The relationship between age, size and shape in the upper thoracic vertebrae of the mouse

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INTRODUCTION

When a biological structure grows it does not merely increase all its dimensions in proportion. Growth is usually disproportionate. Some measurements increase faster than others; shape changes as size increases. After a bony structure has reached adult size remodelling may cause further changes in shape.

In a series of previous papers (Johnson *et al.* 1985; O'Higgins, Johnson & McAndrew, 1986, 1988; Johnson, O'Higgins & McAndrew, 1988) we have shown that Fourier transforms of the outlines of vertebrae can be used to study differences in vertebral shape due to different genetic make-up. In this paper we have used vertebral outlines taken from mice with the same genome but of known sex and at a series of different ages in order to investigate the way in which vertebral shape changes with age and sex.

MATERIALS AND METHODS

The mice used in this study were bred in the animal house of the University of Leeds. Two parental stocks (BALB/c and CBA) were obtained from Charles Rivers. These were crossed BALB/c male × CBA female and *vice versa* to produce an F_1. Sexes were separated at weaning and litters (fed *ad libitum*) were killed by CO_2 overdosage randomly at five day intervals between 25 and 60 days. We aimed for samples of 10 males and 10 females of each age group, but this was not always realised (Table 1). The deep frozen eviscerated cadavers were thawed and skinned and skeletal preparations were made using papain. The cleaned T1 and T2 vertebrae were videodigitised using the technique of Johnson *et al.* (1985), but with uprated hardware (a Video Interface Peripheral [VIP] supplied by Sight Systems, P.O. Box 37, Newbury, Berks) and specially developed software.

Vertebrae were placed on the illuminated base of a dissecting microscope. Images captured by a television camera mounted on the microscope were fed to the VIP and its associated microcomputer (Fig. 1). The image was then cleaned (converted to black and white only, and the outline enhanced by a standard averaging method) and digitised (Fig. 2). To do this the screen is sampled diagonally from one corner until a change from black to white, representing the boundary of the image, is located. The coordinates of this point are recorded and the surrounding area searched to find the nearest change, i.e. the next point on the boundary. This process is repeated until original point of contact with the image is regained. The pairs of coordinates representing the shape are then stored on a floppy disc pending transfer to the mainframe computer.

Table 1. *Numbers of animals used*

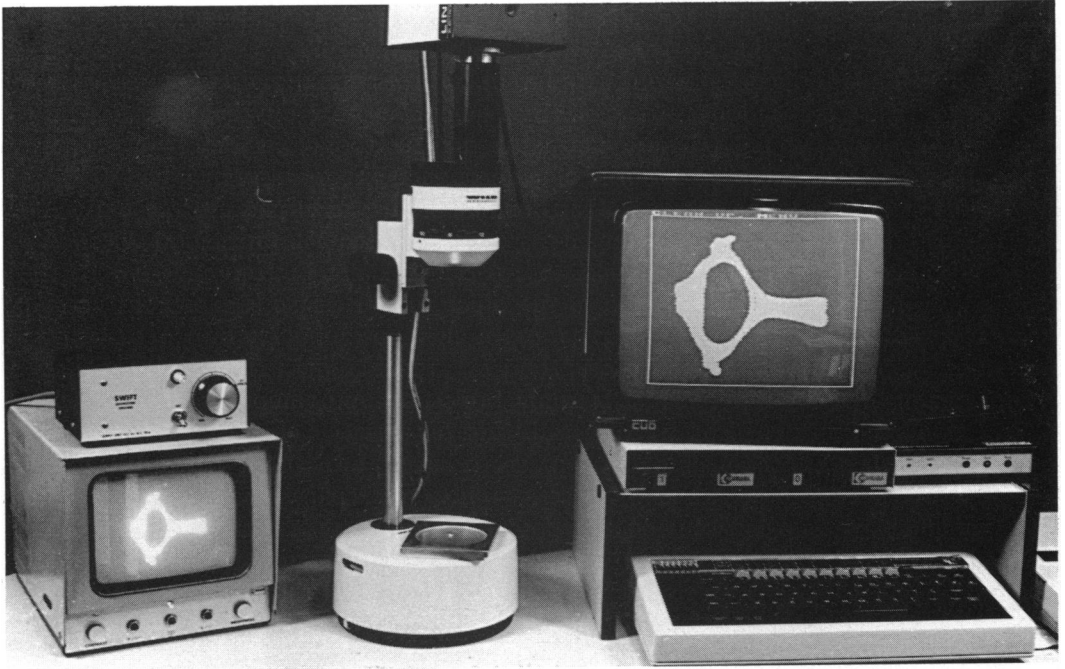| Age in days	25	30	35	40	45	50	55	60
Males	10	13	9	11	13	9	10	11
Females	9	8	10	11	12	9	6	11
Totals	19	21	19	22	25	18	16	22

