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LONGITUDINAL AND CROSS-SECTIONAL OBSERVATIONS OF GROWTH AND BODY COMPOSITION WITH AGE IN LABORATORY POPULATIONS OF THE MALE ANNUAL CYPRINODONT FISH, *NOTHOBRANCHIUS GUENTHERI*

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Abstract—Body weight and length were followed on individual fish throughout major portions of the lifespan in laboratory populations of the male annual fish, *Nothobranchius guentheri*. In addition, these same populations, hatched at different times, were killed on the same calendar day, and one half the fish of each population were analyzed for total body fat, water and protein. Since the proportion of body fat was always low, trends of body weight reflected that of the fat-free body (FFB = body weight minus body fat). If the data are only evaluated cross-sectionally, it appears that (1) growth ceases by 268 days; (2) fish lose FFB and shorten by 460 days; and (3) protein is lost from the FFB by 460 days. However, the availability of the longitudinal data for body length and weight allowed for more critical evaluation of the cross-sectional data; and indicated that the first two conclusions were not valid, and the third could have alternative interpretations.

THE LOSS of fat-free body (FFB = body weight minus body fat) has been considered to be a manifestation of mammalian aging. The data supporting this view come from (1) the apparent loss of body weight in senescence in the human population (Build and Blood Pressure Study, 1969); (2) the premorbid loss of body weight in experimental rat colonies (Everitt, 1957); (3) the apparent loss of muscle mass in rats studied late in life (Lowry *et al.*, 1942; Neumaster and Ring, 1965; Rockstein and Brandt, 1962; Yiengst *et al.*, 1959); and (4) the observed drop in the content of K^{40}, a predominantly intracellular ion of assumed constant concentration in the FFB, with age (Forbes and Reina, 1970; Novak, 1972). In none of the above studies was fat-free body or body fat measured directly, and the conclusions were dependent on the validity of the assumptions within each experiment. In addition, virtually all of the above studies were carried out cross-sectionally, in that young and old subjects were sampled at the same time, in contrast to longitudinal studies, where the same subjects are followed throughout the lifespan. In a previously reported longitudinal study of FFB in the rat, it was observed that in healthy, long-lived animals, there was no loss of FFB with age, and that interpretation of cross-sectional data had to be made with caution (Lesser *et al.*, 1970, 1973).

In the present study, body weight and length were followed through major portions of the adult lifespan in six laboratory populations of the male, annual killifish, *Nothobranchius guentheri*. In addition, these same populations, hatched at different times, were killed on the same calendar day, and one half of the fish analyzed for total body water, fat and protein. The availability of longitudinal data of weight and length allowed for broader interpretation of the cross-sectional data of body composition and led to somewhat different conclusions. The similarities of the observations made for this fish with those in the rat and in man suggest that many aspects of the time course of aging may be similar among the vertebrates.

MATERIALS AND METHODS

All the fish used in the study were the progeny of an initial stock of fish which was purchased commercially (Markofsky and Perlmutter, 1972, 1973). All males were separated from the general population at the onset of sexual maturity (usually between 4–8 weeks of age, see Markofsky and Perlmutter, 1972), and then isolated into separate compartments to allow for weight and length measurements throughout the study period. Population A was born in March, 1969, and isolated at eight months of age. Population B was born in May, 1969, and isolated at six months of age. Population C was born in June, 1969 and isolated at five months of age. Populations D, E, and F were born in October, 1969, and February and April, 1970, respectively. In these populations, each male was isolated immediately at the onset of sexual maturity.

Husbandry for Population A was previously reported (Markofsky and Perlmutter, 1972). Procedures were repeated for all subsequent populations, except that at 4–6 weeks of age, populations B–F were switched to the shrimp, blood worm and beef heart regimen. All fish were housed in the same room during the 15-month experimental period. The day–night cycle was not controlled and varied with the seasons. Mean temperature was 24°C, with a maximum range of 21–25°C.

