
<td>4.42 ± 0.96</td>
<td>2.40 ± 0.19</td>
</tr>
<tr>
<td>\text{X. helleri}</td>
<td>5.55 ± 1.95</td>
<td>1.77 ± 0.24</td>
</tr>
<tr>
<td>\text{X. maculatus}</td>
<td>4.38 ± 2.24</td>
<td>1.99 ± 0.29</td>
</tr>
</tbody>
</table>

Developmental patterns in different species (as discussed below). If all measures were perfectly correlated, the choice would be irrelevant.

**Intraspecific correlations with FAP accumulation**

In order to estimate the precision of the methods used to measure FAP fluorescence, three individual swordtails were subsampled, varying the tissue-storage durations among subsamples so as to maximize possible artifactual variation. The results (Table 2) indicate that the repeatability of the method is high, with coefficients of variation among tissue replicates ranging from 5.3-7.2%. Although in each case there was a slight trend toward increasing fluorescence with increasing time between sampling and assay, the variation is quite small compared to the differences observed among individuals and species. In addition, in the results described below there were no significant correlations between tissue-storage durations for individual samples and any residual variation in fluorescence, after accounting for the effects of general size and chronological age.

In all four species there exist highly significant positive relationships between FAP fluorescence levels and both general body size and chronological age (Fig. 4; Table 3). There is no evidence that these relationships are nonlinear or systematically heteroscedastic. This confirms that both age and size are consistent predictors of FAP levels, and suggests that FAP level itself may be a reasonable proxy for biological age. However, the correlations between FAP level and age (which range from 0.53-0.78) are consistently smaller than those between FAP level and size (0.78-0.85), particularly in the two Xiphophorus species (Table 2).

**Interspecific differences in FAP accumulation**

There are two aspects to the patterns of FAP variation among species that are notable. First, the linear relationships between FAP fluorescence levels and size and age vary considerably among species (Fig. 5; Table 3), although none of the univariate slopes differ significantly from one another. For example, the size-dependent accumulation rate for Poecilia reticulata (2.40 ± 0.19) is 20% greater than the rates for Gambusia holbrooki and Xiphophorus maculatus (1.99 ± 0.21 and 1.99 ± 0.29, respectively), and 36% greater than that for X. helleri (1.77 ± 0.24). Thus, at a given general size, FAP levels tend to be higher in P. reticulata than in the other three species because FAPs accumulate more rapidly with increasing body size.

Second, the species rankings of FAP-accumulation rates differ when they are measured with respect to age rather than size. Poecilia reticulata, which has the largest size-dependent accumulation
Figure 4: Scatterplots of standardized whole-tissue fluorescence level as a function of chronological age and body size, for the four species of poeciliid fishes. Fitted lines are linear regression lines and associated 95% confidence intervals of predicted mean values.
TABLE 4
Mean FAP levels (±1 standard error) in four species of poeciliid fishes, after GLM adjustment for the “treatment” effects of general body size and chronological age.

<table>
<thead>
<tr>
<th>Species</th>
<th>None</th>
<th>Size</th>
<th>Age</th>
<th>Age and size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. holbrooki</em></td>
<td>13.92 ± 1.12</td>
<td>14.20 ± 0.68</td>
<td>13.09 ± 0.85</td>
<td>14.26 ± 0.69</td>
</tr>
<tr>
<td><em>P. reticulata</em></td>
<td>15.23 ± 0.93</td>
<td>17.89 ± 0.58</td>
<td>14.78 ± 0.71</td>
<td>18.02 ± 0.65</td>
</tr>
<tr>
<td><em>X. helleri</em></td>
<td>18.26 ± 1.17</td>
<td>15.68 ± 0.73</td>
<td>18.67 ± 0.90</td>
<td>15.56 ± 0.78</td>
</tr>
<tr>
<td><em>X. maculatus</em></td>
<td>18.38 ± 1.26</td>
<td>16.07 ± 0.77</td>
<td>19.79 ± 0.97</td>
<td>15.90 ± 0.86</td>
</tr>
</tbody>
</table>

rate, has the least age-dependent rate (0.180 ± 0.02), which is 22% less than the largest value of 0.230 ± 0.06, for *X. maculatus*. The primary differences in age-dependent accumulation are in intercepts rather than slopes: at a given age, FAP levels tend to be higher in the two *Xipophorus* species than in *P. reticulata* and *G. holbrooki* despite the variation in accumulation rates because mean FAP levels are somewhat higher at birth.