Fig. 1. The digitising apparatus. A T2 vertebra is placed on the stage of the dissecting microscope (centre) which is fitted with a TV camera. The image of the bone appears on the monitor (left). A digital image of the bone generated by the microcomputer and the interface is displayed on the computer screen (right).

In order to obtain a group mean outline from the outlines of a series of individuals the latter must be superimposed. We do this in three stages. The area within each outline is first calculated and outlines scaled to a standard area. The centre of area (centroid) of each shape is then found by integration and the shape re-expressed as 128 polar coordinates based on the centre of area. Centres of area are then superimposed as fixed reference points.

Since the bones have been placed on the microscope stage in no particular orientation they must also be rotated relative to each other to produce alignment. In practice this is done by making a least squares fit comparison for each outline against a standard. Standard shapes are chosen by trial and error. A suitable standard shape is a simple polygon.

The vertebral shape is tested against the standard and the residual area (Sneath, 1967; Johnson *et al.* 1985) calculated. The vertebral shape is then rotated by one polar coordinate at a time and residual area recalculated. When this quantity is minimal the shapes overlap by the greatest amount, i.e. are similarly orientated. A mean outline of
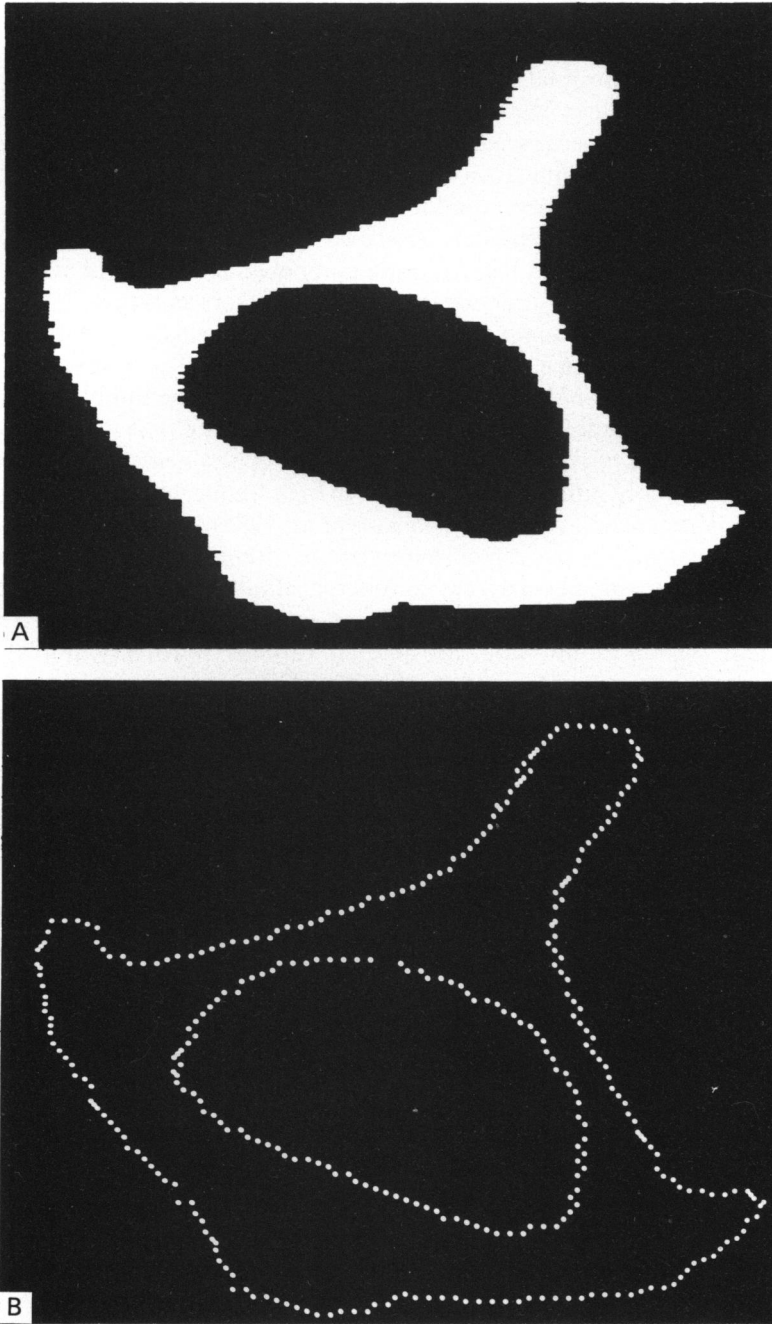
Fig. 2(A–B). The reconstituted image of another T2 vertebra (A) as it appears on screen after having been reduced to black and white only and cleaned and (B) as a series of points representing the edge of the vertebra. Information on the inner outline (the edge of the neural canal) was discarded for the purposes of this study.

