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Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*)

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Abstract

Zebrafish have become a widely used model organism in developmental biology research. In order to initiate an experimental foundation for aging studies, we have determined some basic gerontological parameters for populations of outbred zebrafish, and the *golden sparse* strain. Outbred zebrafish manifested a mean life span of about 42 months, with the longest living individual surviving for 66 months. The *golden sparse* populations had a mean life span of 36 months and a maximum longevity of 58 months. Skeletal length at death increased with age, suggestive of indeterminate growth. A common age-related phenotype was spinal curvature. Radiographic analysis excluded bony changes as the cause of the spinal curvature, suggesting muscle abnormalities as a primary mechanism. These data and a growing abundance of related biological resources suggest that the zebrafish may be a compelling model organism for studies on aging. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Zebrafish; Life span; Phenotype; Senescence

1. Introduction

The bony fishes (Osteichthyes) represent the largest class of vertebrates, with some 24,000 extant species (Liem, 1995). However, only limited data are available on the aging and senescence of just a few of these species (Patnaik et al., 1994). Several small tropical species, such as the guppy (*Lebistes reticularis*) (Reznick, 1997) and a species of annual fish (*Cynelobias bellottii*) (Liu and Walford, 1969), manifest increased mortality with age typical of gradual senescence and definite life span (Finch, 1990). In addition, age-related degenerative changes characteristic of gradual senescence, such as loss of muscle fibers, endocrine abnormalities, decline in reproductive capacity, increased cancer incidence, and increases in various pathological lesions have been documented in other species (Patnaik et al., 1994).

A tropical fish species that has been increasingly used as a biological model is the zebrafish (*Danio*

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rerio). Zebrafish are small fresh water tropical cyprinid fish originating from India that have been exploited for developmental biology studies because of their transparent embryos, rapid extrauterine development, prolific reproductive capacity, and the relatively modest husbandry costs (Eisen, 1996; Streisinger et al., 1981). Much is now known about its early embryonic patterning, cell fate, and lineage determination. The genetic and biological resources for the zebrafish continue to expand with an increasingly dense genetic map, a growing collection of expressed sequence tags, a genome sequencing project (Vogel, 2000a), the use of large-scale mutagenesis to generate thousands of genetic mutants (Driever et al., 1996), and the availability of transgenic methods (Postlethwait and Talbot, 1997) and morpholino-based genetic knockdown techniques (Nasevicius and Ekker, 2000). Thus, the zebrafish is an experimentally expedient organism similar to several invertebrate models with the anatomic and physiological complexities inherent to a vertebrate.

In order to explore the potential utility of the zebrafish as a model for gerontology, we have conducted studies to determine zebrafish life span and senescent morphology. We show here that zebrafish derived from outbred stocks manifested a mean life span of approximately 3.5 years and a maximum life span of over 5 years. The *golden sparse* strain was shorter lived. The most obvious senescent phenotype was spinal curvature, also reported for other fish species (Comfort, 1960; Liu and Walford, 1969). Radiographic and ultrastructural data suggest that the body curvatures are not the result of bone abnormalities, but may be mediated by muscle degeneration. We discuss how these features may make the zebrafish an especially useful model for studies on aging.

2. Materials and methods

2.1. Zebrafish breeding and husbandry

An outbred population of zebrafish was derived by crossing wild-type fish from the University of Washington (generous gift of Mike Rust) and wild-type fish from Lile's Tropical Ponds (Ruskin, FL). The *golden sparse* strain was obtained from Dr Steve Johnson (Washington University of St Louis). Each tank population was derived from a single clutch of eggs. Fish were bred naturally and early stage fry fed with paramecia, then live brine shrimp until 4 weeks of age when dry flake food was introduced. At 10 weeks of age, fish were housed at a density of 40 fish per 10 gallon glass tank with a 14 h light/10 h dark cycle in a central fish facility. At 17 months of age, approximately equal numbers of male and female fish were selected for the life span study. At 22 months of age, fish were transferred to a satellite fish facility for the remainder of the life span study. At this age, the entire population was placed on a feeding schedule of once per day fish flakes (Wardley Corp., Secaucus, NJ), with a feeding of frozen brine shrimp (Novalek, Inc., Hayward, CA) 3 days per week. A second flake food feeding was given on two other days per week.

A number of measures were put into place to ensure adequate water quality and to minimize the risk of cross-tank contamination and introduction of infection. Each tank was treated as an independent unit with its own sterilized nets and containers to minimize risk of cross-tank contamination. Thorough hand washing was required between handling of fish from different tanks and no gravel or other non-essential objects were placed in the tanks. Water temperature was maintained at 26 ± 2 °C with an in-tank automatic aquarium heater (Tetra, Oakland, NJ) and checked daily. Water quality was maintained with a continuously cycling AquaClear Mini power filter (Rolf C. Hagen, Mansfield, MA) that continuously drew water from the tanks through a strainer into a chamber containing foam filter and activated carbon inserts. A 3–4 gallon water change per 10 gallon tank was also performed each week with carbon filtered (Pur Plus II, Minneapolis, MN) tap water to remove chlorine, lead, mercury, and large mircoorganisms. Debris and mural algal growth were removed with each water change. Carbon filters were replaced every 3–6 months and water quality was tested from monthly to quarterly for chlorine, ammonia, pH, and nitrate (Aquarium Pharmaceuticals, Chalfont, PA). Tanks were checked daily for deaths. Carcasses were removed immediately and placed in 10% neutral buffered formalin.

2.2. Survival curve and mortality rate calculations

Length of survival for each individual was calculated from the date of egg fertilization to the date the fish was found dead. Survival curves were plotted for each tank as percent survival from 17 months of age, the age at which fish were selected for the life span studies, until the entire population in the tank had died. To model the mortality characteristics of the population, the logarithm of the age-specific mortality rate was plotted against age, a classical parametric Gompertz approach that results in a linear increase over time in organisms undergoing senescence (Finch, 1990). Age-specific mortality rates were calculated for each 6-month interval beginning at 18 months of age. The interval mortality was defined as the number of deaths that occurred during a 6-month interval divided by the number of individuals alive at the beginning of the interval. The natural logarithm (ln) of the interval mortality was then plotted versus age (in months).