Overall, mean FAP levels are greatest in *X. helleri* and *X. maculatus*, intermediate in *P. reticulata*, and least in *G. holbrooki* (Table 4). However, because the former species tend to be larger than the latter two and the FAP levels are differentially dependent on both size and age, to be comparable the mean levels must be statistically “adjusted” for the two effects via analysis of covariance. The results (Table 4) suggest that, on a per-size and per-age basis, mean FAP levels are actually greatest in *P. reticulata*, least in *G. holbrooki*, and intermediate in *Xipophorus*.

The close similarity of values for the congeners *X. helleri* and *X. maculatus* suggests that FAP levels might be evolutionarily conservative. If so, a parsimonious mapping of FAP levels onto a hypothetical phylogenetic tree would estimate likely patterns of evolution of FAP levels. The mapping of these initial data (Fig. 6) onto a tree provided by conventional systematic criteria (Strauss 1990, 1992) suggests that *Xipophorus* has retained a primitive FAP level, with relative accumulation rates increasing historically in *Poecilia* and decreasing in *Gambusia*. Obviously the data from the study are far too limited to be able to make such statements with any confidence. However, this example illustrates the kinds of evolutionary inferences that might be made with data from a larger sampling of species.

As an issue separate from the identification and comparison of trends, the correlations of FAP levels with age and size are smaller (i.e., the amounts of individual variation are greater) in the two *Xipophorus* species than in *Gambusia holbrooki* and *Poecilia reticulata*. As noted below, this might be accounted for in terms of greater genetic variability and behavioral inhibition of growth in *Xipophorus*.

Multivariate patterns

Because of the imperfect pairwise correlations among FAP levels, body size, and chronological age (Fig. 3), with no obvious causal connections among them, bivariate regression analyses by themselves are insufficient for understanding the relationships among these variables. In particular, the asymmetric dichotomy between dependent and independent variables in predictive-regression models may bias estimates of linear trends among variables that are measured with error (McArdle 1987, Kimura 1992). Multivariate procedures permit (1) the identification
Figure 5: Superimposed regression lines for four species of poeciliid fishes, from the scatterplots of Fig. 3, of standardized whole-tissue fluorescence level as a function of chronological age and body size.

Figure 6: A parsimony hypothesis (shortest tree) depicting the evolution of FAP-accumulation rate, as implied by the adjusted means from this study and a conventional hypothesis of phylogenetic relationships among the four poeciliid species.
of trends of variation and covariation presuming symmetry among variables, and (2) the evaluation of potential latent (unmeasurable) variables as underlying “causes” of observed variation (Bookstein, et al., 1985, Bookstein 1991)

A principal component analysis (PCA) describes the major patterns of variation within and among the four species (Fig. 7), and in this case also provides a decomposition into a “general” component (PC1), reflecting jointly positive covariation among the three variables, and “bipolar” components (PC2 and PC3) that reflect trade-offs (residual positive and negative covariances) among the variables. PC1 thus represents a “biological age” factor, in that individuals having high projection scores on the axis are older and larger – as judged by size, age, and FAP accumulation level – than are individuals having low PC1 scores. However, because this is an exploratory rather than confirmatory analysis, PC1 is not necessarily the best estimate of such a factor if it were postulated a priori.

The analysis supports the bivariate regressions and analyses of covariance but extends their conclusions. Variation within the plane of the first two components (Fig. 7) accounts for 96% of total variation within the dataset and shows: (1) much more variation within the *Xiphophorus* species than in the other two species; (2) slightly larger mean “biological ages” (i.e., mean PC1 scores) for the *Xiphophorus* species; and (3) a notable difference in centroids between the *Xiphophorus* species and the other two species, due primarily to variation along PC2 reflecting a trade-off between variation in age and FAP level at a given “biological age.” This contrast is more evident in the PC2/PC3 plane (Fig. 7), which accounts for the 19% of total variation independent of the PC1 vector and which portrays the trade-offs among all three variables at a given “biological age.”

The concept of a biological-age factor can be modeled explicitly via path analysis, a statistical tool for partitioning the simple correlations among a set of variables according to a particular working model of their causal relationships (Wright 1921, 1934, Child 1990, Wright 1921) Child 1990. Biological age in this sense is a latent (unmeasured) variable estimated from the correlations among the age-related variables that can be observed and measured, such as chronological or calendar age (as measured in days or years), body size (which increases monotonically with chronological age in vertebrates and most other animals), and physiological age (Fig. 8A). Body size is itself a latent variable, estimated from the covariances among a suite of morphometric variables, as is (potentially) physiological age, which might be estimated from a suite of physiological measures correlated with cumulative metabolic rate.