all shapes within a group can now be calculated, together with a figure for the variance of the group.

The graph of polar coordinates is now opened out from its ventral midpoint and regarded as a waveform. Jean Baptiste Fourier (1786–1830) described a method (since used extensively in the physical sciences and telecommunications) which decomposes such a complex waveform into a series of sine and cosine components of varying amplitudes and frequencies. These components, when summed, reproduce the original waveform. A simple shape is adequately described by the earlier members of a Fourier series: a more complex one will require more components to describe it adequately. The earlier members of the Fourier series form a good representation of the shape of a gently curving object such as a vertebral outline. We found that the first 20 cosine and 20 sine components from each outline gave the least number of misclassifications in a repeated discriminant analysis (when successive tenths of the sample are randomly removed and classified against the remainder) and hence the best description of the shapes. This represents a compromise: using fewer components oversimplifies the outline whilst using more introduces errors due to high frequency 'noise' in the system which in turn reduces discrimination (Johnson et al. 1985).

In order to investigate the pattern of shape change within the two vertebrae with age and sex the shape measures chosen were considered simultaneously using multivariate techniques. The mean and standard deviation of each variable from each age group was calculated. Within-group variances of each variable were very similar. It was therefore considered appropriate to perform a Canonical analysis in order to investigate the morphological relationships between group mean shapes. The relationships between individuals and group means were also visualised by two and three dimensional plots.

RESULTS

The rate of vertebral growth was assessed by plotting the mean area of the projected image of the vertebrae (digitised at constant magnification) against age (Fig. 3A, B). This showed a decreasing rate of growth for both bones, and no significant overall difference between the sexes. The dip in both graphs at 35 days is thought to be due to sampling error.

If all the T1 vertebrae in the study are subjected to Canonical analysis using the first 20 sine and the first 20 cosine Fourier components and the results expressed as a three dimensional graph of Canonical axis 1 *versus* 2 *versus* 3 (Fig. 4A) we can gain some idea of how our mathematical representation of shape changes with age. Vertebrae aged 30 days and above form a large cloud, with most of the 25 days old individuals well separated. A concordant separation is seen in T2 (Fig. 4B). If we plot only the group mean shape (Fig. 5A, B) this separation is even clearer. This separation can also be appreciated from the table of Mahalanobis' distances derived from the Canonical analysis (Table 2A and B). Between 30 and 60 days the shape separation averaged 3·65 SDU (T1) and 3·98 SDU (T2) respectively with no clear increasing or decreasing trend with age.