2.3. Radiographic analysis

Radiographs were taken of all formalin fixed fish from the life span studies. Fish were radiographed in groups using a GE Senograph 600 T mammographic system (General Electric Medical Systems, Milwaukee, WI) to allow increased resolution of the gracile skeletal systems. Images were obtained using a magnification technique (27 kVp, 20 mAs) with a dedicated mammographic film screen system (Kodak MinRE, Rochester, NY) to better define skeletal anatomy.

2.4. Skeletal length measurements

As a measure of growth, the length of the skeleton was measured on the images obtained from post-mortem radiographs. The distance along the length of the vertebral column, from the upper jaw tip to the end of the last vertebrae, was traced with a non-distensible but flexible 1 mm diameter filament that could be easily aligned to follow the curvature of the spine. The filament was then straightened and placed against a ruler to determine the skeletal length. Twelve fish without spinal curvature were removed from formalin storage and measured with a ruler for comparison against the length measurements obtained on the corresponding radiographs.

2.5. Spinal curvature measurements

Previous studies on aging fish assessed spinal curvature by subjective visualization (Comfort, 1960; Liu and Walford, 1969). In order to objectively quantify spinal curvature, we used two measurements obtained from the radiographs to calculate a simple ratio to determine the degree of deviation from the linear spinal axis and thus serve as a quantitative index of spinal curvature. Digital images of radiographs were analyzed in a blinded fashion using the public domain NIH Image program 1.62 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on a Macintosh computer. A mean for each of the two lengths for each fish was obtained from triplicate measurements.

The spinal curvature ratio was calculated as the length of the vertebral column (from the first to last vertebra) divided by the straight-line distance from the first to last vertebrae. Without curvature, the length of the vertebral column is almost identical to the distance from the first to last vertebrae producing a ratio close to unity (1.0). As the spine deviates from its normal linear course, the length of the vertebral column does not change but the distance from the first to last vertebra decreases, increasing the ratio (Fig. 5). Thus, the greater the curvature, the higher the ratio. The complexity of deformation in some fish made these simple length measurements preferable to alternative methods of estimating curvature. The spinal curvature ratio was also measured in 11 euthanized 9-month old outbred fish.

2.6. Electron microscopy

Several small (1 mm^3) fragments of tail muscle from a single old (60 months) zebrafish and a single young (6 months) fish were prepared for conventional transmission electron microscopy. Tissue was minced and fixed in 0.5% glutaraldehyde, dehydrated in graded ethanols, and embedded in Epon 812. Thin sections (60–70 nm) were counterstained with uranyl acetate and lead citrate before visualization on a

transmission electron microscope (Phillips EM400, Eindhoven, The Netherlands).

2.7. Statistical analysis

All analyses were carried out using the statistical package S-Plus 2000 (Insightful Inc., MathSoft, Seattle). For determining whether the length at death was different between tanks, a multivariate regression analysis with a dummy variable was performed, which was zero for data from tank 1 and age for tank 2 (Draper and Smith, 1998). To determine differences in survival, the Wilcoxon (two-sided) sum ranked test was used.

3. Results

3.1. Life span

The plots of survival from 17 months of age for two tanks of outbred zebrafish and two tanks of golden sparse fish are shown in Fig. 1. For the outbred fish, the shape of the survival curve for tank 1 is quite rectangular (solid diamonds), while for tank 2 (solid triangles), the population declined earlier. Similar differences are present between the *golden sparse* survival curves as well. Non-rectangularity of the survival curves suggests the possibility that non-aging related mortality may have been a factor. The presence of infectious diseases cannot be excluded since the fish were in part derived from fish purchased from a commercial source, although no evidence of epizootic disease occurred in any of the facilities in which they were housed. Some degree of inter-tank variability is expected since each tank was treated as an independent unit from 17 months of age, done in part to minimize the risk of epizootics. Water quality is another potential variable. No chlorine, ammonia, or nitrate was detectable, and the pH ranged from 7.0 to 7.6, for the entire study except for the first month in the satellite facility, and then again for a duration of approximately 8 weeks, 1 year after transfer to the satellite facility.

The mean life span of the outbred fish was approximately 42 months with the longest lived survivor from these two populations living for 66 months, and the longest lived decile living to 62 months in tank 1 (Fig. 1, solid diamonds) and 54 months in tank 2 (Fig. 1, solid triangles). For the relatively inbred zebrafish strain, *golden sparse*, the mean life span was approximately 40 months, with the longest lived individual and longest lived decile surviving to 59 months and 51 months, respectively, in tank 1 (Fig. 1, open circles) and 45 and 48 months, respectively, in tank 2 (Fig. 1, open squares). The

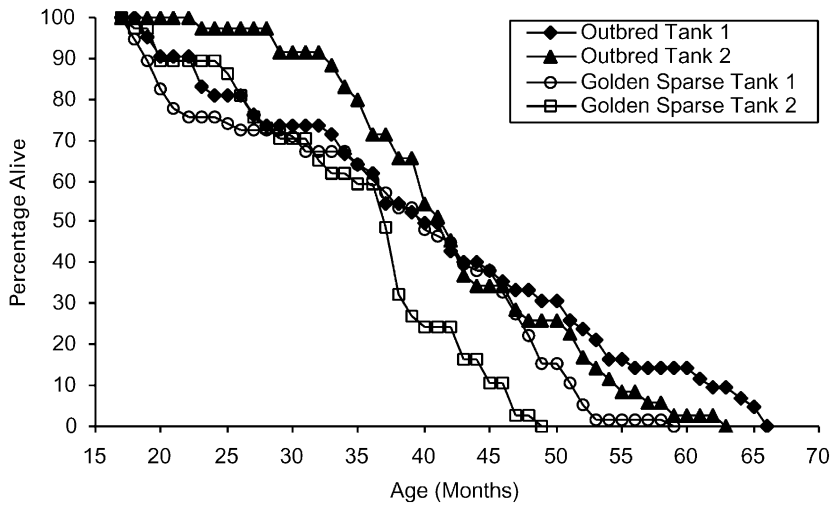

Fig. 1. Survival of outbred zebrafish from 17 months of age shown in solid symbols ($n = 42$, tank 1; $n = 35$, tank 2). Survival of the inbred *golden sparse* strain from 17 months of age shown in open symbols ($n = 55$, tank 1; $n = 36$, tank 2).

