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ORIGINAL PAPER

Is the vertebral canal prepared to host the aged spinal cord? A morphometric assessment

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Abstract Although the interaction between the growing spinal cord and the vertebrae has been widely demonstrated for mammal's prenatal and early postnatal life, there is no extensive knowledge about this interaction during late postnatal stages. It has been shown that spinal cord injuries are causally related to significant degenerative changes in bone properties. Nevertheless, information about a possible influence of the spinal cord on bone remodelling in adult healthy animals is missing. The aim of this research work was to assess possible morphological changes of the cervical vertebral canal of juvenile and aged rats during the ontogenetic period of adulthood that would justify the suggested influence. Since the spinal cord of rats increases its size with ageing, we analysed whether morphometric changes are occurring in the vertebral canal that would indicate bone remodelling in response to said growth. To this end, we used three complementary morphometric methods to describe the canal of the cervical and the first thoracic vertebrae. Geometric morphometric analyses evidence scarce variation in size and shape between juvenile and aged rats suggesting that, in general terms, the canal morphology of cervical vertebrae is already prepared in early adulthood to host the growing spinal cord. C3 was the only vertebra that showed consistent variation for the variables of canal thickness, perimeter, height and area. This regional variation may be linked to the patterns described for the changing spinal cord.

Keywords Vertebra · Morphometry · Rat · Remodelling · Ageing · Vertebral canal

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Introduction

The vertebral canal size has been studied in different species such as rats and humans (Flynn and Bolton 2007), red deer (Kumar et al. 2000, 2002), mouse, shrew, bandicoot, rabbit, grey squirrel (Kida et al. 1999) and several primates (Manfreda et al. 2006). These works were carried out using

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either conventional morphometry (Flynn and Bolton 2007; Tatarek 2005) or geometric morphometric methods (Manfreda et al. 2006). With the latter, it was established how the first cervical vertebra adapts to different forms of locomotion (Manfreda et al. 2006). Furthermore, Morishita et al. (2009) and Song et al. (2009) have linked morphometric data of the vertebral canal to the development of different spine diseases in humans.

Few studies have dealt with vertebral growth over the mammal's lifespan. Some authors carried out allometric studies in giraffes (van Sittert et al. 2010) and sheep (Ghazi and Gholami 1994), establishing their cervical spine growth during prenatal and early postnatal ontogeny. Nevertheless, no morphometric studies have focused on changes of the vertebral canal in aged animals.

In previous works, we have shown that rat spinal cord increases its size with ageing. It was observed that removal of the aged spinal cord from the vertebral canal is more difficult in comparison with juvenile specimens, since it is tightly adhered to its inner wall (Fontana et al. 2009; Portiansky et al. 2011). This pattern may suggest that in juvenile female rats, the vertebral canal is already prepared to host the larger size of the aged spinal cord and that it does not undergo further modification as the spinal cord grows. Nonetheless, there is no clear evidence about the potential existence of modifications in the vertebrae that may be the result of the interaction between the bone and the nervous tissue.

It has been demonstrated that spinal cord injuries are causally related to significant changes in bone properties of adult humans and rats (Jiang et al. 2007; Maïmoun et al. 2011). All these changes are regressive and include bone loss and alteration of bony structure and microstructure. In those cases, the effect of neural factors on bone remodelling in favour of bone resorption becomes evident. To our knowledge, no data related to the influence of spinal cord on vertebral bone in normal adults is available. Taking into account the expansion of the spinal cord previously reported in normal rats (Fontana et al. 2009; Portiansky et al. 2011), it would be feasible to find remodelling in vertebral bone due to that spinal growth. In particular, we are interested in the vertebral canal, which is in close contact with this nervous growing organ.

The aim of this article was to assess morphological changes of the cervical vertebral canal of juvenile and aged rats during the ontogenetic period of adulthood. For this purpose, we used three different and complementary morphometric methods. Results were then discussed in relation to the morphometric changes of the spinal cord to establish whether the vertebral canal reaches its final morphology in juvenile animals or suffer remodelling events that accompany the spinal cord growth during ageing.