Longitudinal study

Individual fish, after isolation into separate compartments, were measured for weight and length at approximately monthly intervals until termination of the study. The techniques for measuring length and weight have previously been published (Markofsky and Perlmutter, 1973). For each population, the growth data are presented for the same fish studied repeatedly. Any fish which died or was accidentally lost prior to termination of the study in June 1970, was not included in the growth data. The survival curves of Populations A and B are virtually identical (Markofsky, 1971; Markofsky and Perlmutter, 1972) and there was virtually no mortality in the other populations. Growth data for fishes of differing longevities were presented and discussed in an earler publication (Markofsky and Perlmutter, 1973).

Cross-sectional study

On 29 June 1970, all the fish in all the populations were measured for weight and length and killed. Within each population, fish were paired for weight and length, and one member of each pair placed in Bouin's solution for histopathological examination (to be reported later). The other member of each pair was prepared for body composition analyses. As a result, growth data are available for all fish, while chemical analyses are available for only one half of the populations. The fish used for chemical analyses were weighed, killed by decapitation, and minced with an iris scissor. The entire minced fish was then dried to constant weight in a vacuum oven at 60°C and the weight of the water was determined by the difference. The dry fish was transferred quantitatively from the drying bottle to a glass homogenizer of known volume (range 17–20 ml). Any ether-soluble material in the drying bottle was quantitatively transferred to the same glass homogenizer by washing the residue three times, each time with 3 ml of ethyl ether. The homogenizer was covered with gauze, and the ether evaporated overnight at room temperature. The fish was then ground to a fine residue in the glass homogenizer in approximately 5 ml of distilled water. The pestle was washed with distilled water into the homogenizer, and the homogenate made up to a known volume with distilled water. A magnetic stirrer was used to mix the homogenate, and 3 ml aliquots were removed for fat and nitrogen analyses.

A 3 ml aliquot of the homogenate was transferred volumetrically into a Whatman 10 × 50 mm micro-Soxhlet extraction thimble and dried at 60°C. The thimble was then placed in a micro-Soxhlet extraction apparatus and the ether-soluble material repeatedly extracted for 24 hr (Love, 1957). After extraction, the ether was evaporated from the flask and the extracted fat dried to constant weight. All analyses were performed in duplicate. The mean weights for the duplicate samples of extracted fat never differed from either of the two values by more than one mg. Since fat contents were low, an error of as high as 100% of body fat would lead to a maximum error of less than two to three per cent of FFB. Since analytical errors appeared to be random, mean values should be reliable.

Total nitrogen was determined, in duplicate, on 3 ml aliquots of the homogenate by the micro-Kjeldahl procedure (Sunderman, 1964). Total N was multiplied by 6·25 to derive a value for protein (Love, 1957).

For all values, means and standard errors of the means were calculated. A Student t-test was used to test for differences between population means.

RESULTS

Cross-sectional

As noted in the methodology, all the fish were killed on the same calendar day, measured for length and weight, and one half of each population was analyzed for total body fat, water and protein. These populations covered a major segment of adult life from just post-

TABLE 1. BODY LENGTH AND WEIGHT OF THE MALE *N. guentheri* WITH AGE ($\bar{X} \pm$ SE). CROSS SECTIONAL OBSERVATIONS

Population age (days)	Pop. F 90	Pop. E 133	Pop. D 268	Pop. C 371	Pop. B 405	Pop. A 460
n	15	17	6	5	13	21
Length (mm)	24·0	32·6	43·2	44·2	43·8	40·1
	1·1	0·6	0·6	1·0	0·8	0·7
Weight (mg)	187	483	1145	1210	1169	982
	17	29	40	75	65	51

TABLE 1(a). FAT, WATER AND PROTEIN CONTENTS OF THE MALE *N. guentheri* WITH AGE ($\bar{X}$) $\pm$ SE). CROSS SECTIONAL OBSERVATIONS

n	7	11	4	3	6	9
Fat/wt. (%)	2·26	3·38	1·63	1·80	2·14	0·73
	0·85	0·19	0·46	0·76	0·62	0·17
H_2O/FFB (%)	81·10	78·73	79·29	78·03	79·56	78·82
	1·57	0·25	1·19	0·69	1·30	0·18
Protein/FFB (%)	12·27	14·70	14·25	14·64	13·67	13·63
	0·59	0·26	0·79	0·38	0·72	0·32