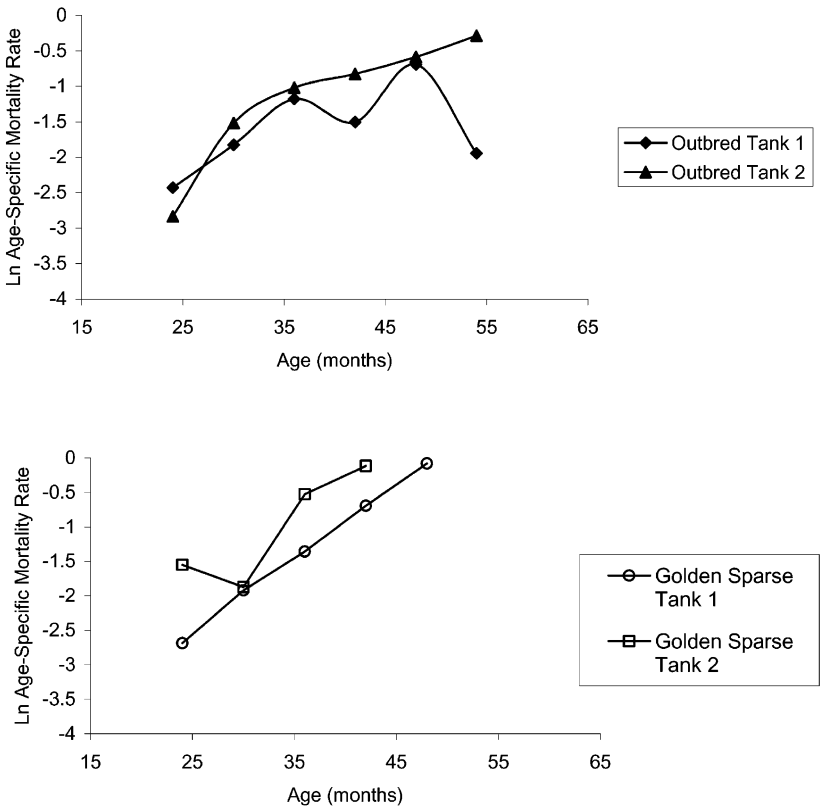

Fig. 2. Gompertz-type mortality rate analysis for survival data shown in Fig. 1. Natural log (ln) of age-specific mortality rate vs. age for outbred zebrafish shown in solid symbols; natural log (ln) of age-specific mortality rate vs. age for the inbred *golden sparse* strain shown in open symbols. Age-specific mortality rates were calculated from 18 months of age at 6-month intervals.

outbred fish were significantly longer lived than the *golden sparse* fish ($P = 0.0366$). The more robust life span of the outbred fish is consistent with a hybrid vigor effect relative to the inbred line.

3.2. Mortality rate

The age-specific mortality rate curves for the two populations of outbred zebrafish and two populations of golden sparse fish are shown in Fig. 2. For the outbred fish, the plot for tank 2 (Fig. 2A, solid triangles) exhibited a biphasic shape with a decreased slope occurring of an inflection point of approximately 32 months of age. This shape is similar to the two-stage Gompertz model reported for a number of species, in which death rates decline after a certain age (Vaupel et al., 1998), and is consistent with a sub-population of fish that died at a more rapid rate at younger ages. Early mortality in aging guppy populations was in part attributed to infectious epizootics (Comfort, 1961). While we did not experience epizootics in the zebrafish population, infectious disease would be a likely pathological process leading to mortality at younger age groups. Outbred tank 1 (Fig. 2A, solid diamonds) exhibited a similar initial slope to outbred tank 2 but two of the data points at later ages were more divergent.

The two *golden sparse* curves were more linear and exhibited higher age-specific mortality rates at older ages (Fig. 2B). For *golden sparse* tank 2, the initial mortality rate data point is higher than tank 1, while the second data points are similar in both tanks. This decrease in mortality rate in tank 1 is consistent with increased deaths occurring at the time of transfer of the fish to the satellite facility. Ammonia and nitrate

were detectable during this period of tank establishment and likely contributed to the stress of transfer for these fish. Variation in mortality kinetics among the tanks throughout the study could be impacted by environmental factors such as water quality since continuous monitoring was not performed.

3.3. Senescent phenotypes

The purpose of the present study was to determine the life span of zebrafish, rather than to carefully document morbidity or causes of death. Determining causes of death in aquatic species kept at warm temperatures is problematic because tissue autolysis occurs rapidly under these conditions (Comfort, 1961). Future studies using a cross-sectional design in which living fish are euthanized will likely be required to accurately determine the pathology distribution in aging zebrafish.

Several pre-morbid abnormalities were observed in fish of any age that usually signaled eventual demise. A cardinal sign of illness was descent to the bottom of the tank. Two other common features of sickness, often preceding residence on the tank bottom, were emaciation or wasting, and equilibrium disturbance, which were noted in fish of any age and have been reported in other fish species (Comfort, 1961; Liu and Walford, 1969). A common morphological phenotype that was not seen in young fish was spinal curvature, as described for the guppy (Comfort, 1960). Some older fish maintained a relatively linear contour (Fig. 3A), while others manifested curvatures ranging from a mild bend or arch (Fig. 3C and D) to severe distortions (Fig. 3B and C). Strikingly similar senescent appearances were reported in the guppy (Comfort, 1960), reproduced in Fig. 4A and B, and in *Cynolebias bellotti* (Liu and Walford, 1969), reproduced in Fig. 4C.