Classification by age and sex (Table 3A and B) showed some separation between sexes at all ages (Mean for T1 4·89 SDU, for T2 6·10 SDU) again with no clear trend, but with a rather large distance between male and female T2s at 60 days.

In the case of the T1 vertebrae 81 % of the total variance was expressed in the first two Canonical axes and only 87 % by the first three (T2: 75 %, 83 %). Plots of the first two Canonical axes thus sacrifice little information about shape difference whilst
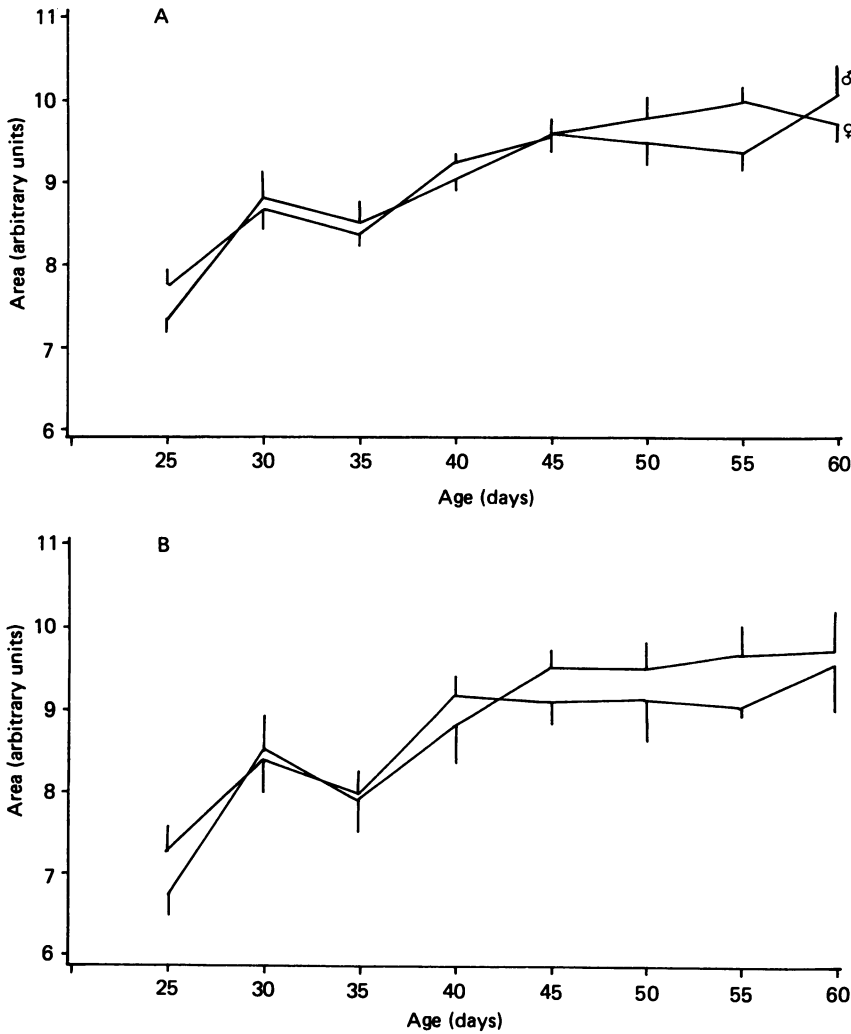
Fig. 3(A–B). Mean area (in arbitrary units) of the vertebrae used in this study plotted against age. Bar equals ±2 s.d. (A) T1, (B) T2.