sexual maturity (Population F) to "senescence" (Population A). The selection of all subjects for analysis at a fixed point in time provides the conventional cross-sectional data common to most aging studies. There were progressive and significant increments of weight and length between 90 and 268 days ($p < 0{\cdot}001$). No further increase of body length or weight could be demonstrated statistically between 268 and 405 days. Both these measurements then dropped significantly by 460 days ($p < 0{\cdot}01$ for body length and $p < 0{\cdot}05$ for body weight) (Table 1). Since body fat at all ages is relatively low, trends of body weight closely paralleled those of the FFB. Since these animals were of different body size, it was necessary to normalize the fat, water and protein data. In normalizing the data, it is assumed that the proportions of fat, water and protein of the body are independent of body size, and any changes in the proportions of fat, water and protein as a function of age are not attributable to the absolute size of the animal. By convention, fat was normalized to body weight (fat/wt.), and protein and water to FFB (protein/FFB and water/FFB). Maximum fat content (fat/wt.) occurred at 133 days, dropped significantly at 268 days ($p < 0{\cdot}01$), remained stable to 405 days, and dropped significantly again at 460 days ($p = 0{\cdot}05$) (Table 1a). Total body water, when referred to the FFB, remained stable throughout the lifespan (Table 1a). The apparently high value at 90 days was the result of one animal with an unusually high value. Total body protein, when referred to the FFB, rose significantly between 90 and 133 days of age ($p < 0{\cdot}01$), remained stable to 371 days and showed lower values at 405 and 460 days (Table 1a). A significant drop in protein at 405 and 460 days can only be demonstrated if the data for Populations C, D, and E are pooled and compared to Populations A and B ($p < 0{\cdot}05$). It should be noted that at all ages there were animals with protein/FFB values similar to that observed at 405 and 460 days of age. Since longitudinal data are not available for protein/FFB it is not known whether the drop in protein reflects a true loss of protein or merely a selected survivorship of animals with lower protein content.

Longitudinal

The longitudinal data for body length and weight are found in Figs. 1 and 2 and in Tables 2 and 3. It is apparent that the populations, hatched at different times, showed

different growth histories. At every time period at least one of the populations was significantly different as to weight and length from one or all of the others. In contrast to the cross-sectional data, growth did not cease for Population A until approximately 400 days, and at least to this age for Population B. In addition, while the cross-sectional data imply a loss of length and weight, and consequently of FFB, between 400 and 450 days of age, the longitudinal observations of the long-lived survivors of Population A indicate stability of length, and probably also of weight between these two intervals.

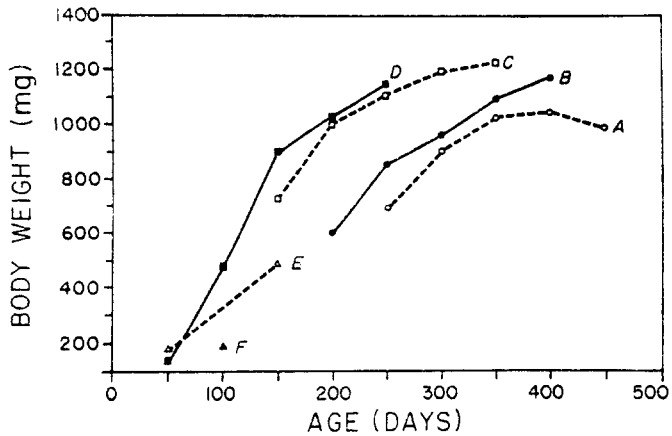

FIG. 1. Body length (mm) of different populations of the male *N. guentheri* with age ($\bar{X}$). Longitudinal observations.