Materials and methods

Animal procedures

Eleven juvenile (4–5 mo.) and eight aged (30 mo.) female Sprague–Dawley rats, from the INIBIOLP (School of Medicine, National University of La Plata) ageing rat colony, were used. Animals were housed in a temperature-controlled room (22 °C) on a 12:12 h light/dark cycle. Food and water were available ad libitum. All experiments with animals were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of INIBIOLP's Animal Welfare Assurance No A5647-01.

Rats were placed under deep anaesthesia, perfused with PBS and then with phosphate-buffered p-formaldehyde 4 %, pH 7.4, fixative solution. After fixation, the entire spine was dissected. Perivertebral muscles were removed by gross dissection, and the column was left macerating in water for a month. Putrid water was periodically replaced with fresh water. When macerating water remained clean, vertebrae were cleaned by fly larvae colony (*O.* Diptera, Fam. Calliphoridae) for 7 days. Each vertebra of the cervical region (C1–C7) plus the first thoracic vertebra (T1) were then separated and boiled in 2 % NaClO and bleached in 35 % H_2O_2 boiling water.

Morphometric analysis

Digital measurements

Vertebrae of juvenile and aged rats were scanned at 600 dpi using a flat bed scanner (Genius HR6X, USA) (Fig. 1). For this purpose, each vertebra was fixed on the scanner so that the entire light of its central canal would be expressed on the final image. Scanned images loaded into an image analyser (ImagePro Plus, v6.3, Media Cybernetics, MD, USA) were thresholded and filtered using "skeletonising" algorithms (thinning and pruning filters), establishing the midpoints of the entire vertebral structures to assess bone mass variation (Fig. 2). The aim of the skeletonisation (i.e., a one pixel width skeleton mask extraction from a digital binary image) is to obtain a region-based shape feature representing the general form of an object. This process is commonly used in pattern recognition. Measurements of the area, height, width and perimeter of the skeleton mask's canal were performed using the automatic Count/Size function of the image analysis program.

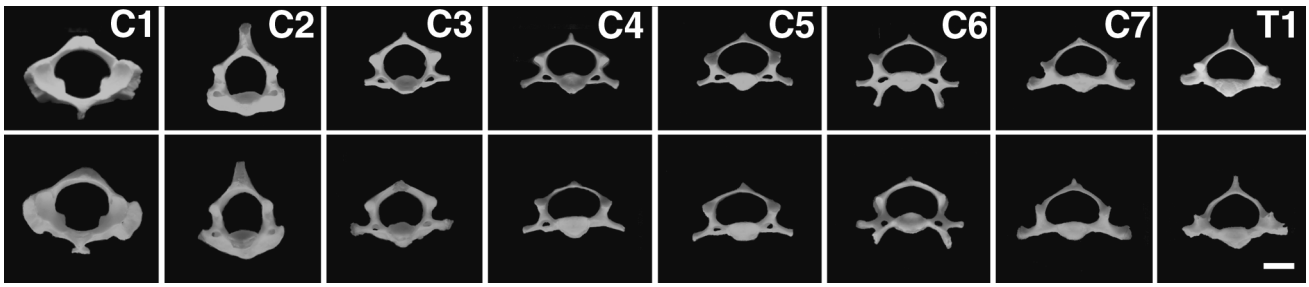


Fig. 1 Macroscopic aspect of vertebral bodies. Juvenile (*upper row*) and aged (*lower row*) vertebral bodies were scanned at 600 ppi. The seven cervical vertebrae and the first thoracic body are represented. *Bar* 3 mm

Fig. 2 Skeletonisation of a vertebra. A one pixel-thick skeleton (**b**) obtained by thresholding the original scanned image of a vertebra (**a**) is superimposed on it to show the middle point of the osseous mass (**c**)