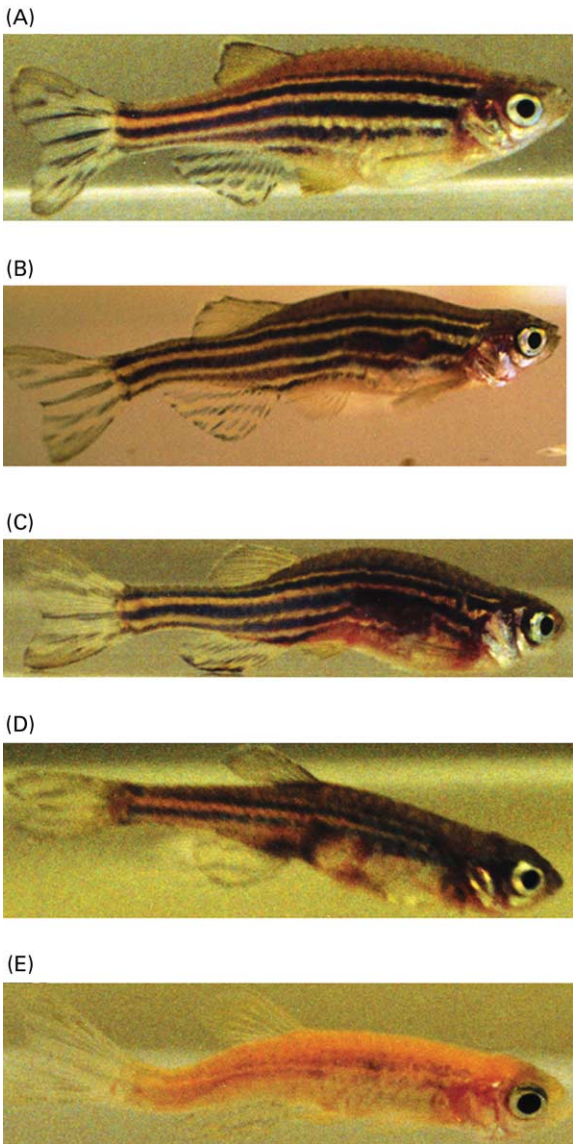

Fig. 3. Common senescent phenotypes of old zebrafish. (A) Old (52 months) outbred zebrafish with very mild bend to otherwise linear contour. (B) Old (52 months) outbred zebrafish with mild to moderate spinal curvature. (C) Old (52 months) outbred zebrafish with mild to moderate spinal curvature. (D) Emaciation in an old (52 months) outbred zebrafish. (E) Emaciation and spinal curvature in an old (44 months) *golden sparse* zebrafish.

3.4. Radiography

In order to determine whether significant bony changes were associated with the senescent spinal curvature, radiography was performed on all fish from the life span studies described above. Since mammography units are optimized to detect fine microcalcifications, formalin fixed fish were X-rayed using a GE Senograph 600 T mammographic system (General Electric Medical Systems, Milwaukee, WI) to allow better visualization of the gracile zebrafish skeletal systems. Radiographs performed on live anesthetized fish were not as clear, likely due to motion artifact

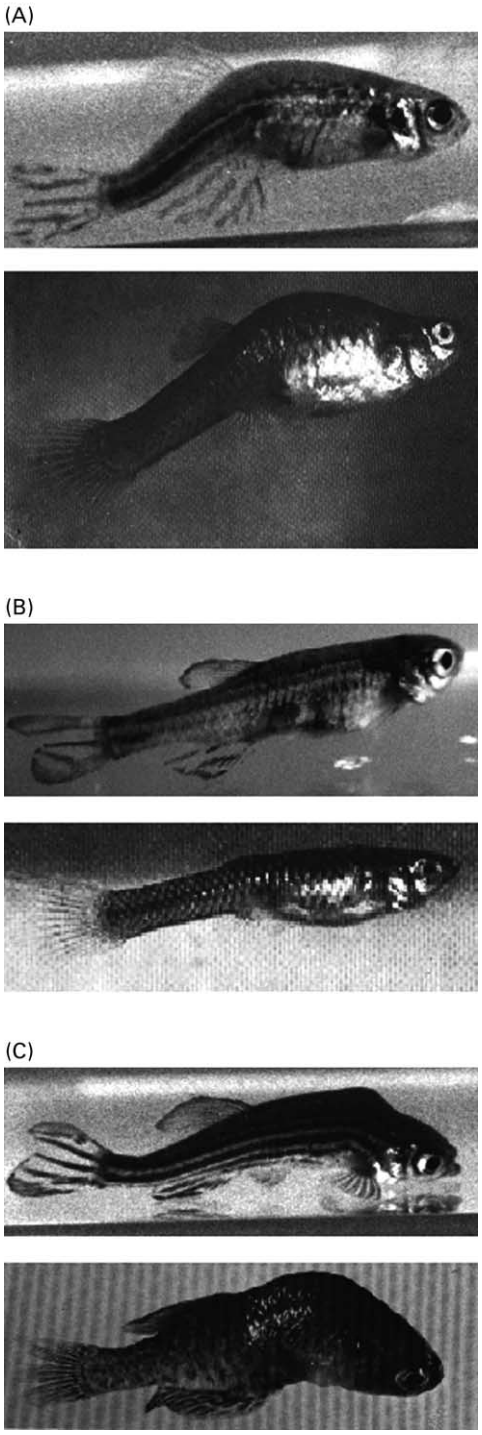

Fig. 4. Similar senescent changes in zebrafish, guppy (Comfort, 1960), and *Cynolebias bellottii* (Liu and Walford, 1969). (A) Old (52 months) outbred zebrafish (top) and old (45 months) guppy (reproduced from Comfort (1960) with permission from S. Karger AG, Basel, Switzerland). (B) Old (52 months) old outbred zebrafish (top) and old (55 months) old guppy (reproduced from Comfort (1960) with permission from S. Karger AG, Basel, Switzerland). (C) Old (52 months) old outbred zebrafish (top) and old (14 months) *Cynolebias bellottii* (reproduced from Liu and Walford (1969) with permission from Wildlife Conservation Society, headquartered at the Bronx Zoo).