Table 2. *Mahalanobis' distances between group centroids of different age groups*

Age	25–30	30–35	35–40	40–45	45–50	50–55	55–60
				(A) Bone T1			
Distance	7·63	2·72	3·26	2·74	3·01	3·45	2·73
				(B) Bone T2			
Distance	6·51	3·48	4·27	2·77	3·59	3·41	3·85

allowing a simpler representation of the pattern of shape change with age. An attempt was made to model shape change with age by fitting a straight line (Fig. 6A, B). Straight lines which included all the data and all data minus that from 25 day-old vertebrae were fitted to the plots of Canonical axis 1 against 2.
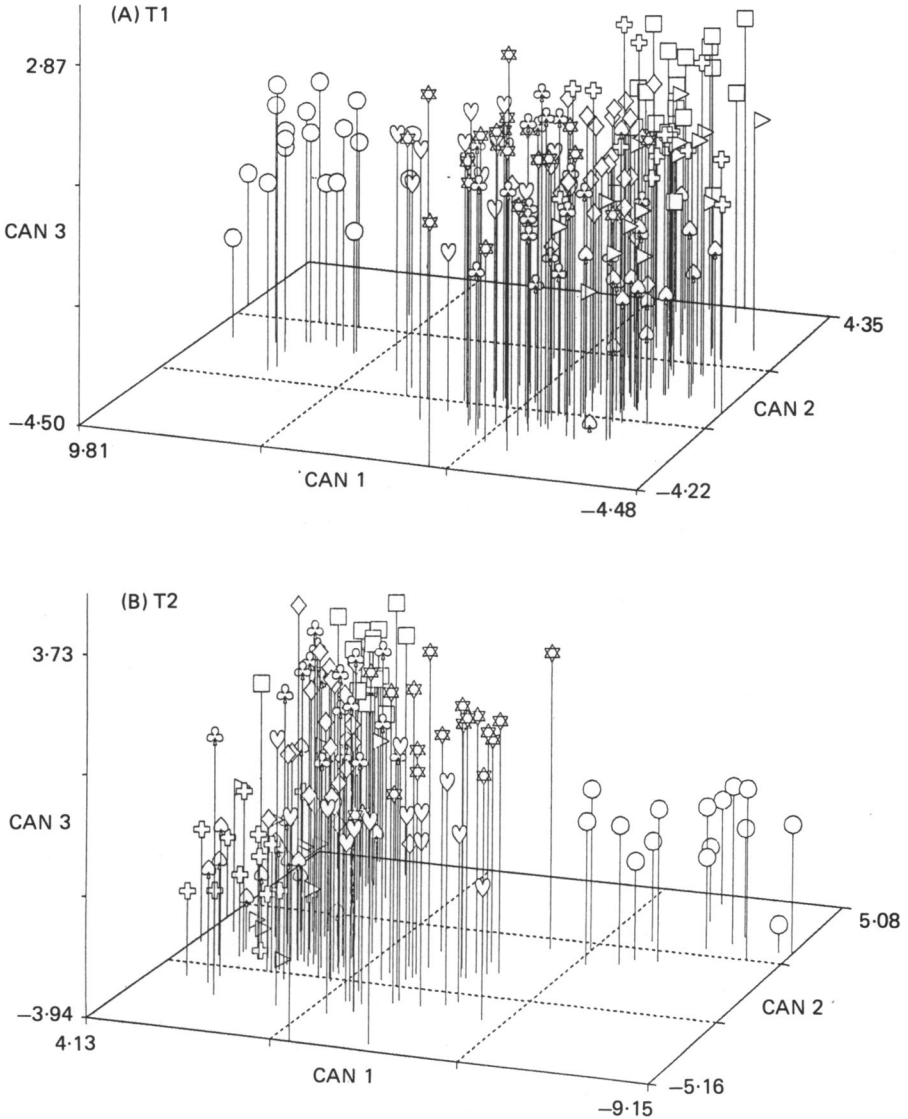
Fig. 4(A–B). (A) Three dimensional plot of the relationship of all T1 vertebrae used in the study. Symbols used in this and all subsequent Figures to denote age groups are as follows: balloon = 25 days; star = 30 days; heart = 35 days; club = 40 days; diamond = 45 