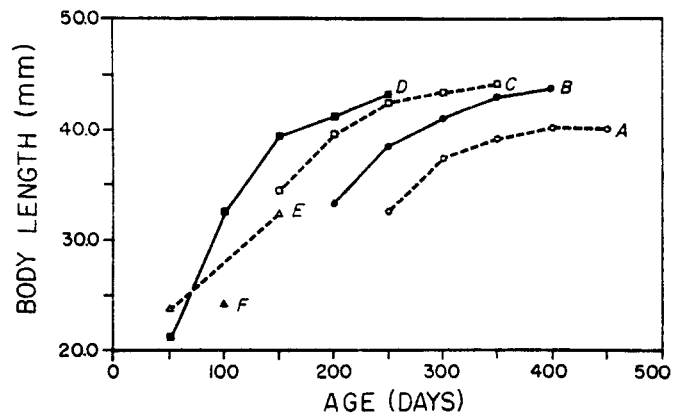

FIG. 2. Body weight (mg) of different populations of the male *N. guentheri* with age ($\bar{X}$). Longitudinal observations.

DISCUSSION

The use of fishes for aging research has now been extended to the annual fishes of the genus *Nothobranchius* (Markofsky and Perlmutter, 1972, 1973) and genus *Cynolebias* (Liu and Walford, 1969, 1970, 1975; Walford and Liu, 1965). The selection of these fishes was based on their short lifespans observed in nature, although the cause of death in nature has been reported to be due to lack of food, and the drying out of the temporary pools (Turner,

TABLE 2. BODY LENGTH (mm) OF DIFFERENT POPULATIONS OF THE MALE *N. guentheri* WITH AGE ($\bar{X} \pm$ SE). LONGITUDINAL OBSERVATIONS

Age (days)	50	100	150	200	250	300	350	400	450
Pop. A	—	—	—	—	32·6	37·5	39·2	40·2	40·1
n = 21					1·0	0·7	0·6	0·6	0·7
Pop. B	—	—	—	33·2	38·5	41·1	43·0	43·8	—
n = 13				1·2	0·6	0·7	0·8	0·8	
Pop. C	—	—	34·6	39·4	42·3	43·4	44·2	—	—
n = 5			1·7	1·4	1·3	1·0	1·0		
Pop. D	21·0	32·3	39·3	41·2	43·2	—	—	—	—
n = 6	1·2	0·8	0·7	0·4	0·6				
Pop. E	23·7	—	32·6	—	—	—	—	—	—
n = 17	0·5		0·6						
Pop. F	—	24·0	—	—	—	—	—	—	—
n = 15		1·1							

TABLE 3. BODY WEIGHT (mg) OF DIFFERENT POPULATIONS OF THE MALE *N. guentheri* WITH AGE ($\bar{X} \pm$ SE). LONGITUDINAL OBSERVATIONS

Age (days)	50	100	150	200	250	300	350	400	450
Pop. A	—	—	—	—	683	900	1020	1040	982
n = 21					58	54	49	47	51
Pop. B	—	—	—	597	850	958	1091	1169	—
n = 13				60	40	48	56	65	
Pop. C	—	—	724	1002	1100	1192	1210	—	—
n = 5			140	117	107	96	75		
Pop. D	135	480	898	1028	1145	—	—	—	—
n = 6	20	25	29	41	40				
Pop. E	175	—	483	—	—	—	—	—	—
n = 17	11		29						
Pop. F	—	187	—	—	—	—	—	—	—
n = 15		17							

1964). When introduced into the laboratory, the life expectancy of these fishes was variable and dependent on factors such as temperature (Liu and Walford, 1969, 1970, 1975) and the onset of sexual maturity (Markofsky and Perlmutter, 1972). Nevertheless, they exhibited a shorter lifespan than other aquarium fishes (Comfort, 1961; Felin, 1951; Markofsky and Perlmutter, 1972).

The reasons for the growth differences between the populations are not yet understood. It has been reported that temperature has a significant effect on the growth of annual fishes. At 15°C the authors reported that growth was more rapid and ultimate body size was greater when compared to fishes raised at 20°C (Liu and Walford, 1970; Liu *et al.*, 1975). Unfortunately, individual animals were not studied longitudinally, and only population means were available. In the present study, each population was housed in the same room, under similar conditions, and temperature is not felt to be a significant variable. It has been reported that there is variability in the onset of sexual maturity in the male *N. guentheri*, and that growth characteristics differ between early and late maturing fish (Markofsky and Perlmutter, 1972, 1973). While there were differences between the present populations as to the onset of sexual maturity, there were no consistent differences which could account for the differences in growth. The qualitative change in the diet between 6–14 weeks of age for Population A only, could possibly have affected ultimate body size. However, in the guppy, after food restriction of many months, realimented fish obtained similar ultimate body size as their *ad libitum* controls (Comfort, 1960).