from cardiac activity and due to the presence of the radiolucent air-filled swim bladder (data not shown). The spinal distortions were primarily kyphotic deformities (inferior deflections of the caudal portion of the spine), although some fish showed associated areas of lordosis (superior deflections of the caudal portion of the spine causing a 'U' shape), and lateral scoliosis or bending (data not shown). An extreme example of spinal curvature in a *golden sparse* fish is shown in Fig. 5. Despite the severe deviations in the skeleton, vertebral bodies showed no evidence of bone demineralization, osteophyte formation, or compression deformity. Some minor erosive changes were occasionally present in vertebral bodies at points of deflection, although these were consistent with changes secondary to the mal-alignment (data not shown). These findings suggest that bony changes are not the primary cause of the abnormal body curvatures.

3.5. Skeletal length

The length of the skeleton, from the snout to the last vertebrae, was obtained from radiographs of formalin fixed fish between the ages of 24 and 59 months, the age of the longest lived *golden sparse* fish. Because many fish had some degree of spinal curvature, a flexible filament was used to follow the linear path of the vertebral column in order to determine the actual length of the skeleton. This approach was used on 12 fish with relatively linear contours and compared against the length measured with a metric ruler on the formalin fixed carcasses (data not shown). The mean lengths and variances with the two methods differed by less than 0.025 cm with a correlation coefficient $r^2 = 0.89$.

The relationship of length of skeleton at death versus age at death is shown in Fig. 6. For both

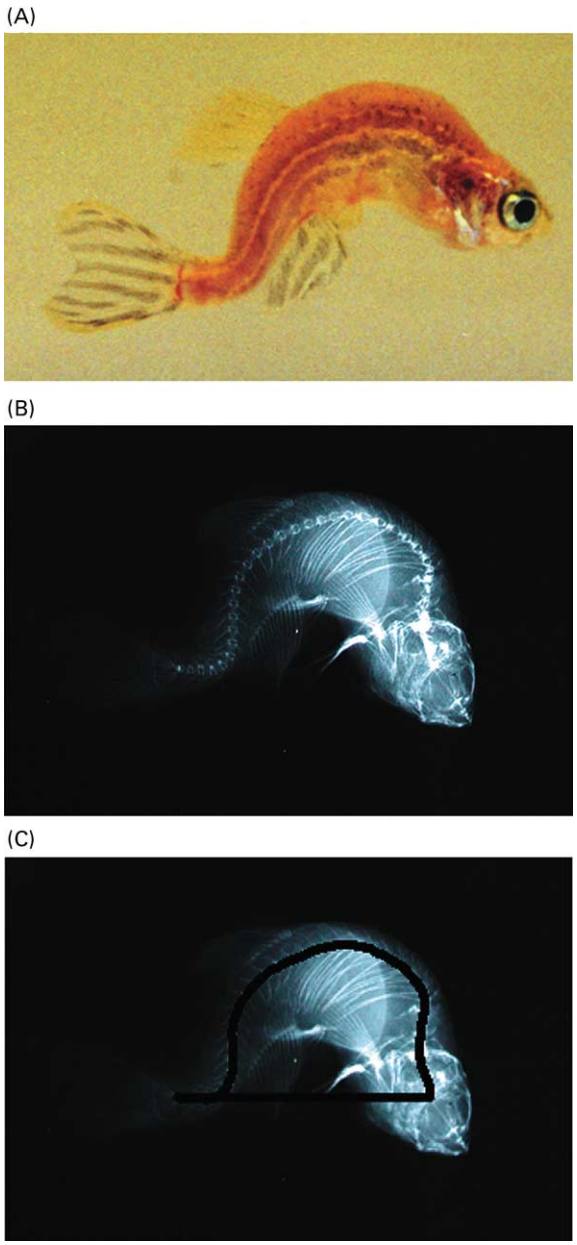

Fig. 5. Radiography of a severe spinal curvature. (a) Severe spinal curvature in a *golden sparse* zebrafish. (b) Radiography of fish shown in A. The severe curvature corresponds to a normal skeletal anatomy suggesting a non-bony cause to the deformation, such as skeletal muscle. (c) Radiography of fish shown in A with lines indicating the measurements taken for the calculation of the spinal curvature ratio. The ratio is defined by the length of the spinal column (curved line) divided by the distance from the first to last vertebra (straight line).

outbred fish and golden sparse fish, the length increases with age, suggesting that growth continues even with advanced age, as reported for the guppy (Comfort, 1960). Three of the tanks exhibited parallel growth rates while one tank of outbred fish (Fig. 6, Outbred Tank 2) had a significantly lower slope ($p < 0.00002$). The mean length of the *golden sparse* fish between the ages of 24 and 36 months ($n = 25$) was 2.64 ± 0.10, and 2.91 ± 0.06 cm between the ages of 36 and 48 months, which were significantly less ($p < 0.002$) than the mean length of either outbred population at these two age ranges (data not shown). The analysis was not extended beyond this age due to the insufficient numbers of surviving golden sparse fish.

3.6. Spinal curvature

A quantitative assessment of spinal curvature was measured as the ratio of the length of the spinal column from the first to last vertebra divided by the straight-line distance from the first to last vertebra. The spinal curvature ratios are plotted versus age at death in Fig. 7. For both outbred and *golden sparse* fish, an increase in the ratio occurs with age ($p < 0.00001$). In contrast, spinal curvature ratios measured on radiographs from 11 euthanized 9-month old outbred fish were all between 1.01 and 1.03. In addition, visual inspection of over 1100 live adult outbred fish up to 1 year of age revealed only one fish with overt spinal curvature (data not shown).