days; spade = 50 days; cross = 55 days; flag = 60 days. Axes in Figures 3–5 inclusive are first three Canonical axes. Figures 3–5 are based upon the first 20 sine and first 20 cosine components. 87·4 % of the variance in this plot was incorporated into the first three axes. (B) Three dimensional plot of the relationship of all T2 vertebrae used in the study. Key as in (A). 83·5 % of the variance in this plot was incorporated into the first three axes.
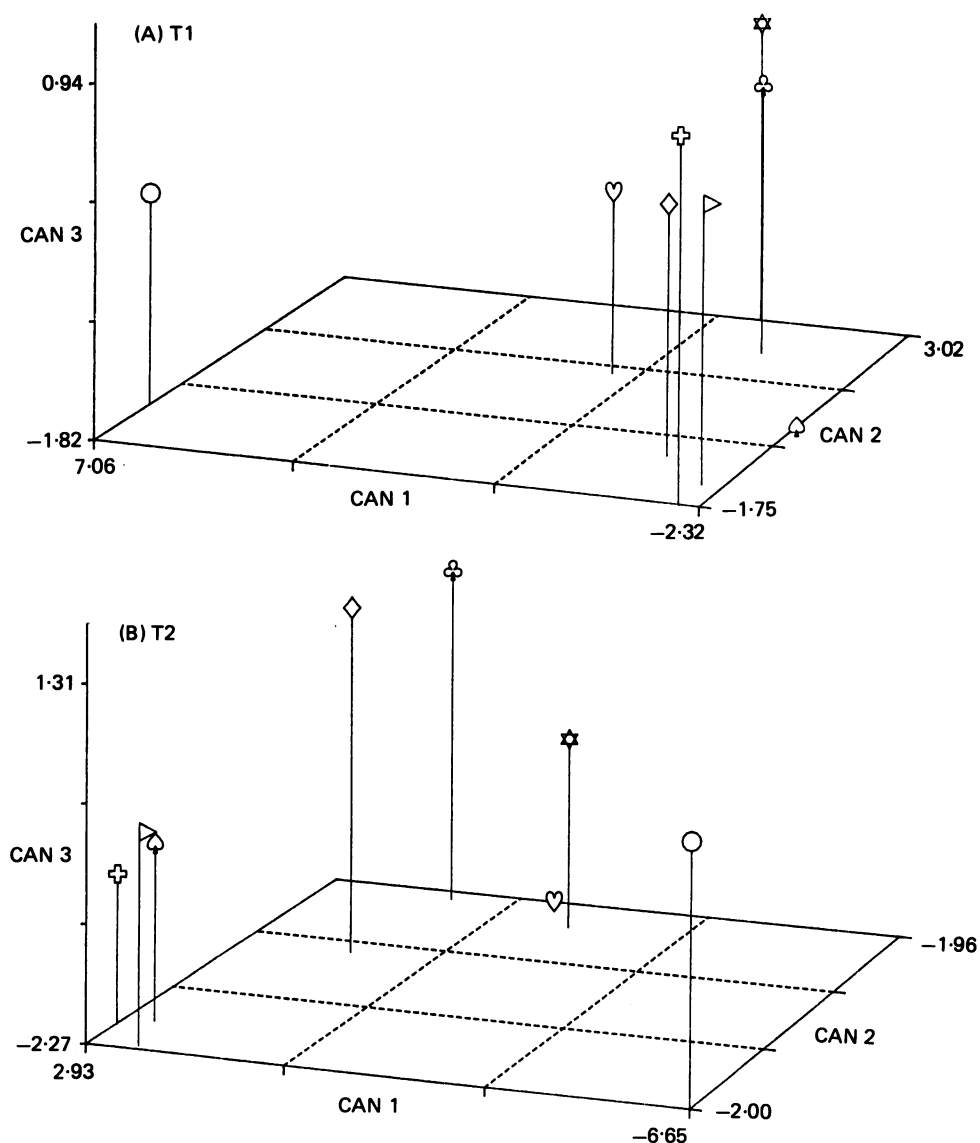
Fig. 5(A–B). (A) Three dimensional plot of the relationship of T1 vertebrae grouped by age. Key as in Fig. 6(A). 87·4% of the variance in this plot was incorporated into the first three Canonical axes. (B) Three dimensional plot of the relationship of T2 vertebrae grouped by age. Key as in Fig. 4(A). 83·5% of the variance of this plot was incorporated into the first three Canonical axes.