The duration of embryological development may also have an effect on growth. The developmental patterns of annual fishes varies from that of rapid development in 2–3 weeks, to that of long periods of developmental arrest up to several months (Lesseps *et al.*, 1975; Markofsky, unpublished observations; Peters, 1963; Wourms, 1972). No attempts were made to separate fish which underwent different rates of embryogenesis. Studies are now underway to better understand the embryology of these fish, and how it may affect the aging process. Other factors which may have affected the growth patterns of the different populations were the age at which the males were isolated from the general population, and the age at which they were isolated into separate compartments. For instance, Population D was isolated immediately at the onset of sexual maturity and attained 39·3 mm at 150 days, while Population A, isolated at 250 days, attained 39·2 mm at 350 days of age. In contrast, however, Population E, which was isolated at the onset of sexual maturity, was the same length as Population C at 150 days of age, yet Population C was not isolated until about 120 days post sexual maturity. There may also be seasonal factors affecting growth, and these are currently under study. Additional studies of the above variables would help to clarify the contribution of each to growth and aging.

As noted, when the data are evaluated cross-sectionally, results based on absolute values for body mass cannot necessarily be adequately interpreted. If it can be assumed that fat/wt., water/FFB and protein/FFB are independent of body size and only a function of age, then these factors may be tested as indicators of physiological aging. In the present study, fat/wt. increased significantly between 90 and 165 days of age. This was similar to that reported in man and the rat, in that a disproportionate amount of body fat was deposited after sexual maturity (Lesser *et al.*, 1970, 1971, 1973). However, in contrast to the rat and man which continue to amass disproportionate amounts of fat with age, the fish ceased accumulating body fat by 133 days of age. Since body fat was not measured longitudinally, the life history of body fat for individual fish remains uncertain. The significantly lower body fat for the 460 day populations as compared to all earlier populations (Table 1) could represent (1) gradual senescent loss of fat for most individual fish, similar to the premorbid weight loss described for the rat (Everitt and Webb, 1957); or (2) major loss of fat for a few premorbid individuals; or (3) a selective longevity for individuals of life-long lower body fat content.

The apparent loss of weight, and consequently FFB, at 460 days of age based on the cross-sectional data, must be reevaluated in light of the length-weight history of each population. Of the 21 fish studied longitudinally in Population A, only two showed minor losses in length of 1–2 mm and one of the fish was deformed. In addition, only 6 of 21 fish showed weight losses of no more than 100 mg. If the decline of body weight at 460 days does in part represent the loss of FFB, the decrement in any case is much less than what was observed from the cross-sectional data.

Total body water followed the same trend as the FFB, and when referred to the FFB (TBW/FFB), showed no significant changes with age (Table 3). This is consistent with observations in mammals that water is a constant proportion of the FFB (Babineau and Pagé, 1955; Lesser *et al.*, 1970; Pitts, 1962). The values observed in the present study of 78·2–81% were consistent for data reported for fish (Vinogradov, 1953), but higher than the 71–74% reported for mammals. These data are also consistent with the concept that "chemical maturity" of the FFB is attained relatively early in life, and that growth thereafter represents proportionate quantitative increases within the FFB (Moulton, 1923).

The increase of the protein content of the FFB between 90 and 133 days of age indicated

that "chemical maturity" of the protein content of the FFB does not occur at sexual maturity, but at some time thereafter. Protein was a constant proportion of the FFB between 133 and 371 days of age, and showed a small decline at 405 and 460 days. If in the present study, there was a selective loss of protein from the FFB, it would be consistent with those reports in the literature suggesting a loss of muscle protein with age (Neumaster and Ring, 1965; Sobel *et al.*, 1968; Yiengst *et al.*, 1959). However, the observation may also represent differences within sub-populations. It is possible that these fish of lower protein content at late ages were a select long-lived sub-population, and that the apparent loss of protein in the FFB primarily reflects a survivorship of animals with a relatively low protein content.

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