3.7. Electron microscopy

With the normal radiographic findings largely excluding bone as a cause of the spinal curvatures, we hypothesized that a loss of skeletal muscle was mediating the spinal curvature. Because the number of old fish was small, and the primary purpose of the study was life span, only a single, healthy appearing, 60-month old outbred zebrafish with moderate spinal curvature was sacrificed to obtain freshly harvested tail skeletal muscle for ultrastructural analysis by electron microscopy. Thus, the precious nature of such old individuals necessitated a limited sample size. Examination of low power sections from the tail skeletal muscle from a young (6-month old) control fish showed no abnormalities, displaying a normal

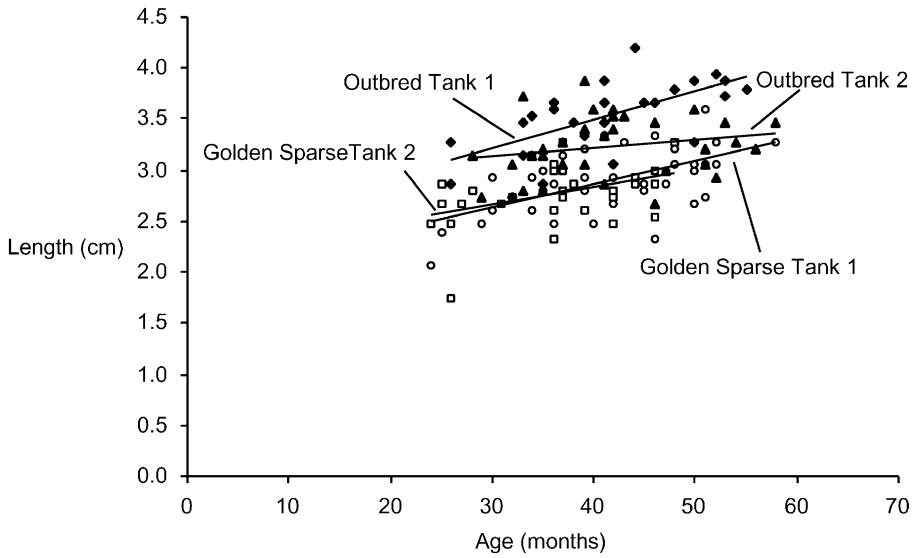

Fig. 6. Skeletal length at death versus age from 24 to 59 months for outbred and *golden sparse* zebrafish fish shown in Fig. 1. The linear regression lines have positive slopes suggesting that growth continues throughout life. Three of the four tanks have parallel regression lines ($p < 0.0001$) suggesting equivalent growth rates.

distribution of myofibrils and mitochondria (data not shown). Higher power (9800 ×) magnifications revealed highly organized myofilaments consisting of the characteristic tight clustering of parallel arrays of actin and myosin filaments adjacent to normal appearing mitochondria admixed with lipid storage droplets (Fig. 8A). Individual sarcomeres with distinct Z discs and central M lines, with thin actin and thick myosin filaments are readily apparent.

In the sections examined from the skeletal muscle of the old fish, areas of severe myofiber disorganization and degeneration were present, without recognizable features of myofibrils (Fig. 8B). Mitochondria were sparse and were morphologically abnormal with thickened, abnormally shaped cristae, and absent lipid storage droplets. In areas where normal myofiber organization was present, the mitochondria still had an abnormal morphology (data not shown). These data indicate that muscle abnormalities were present in a single old fish with spinal curvature. In the guppy, senescent spinal curvature was reported to result from muscular degeneration and that 'the most striking degenerative change was an almost complete loss of skeletal muscle' (Comfort, 1961). These observations are consistent with our limited ultrastructural data supporting a primary role for muscle degeneration in the development of spinal curvature seen in aging zebrafish. Future studies with a larger sample size will be required to confirm these findings.

4. Discussion

Woodhead (1978) has previously cited a number of advantages for the use of fish species as models for the study of aging including the availability of large cohorts of offspring from single matings, the ectothermic nature of fish facilitating modulation by external environmental changes, and their reasonably short life span relative to many mammalian species. Other advantages of fish as gerontological models have also been discussed by Patnaik et al. (1994), such as the lower costs for breeding and maintenance, the ability to manipulate aging by both temperature reduction and food restriction, and the large variety of species available as potential models.

While these attributes make fish an attractive class of organism for studies on aging, zebrafish are unique among the tremendous variety of fish species for its strong experimental foundation, primarily in developmental biology. These same features may also be advantageous for gerontological investigations. Large