Because FAP level was the only age-dependent physiological variable measured, Figure 8B is the version of the general path model implemented in this study. The $d_1...d_{43}$ represent the 43 morphometric distance variables measured on each specimen (Fig. 1). All measured variables were “group-centered” for this analysis and so are not confounded by interspecific differences. The path coefficients (the values on the vectors emanating from latent variables) thus are pooled within-group correlations reflecting only intraspecific variation, averaged across species. In particular, the path coefficients $a_1 ... a_{43}$ represent the proportions of variation of the corresponding morphometric variables that are attributable to general body size, and are proportional to the allometric coefficients of Figure 2; thus they are not portrayed in the figure.

The results suggest that body size and FAP-accumulation levels are most highly correlated with biological age in these species (with standardized correlations of $0.86 \pm 0.04$ and $0.89 \pm 0.04$, respectively), with chronological age being somewhat less correlated ($0.74 \pm 0.06$).

**DISCUSSION**

This study has shown levels of FAP accumulation to be positively correlated, both within and among species, with chronological age and body size; however, the correlations among these three variables, though statistically significant, vary widely in magnitude (0.55-0.93). Imperfect correlations between age and various measures of body size (weight, standard length, total length) in these and other poeciliid fishes have been demonstrated to be due to indi-
Figure 7: A principal components analysis (PCA) of variation in FAP level, chronological age, and general body size among the four poeciliid species. *Upper panels*: scatterplots of individuals on the first three components. Polygons (convex hulls) delimit the extents of species variation. Ellipses represent 95% confidence intervals about the corresponding centroids (solid symbols, identical in shape to the corresponding hollow symbols of the convex hulls). *Lower panels*: vector plots (biplots) corresponding to the scatterplots and indicating the directions of maximum variation in the three variables within the spaces of the principal components.
vidual and genetic variability in growth rates and to social inhibition of growth (Snelson, Jr. 1989); thus, the correlations between FAP levels and other measures of age and size are likely to be smaller in wild populations, in which growth rates are likely to be more variable than in this laboratory study. Assuming that FAP levels do accumulate in proportion to cumulative metabolic rate, however, the low correlations (high individual variabilities) undoubtedly have both artifactual and biological components. The artifactual component might include some of the methodological problems noted by Hill & Womersley (1991), including temperature-dependent FAP accumulation and the contamination of brain-tissue extracts with fragments of blood and muscle tissues. Contributions to biological variability might include individual differences both in brain-tissue cellular density and mitotic activity (the latter which would tend to dilute intracellular FAP levels), and in rates of FAP degradation and excretion.

The relationships among size, age, and FAP levels vary considerably among species. Overall, mean FAP levels appear to be greatest in Xiphophorus, but when adjusted for covariation with size and age, mean FAP levels occur in the sequence Poecilia reticulata > Xiphophorus spp. > Gambusia holbrooki. A simple prediction would be that species-specific metabolic rates vary in the same sequence, but this has not yet been examined.

Several caveats to this study should be noted. First, these species were raised under a common and constant set of laboratory conditions, and little is currently known about the norms of reaction of FAP accumulation, i.e., about how accumulation rates might be affected by the various environmental factors that have been shown to affect instantaneous metabolic rates and average lifespan: temperature, caloric intake, photoperiod, population density, ionizing radiations, etc. (Sacher 1977, Lindstedt and Calder 1981, Moore, et al. 1995). Studies testing the effects of temperature and caloric intake are currently in progress, and preliminary results suggest that the amount of variation in FAP accumulation accounted for by these factors is small (on the order of 10-15%) compared to interspecific differences. The four species seem to vary in their maximum lifespans in direct proportion to their maximum body sizes (in order from small to large: Poecilia reticulata, Gambusia holbrooki, Xiphophorus maculatus, Xiphophorus helleri). Thus it is not yet known whether the observed interspecific differences might represent extrapolations of intraspecific trends of variation.

Second, FAP levels might not be strictly monotonic across the lifespan of an individual, particularly in larvae. Hill & Womersley (1991) observed an initial decrease in apparent weight-specific FAP concentrations in the brain tissue of juvenile tilapia (Oreochromis mossambicus) that appears to correspond to a period of rapid initial brain growth, and they suggested that this is due to the growth rate of the organ exceeding the rate of FAP accumulation in young fish. A similar effect was observed in adult males of a lizard species (Manabu and Patnaik 1997). Such transient decreases in FAP concentration were not observed in any of the poeciliid species. However, because the growth patterns of poeciliids undergo relatively rapid transformational shifts (“metamorphosis”) from larval patterns to juvenile-adult patterns at about the time that they are hatched and released by the female parent (Strauss 1990, 1992), such changes in FAP concentrations might be detected in late-stage embryos or pre-natal larvae.