Table 3. *Mahalanobis' distances between sexes at different ages*

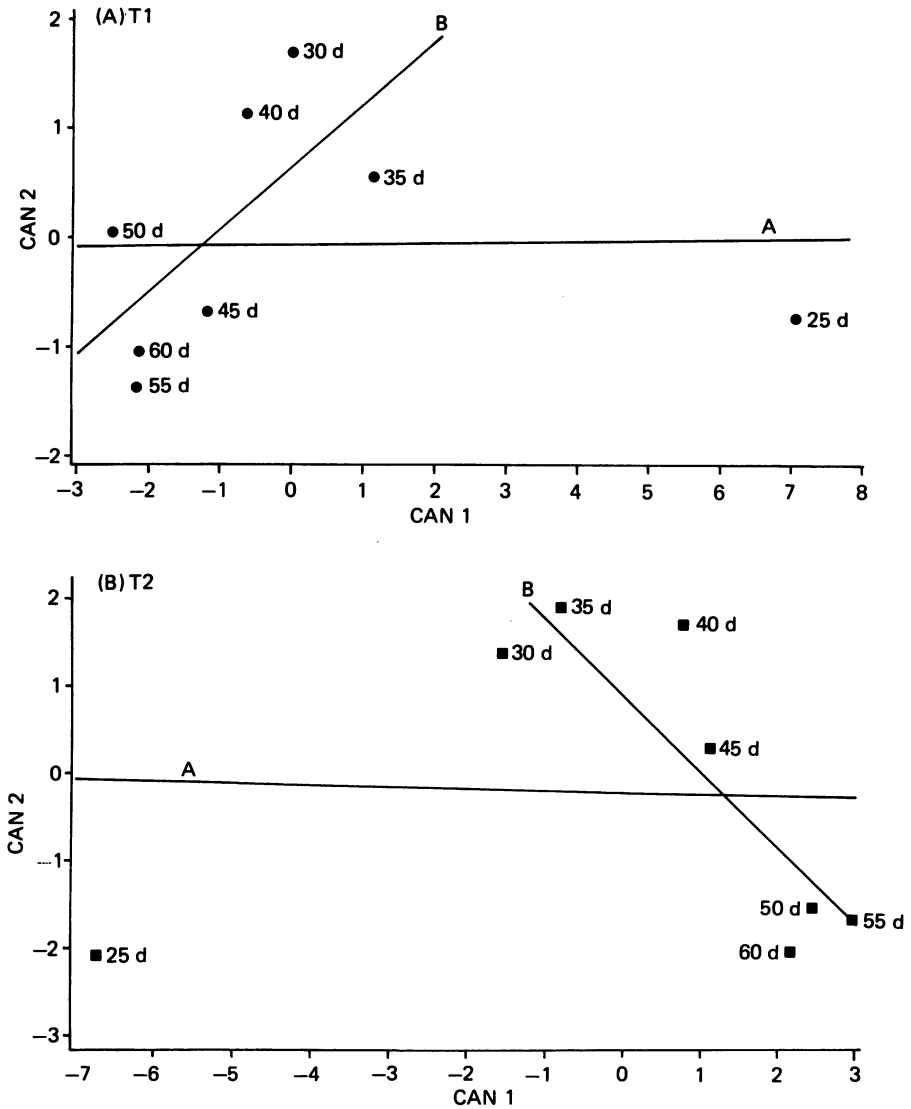| Age in days	25	30	35	40	45	50	55	60
				(A) Bone T1				
Male–female distance (SDU)	3·54	4·28	4·18	3·32	3·66	4·76	5·96	4·54
				(B) Bone T2				
Male–female distance (SDU)	3·72	5·18	4·04	4·12	6·14	4·54	5·07	9·89

Fig. 6.(A–B). (A) Two dimensional plot of the relationship of T1 vertebrae grouped by age, sexes pooled. The first two Canonical axes accounted for 82% of the variance. Line A is fitted to all data; line B is fitted to 30–60 day data only. (B) Two dimensional plot of the relationship of T2 vertebrae grouped by age, sexes pooled. The first two Canonical axes accounted for 75% of the variance. Line A is fitted to all data; line B is fitted to 30–60 day data only.