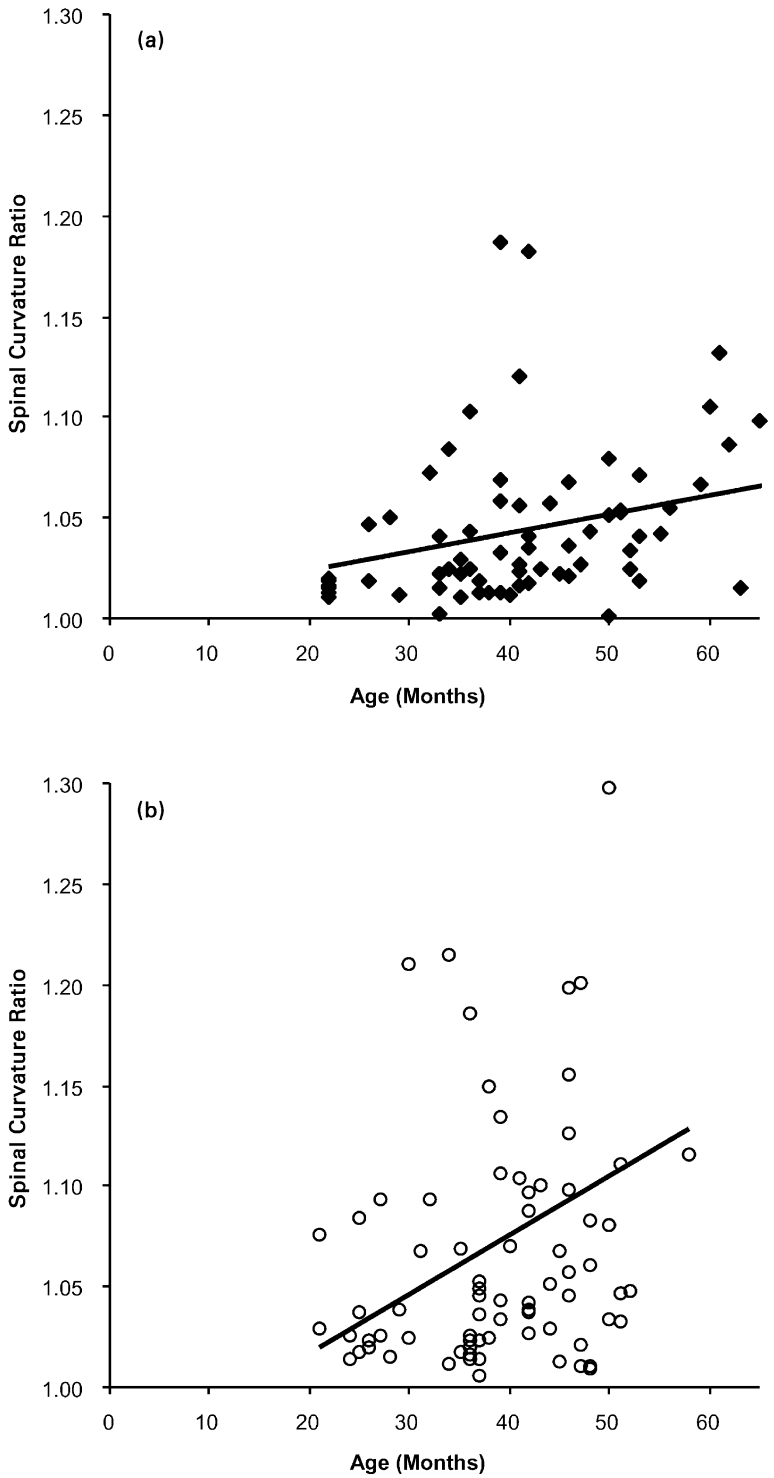

Fig. 7. An index of spinal curvature versus age at death for outbred (a) and *golden sparse* (b) zebrafish. The ratio of the length of the vertebral column divided by the straight-line distance from the first to last vertebra was calculated as an index of spinal curvature. The linear regression lines have positive slopes ($p < 0.0001$) suggesting that spinal curvature increases with age.

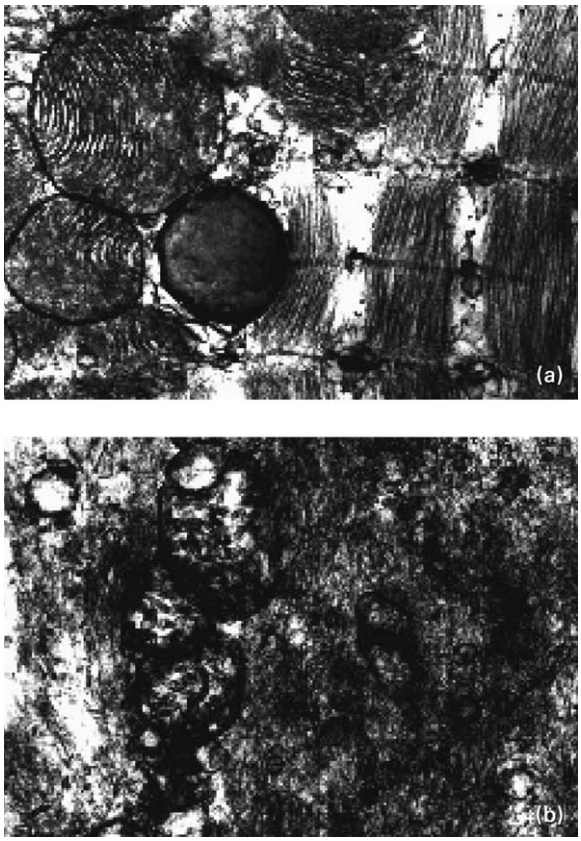

Fig. 8. Transmission electron micrographs from young and old outbred zebrafish. (a) 9800 × magnification of tail skeletal muscle from young (6 months) zebrafish. Normal myofiber architecture is present. (b) 9800 × magnification of tail skeletal muscle from old (60 months) zebrafish. Extensive myofibril disorganization and mitochondrial abnormalities are present.

populations may be easily maintained at relatively low expense. The expanding genetic database such as the cloning of aging related genes (Gerhard et al., 2000) significantly enhance the potential for aging studies. The generation of viable mutagenized animals should allow for the identification of mutated genes conferring age-related phenotypes. Several potentially relevant satiety and temperature mutants have already been described (Vogel, 2000b) and mutant screens for age-related processes such as cancer may be feasible (Beckwith et al., 2000; Cheng and Moore, 1997). The effects of both food restriction and temperature modulation on life span may also be easily studied in this vertebrate organism.

Other fish species have been studied from a gerontological perspective including *Cynolebias bellottii* (Liu and Walford, 1969), *Nothobranchius guentheri* (Markofsky and Perlmutter, 1972), *Oryzias latipes* (Egami and Etoh, 1969), *Xiphophorus maculatus* (Schreibman et al., 1983), *Betta splendens* (Woodhead, 1974), and *Ophiocephalus punctatus* Bloch (Satapathy and Patnaik, 1980), among others (Beverton, 1987; Patnaik et al., 1994). Comprehensive investigations on the mortality characteristics of the guppy were performed more than 40 years ago (Comfort, 1961), and more recently from an evolutionary perspective (Reznick et al., 2001). However, as stated by Woodhead more than 20 years ago, "There have been few studies which have set out specifically to investigate the aging process throughout the lifespan of any fish" (Woodhead, 1978). Little has changed since. Our primary goal was to perform an initial life span study for zebrafish. Due to the variability in the data and the lack of corroborating studies, the life spans reported here should be considered minimum potential longevities. While the total numbers of fish studied were relatively small, the sample sizes were close to that recommended for life span studies in test animal models (Warner et al., 2000) and the populations analyzed represented survivorship from 17 months, not from reproductive maturity. Future more comprehensive studies will be needed to provide a more complete understanding of the biology and mechanisms of aging in *Danio rerio*.