Third, Hill & Womersley (1991) noted a marked difference in rate of FAP accumulation between males and females of O. mossambicus, an effect also observed in the crustacean Nephrops norvegicus (Belcher, et al., 1994). Such dimorphisms, which are presumably due to sexual differences in intrinsic growth rates, were not observed in this study because only juveniles and immediate post-juveniles were studied.

The estimation of biological age
Reiss (1989; see also Lindstedt and Calder 1981) suggested that a useful measure of ‘intrinsic age’ would be a “metric of developmental time that for any species correlates closely with developmental stage but is general enough to allow comparisons between different species.” He proposed that such a metric should satisfy seven criteria: (1) it should be
independent of morphology; (2) it should be causally independent of body size; (3) it should be standardized or calibrated with respect to only a single event, such as fertilization, that can be judged to be homologous among species; (4) its rate should be temperature-independent; (5) homologous events should occur at similar developmental stages as measured by the metric; (6) it should increase monotonically with chronological time; and (7) it should be operationally defined with respect to a physical measurement.

Reiss proposed as such a metric the mass-specific metabolic rate (energy metabolized per mass per time), integrated over chronological time from fertilization (or other initial event) to the time of interest, and proposed the name "physiological time" unit (a term first used and refined by Carrel [1931], Brody [1945], and Hill [1950]) for the metric when measured in units of cal/(g dry wt)/day. He showed that this metric satisfies the first, second, sixth, seventh, and probably fifth criteria.

An important problem with Reiss' proposed measure of physiological time is that it would be extremely difficult to assay for an individual organism, although it can be approximated for a cohort or other group by cross-sectional sampling at regular age intervals. FAP accumulation seems to be directly correlated with Reiss' measure, and would in addition provide age estimates for individuals as well as cohorts. It satisfies the same criteria as Reiss' measure. It certainly does not satisfy the fourth criterion (temperature-independence) in fishes and other ectotherms, but the rationale for this criterion in such animals is unclear.

Despite the evidence that FAP accumulation may represent a reasonable measure of the cumulative "rate of living" (cumulative metabolism) of an organism, and thus might be used as the basis for comparison of populations or species, it is nevertheless a proxy, and is therefore only one of a variety of possible operational proxies of "biological age", the actual scale onto which ontogenetic events should be mapped (Strauss 1987). In this sense biological age is an unobserved, intrinsically unmeasurable latent variable (like "intelligence" [Hunt 1983], "develop-
mental integration" (Zelditch 1987), or "body size" (Bookstein, et al., 1985, Bookstein 1989) that can be estimated as an explanatory correlation among sets of possible measurements (such as test scores, expressed preferences, or distance measures, respectively). To the extent that different estimates of biological age are highly correlated with one another, our best operational measure should be a multivariate factor that best summarizes their joint correlations or covariances.

Such a factor can be estimated in various ways, such as by the first within-species principal component of the correlation or covariance matrix among estimates, with or without subsequent factorial rotations (Zelditch 1987). In this study (Fig. 8) it was expressed as a confirmatory factor model based on Wright's (1921, 1934, 1954) method of path analysis for the analysis of covariance structures (Bookstein, et al., 1985, Crespi and Bookstein 1989, Shipley 1997). Here biological age is taken to be a latent variable that optimally and causally predicts the values of other latent or measured variables. Just as there are many different measurable morphological variables (the distance measures \(d_1 \ldots d_n\)) that contribute to an organism's general body size, so there are many possible physiological variables \((p_1 \ldots p_m)\) that might contribute to its general biological age. Presumably there is only a single possible measure of chronological age, which might be measured in various units (e.g., seconds, months, years). A general conceptual path model of the biological age of an organism (Fig. 8A) thus depicts biological age as a latent variable that determines the organism's chronological age as well as it's physiological age and body size. The latter two are latent variables that in turn determine traits that can be measured. Other latent variables (such as behavioral age or ecological age) determined by biological age might also be added to such a path model.

Comparative studies of the morphology, physiology, ecology and evolution of fishes and other organisms are often limited by an inability to identify a common and consistent measure for the ontogenetic state of an individual (Fuiman, et al., 1998). Investigators in various disciplines use differing standard but arbitrary measures of developmental progress, often tied to the occurrence of discrete events or stages during development (Balon 1979, Kimmel, et al., 1995). In evolutionary biology, developmental variability is important to systematists who have attempted to related variations in ontogeny to methods of assessing phylogenetic relationships and understanding patterns of morphological evolution (Strauss 1992, Mabee and Trendler 1996, Reilly, et al., 1997). A general, universally applicable measure of biological age such as that suggested here would provide for more rigorous interspecific comparisons of developmental timing, assessment of individual variation in development, and testing of theories of developmental timing and change.

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