DISCUSSION

In this paper we have attempted to answer the questions 'How much does shape in mouse vertebral outline change as the mouse gets older?' and 'Is there any difference in shape between vertebral outlines from mice of a given age, but of different sex?'

Our samples were in the form of pseudo-longitudinal data, i.e. mice that were as similar as we could make them genetically and environmentally, killed at fixed ages. Since the age interval chosen, five days, was quite small we should expect some overlap between groups, with the fastest growing 30 day individuals, for example, resembling

the slowest growing 35 day mice. This is seen most clearly in T2 vertebrae (Fig. 4B).

There is an intuitive feeling that underlying the discontinuous data there should be a continuous, gradual, seamless, linear shape change with younger bone shape changing smoothly into that of older mice. This indeed seems to be so (Figs. 4, 6), with the exception of 25 day-old mice which are well separated from the rest.

Twenty five days is the youngest practicable age for the papain technique that we used to prepare dried bones. In mice aged 20 days or less from our stock vertebrae have not fully ossified and tend to fall into two lateral halves after papain treatment. We were therefore not surprised that the greatest shape differences we observed were between mice aged 25 and 30 days, both vertebrae behaving concordantly.

Separating age groups further by sex brings an inevitable decrease in group size and decreasing confidence in the spatial orientation of the group centroids. The mean distance between centroids of groups of the same age classified by sex again seems to be constant throughout. 60 day T2s (Table 3B) appear to be widely separated: we take this to be artefactual.

Most of the variation between group means of unsexed data can be resolved into two axes with little loss of discrimination, since the variation in Canonical axis 3 is small (Fig. 6). For each vertebra, as before, the 25 day group is widely separated from the rest. Suspecting a smooth transition of shape with age we could fit a straight line to all the data (line A, Fig. 6A, B) but this fits points representing the 30–60 day age groups very poorly. A much more convincing fit (line B) is obtained by fitting a line which excludes the 25 day individuals, i.e. by considering them as qualitatively as well as quantitatively different and as representing an immature vertebral shape.

The Fourier technique can only give an idea of how different two groups of vertebral shapes are, and can give no information as to where bones differ in shape. Reconstructed outlines of mean bone shape show clearly that in the 25–30 day interval we are recording change in localised shape, mainly in the vertebral arch and spinous processes. It is clear from Figure 3 that the bones are also growing most rapidly at this time.

The definitive shape of T1 and T2 is attained by 30 days. Thereafter change could be of two theoretical types. Change in the sites of remodelling from age group to age group should produce different directions in shape change. A constant pattern of remodelling would be expected to produce a more uniform pattern. In fact the latter seems to occur and we would predict two basic patterns of vertebral growth over the periods 25–30 days and 30–60 days respectively. From 30 to 60 days there is little change in size: despite this the shape of the bones is changing by a constant amount (measured as Mahalanobis' distance between mean shapes; Table 2) and in a linear manner in classification space. We feel that this change implies that the same 'factors' (whatever they may be) influence post-growth shape change in the period under consideration.

There is no evidence of increasing or decreasing sexual dimorphism in our sample, although male and female bones were of different shape throughout.

SUMMARY

The shapes of the first two thoracic vertebrae of F_1 mice produced by crossing BALB/c and CBA inbred strains have been examined at 25–60 days by Fourier analysis. The greatest shape change during this period is between 25 and 30 days and

is related to the finalisation of the form of the neural arch and spinous process. Between 30 and 60 days there is continued linear shape change not associated with further increase in vertebral area, and probably due to a constant pattern of localised shape changes. Both vertebrae and both sexes behave similarly.

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