The life span data presented here for the zebrafish also do not include sex-specific longevities or mortality rates. This is of interest to determine for zebrafish, since male fishes of a number of species have been reported to have shorter life spans than females (Das, 1994). Although approximately equal numbers of visually identified males and females were used in the initial populations, accurate sex determination based upon such subjective morphological criteria (Westerfield, 1994) is difficult to ascertain with certainty in zebrafish. Accurate sex determination through histological confirmation was not possible due to autolysis. Therefore, whether a sex differential in longevity exists for zebrafish cannot be determined from the present study. The design of subsequent studies will need to include objective measures, such as histology or perhaps yet-to-be

developed genetic methods, to establish the genders of individual fish.

We also did not document causes of death or presence of specific pathologies because of the effect of warm water on tissue autolysis and the longitudinal nature of the study. In previous studies on aging in the guppy, a small number of fish euthanized when seen to be in extremis were found to suffer from infections from various microorganisms (Comfort, 1961). The use of live food was noted as a potential source of pathogens responsible for epidemic deaths due to epizootic incidents that appeared to be related to the amount of food provided (Comfort, 1963). We used commercially obtained frozen brine shrimp as a component of the diet, which may have also contained potential pathogens. However, we did not experience any significant clusters of deaths that were regarded as infectious epizootics, although almost certainly infectious diseases were present and may have played a major role in the mortality of our fish. Another potential advantage of the zebrafish as an externally fertilizing egg laying species is that eggs and early stage embryos may be treated with a dilute bleach solution to eliminate many potential microbial pathogens (Matthews and Trevarrow, 2000; Westerfield, 1994). Colonies of specific pathogen-free fish may thus be possible for future aging studies.

Other environmental factors could also have major influences upon life span. A number of such variables are present in fish husbandry practices, foremost of which is water quality. With the use of municipal water supplies as the primary source of tank water, levels of chlorine, ammonia, nitrite, oxygen, alkalinity, hardness, particulates, and contaminants such as lead and mercury, are all extremely important factors to control (Klontz, 1995). We used water filtered to remove key heavy metals, chlorine, and particulates, and established a biological filtration system with an automated continuously recirculating filter containing activated charcoal and a biological filter bed to maintain optimum and consistent levels of oxygen, ammonia, and nitrite, which were periodically measured to ensure quality and stability. Weekly partial water changes were also used to maintain water quality. Despite these measures, we experienced two brief episodes of detectable ammonia and nitrate levels. However, our water quality practices were in stark contrast to those reported for the guppy (Comfort, 1961), in which 'no attempt was made during the experiments to standardize the pH, composition, or algal flora of the tanks and jars.' The best survival of guppies was in tanks that were left alone, without 'hygienic interference intended to produce standard conditions.' This included no aeration, continuous application of artificial light, and water changes only when tanks became visibly contaminated. These conditions were likely necessary to maintain the microbial presence necessary to control ammonia and nitrite levels in the water and promote fish well-being. The availability of recirculating filtration systems and the extensive knowledge gained over the past 40 years in fish husbandry and aquaculture has enabled more standardized and optimized conditions for maintaining fish in captivity.

The spinal curvature that was prevalent in old zebrafish was also reported as an age-related finding in the guppy (Comfort, 1961) and in *Cynolebias bellottii* (Liu and Walford, 1969). Data from the guppy and the data presented here indicate that a presumably common mechanism underlying this age-related phenotype is skeletal muscle degeneration. The zebrafish may therefore be a useful model for human sarcopenia, the loss of muscle mass and strength that occurs with normal aging (Evans, 1995; Roubenoff and Hughes, 2000). Sarcopenia in humans is likely the result of a complex and multifactorial process(es). The loss of muscle that appears to correlate with spinal curvature in zebrafish is also likely to result from several causes, including those that may not be age-related and could affect fish at younger ages. The use of zebrafish in mutational screens for genetic mechanisms underlying sarcopenia may be feasible, although much more information regarding age-related skeletal muscle degeneration in zebrafish must first be obtained before induced mutants with similar phenotypes can be sought.

Zebrafish should also be amenable to studies that modulate aging, i.e. caloric restriction and temperature reduction. A caloric restriction regimen was reported in guppies that consisted of feeding once every 2 weeks for the first 600 days of life, then switching to the control schedule of once per week feeding (Comfort, 1963). This severe restriction regimen that was imposed for only a portion of the life span resulted in a slight prolongation of both mean and maximal longevities, although this effect may

have been due to amelioration of early mortality and not slowing the rate of aging. We have performed short-term pilot studies (Gerhard, 2000) in zebrafish using a caloric restriction regimen more akin to those used in rodent and primate studies (Pugh et al., 1999). Full life span studies will be needed to fully explore the effect of caloric restriction on zebrafish aging.

Temperature reduction was studied in *Cynolebias bellottii* and showed dramatic increases in mean and maximum life spans with modest (6 °C) reductions in tank temperatures (Liu and Walford, 1966). Interestingly, fish kept at the higher temperature for the first 8 months of life then switched to the lower temperature had life spans even longer than fish kept continuously at the lower temperature, which in turn lived longer than fish kept continuously at the higher temperature (Liu and Walford, 1975). Growth was also faster at the lower temperature. A potential inter-relationship between growth and aging in fish dates back to Bidder's original hypothesis that senescence was a by-product of determinate growth. While this hypothesis has been disproven, delayed senescence may evolve more readily in organisms that manifest indeterminate growth, such as fish (Reznick et al., 2001). How growth rate affects aging within a given fish species, among factors such as temperature and calories that also affect growth, is not yet well defined.

The longevity characteristics and senescent morphology reported here provide initial gerontological data for zebrafish. Future studies to confirm and extend these results will be important to set the stage for exploiting other approaches to aging, such as large-scale mutagenesis. The application of such approaches may begin to realize the potential power of this model organism for experimental gerontology.

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