Geometric morphometric analysis

For each scanned vertebra, the 2D coordinates of four landmarks and eight semilandmarks were digitised in the contour of the canal (its most external rim) to describe their morphology. Landmarks correspond to the most superior, inferior and lateral (right and left) points of the canal and are included under the type II of Bookstein's classification (Bookstein 1991). Two semilandmarks were evenly spaced and placed between each pair of landmarks. In order to eliminate variation due to measurement error, an intra-observer analysis of the error in landmark placement was carried out, and only one of the authors collected the morphometric points. On a subsample of 30 randomly chosen vertebrae, landmarks and semilandmarks were placed three times and measurement error was evaluated by means of repeated measures ANOVA. Once measures showed consistency among successive series, definitive digitisation was carried out. Semilandmarks were sliced using minimum bending energy (Bookstein 2002). From these raw coordinates and after sliding the semilandmarks, a size variable (centroid size) and a set of shape variables (Procrustes coordinates) were obtained using the Generalised Procrustes Analysis as the superimposition technique (Rohlf and Slice 1990). Using this procedure, it is possible to obtain pure shape data, defined as the geometric information that remains after the effect of scale (size) is eliminated. For data collection and semilandmarks' treatment, the software TPS series was used (Rohlf 2011). Generalised Procrustes Analysis was carried out for each vertebra (from C1 to T1) separately. For geometric morphometric analyses, the free software MorphoJ was used (Klingenberg 2011).

Manual measurements

To evaluate axial changes, thickness of vertebrae was manually measured by two of the authors using a digital calliper (Stronger®, Argentina). Vertebral arch thickness measurements were recorded just above the body of each vertebra.

Statistical analysis

Different statistical approaches were used to assess morphometric differences between both age groups. Thickness means as well as those of area, height, width and perimeter of skeleton masks were compared using a two-tailed Student's *t* test.

Geometric morphometric variables were analysed separating size (centroid size) and shape (Procrustes coordinates) aspects. Differences in centroid size between juvenile and aged rats were assessed with a two-tailed Student's *t* test. As shape variables are numerous, two multivariate statistical techniques were applied. A principal component Analysis (PCA) was performed on Procrustes coordinates to obtain axes that resume a large amount of variation. The first two principal component (PC) axes were plotted to point out whether both age groups differed in the main aspects of shape. In addition, Procrustes coordinates were compared using Procrustes ANOVA, which establishes variation in group's mean shape including all the Procrustes coordinates in the analysis (Klingenberg and McIntyre 1998). The probabilities of all the mentioned statistics tests were obtained through bootstrap using 1,000 iterations to cope with the reduced sample size.

Bootstrapping methods are based on resampling the original sample a large number of times. Therefore, resampling represents a close picture of what one would get when sampling the actual population (Hesterberg et al. 2003). Due to their properties, these procedures are preferable in contexts where sample sizes are not very large, such as in this experimental study. Statistical analysis of these processes was carried out using R (R-Development Core Team 2012).

Results

Differences between juvenile and aged groups were found in the thickness of the vertebra (Table 1). In particular, significant variations in C1, C3 and C6 were observed.

Digital measurements, performed on skeleton masks, showed that even though aged rats seem to present larger values, differences for the area and perimeter were only observed at C2, C3 and C4, while differences in height were computed only at C3. No differences were registered in width (Fig. 3). Data concerning measured height and width together with the visual aspect of the central canal (Fig. 1) clearly show its horizontal ovoid aspect in agreement with the morphometric aspect of rat spinal cord (Fontana et al. 2009; Portiansky et al. 2011). Summarising, a result that deserves attention is that C3 was the only vertebra that showed consistent variation for thickness, perimeter, height and area. C2 and C4 just varied for only one morphometric parameter.

Geometric morphometric analyses showed that the cervical spine canal of juvenile and aged rats is similar in size since no differences were found when the mean of the centroid size was compared (Table 2). According to this, canal size remains stable during late postnatal life in rats. Regarding shape, the dimensionality of the Procrustes coordinates was reduced through PCA in order to obtain the main trends of variation. In all vertebrae, the first two PCs resumed more than 50 % of total shape variation. In Fig. 4, the overlapping of juvenile and aged rats along PC1 and PC2 becomes evident. None of the observed distributions showed a segregation of individuals related to age. This means that there is no clear separation between both age groups regarding shape variation. In line with these results, Procrustes ANOVA showed that there are not significant differences in shape among groups when all shape variables are compared with the exception of T1 (Table 2). In conclusion, according to geometric morphometric analyses,

Table 1 Manual measurement differences between young and aged groups in vertebral thickness

Vertebra	*t*	*p*
C1	2.885	0.012*
C2	0.365	0.720
C3	3.132	0.006*
C4	0.716	0.484
C5	1.668	0.115
C6	5.649	0.001*
C7	0.851	0.408
T1	0.158	0.876

* Significant value ($p < 0.05$)

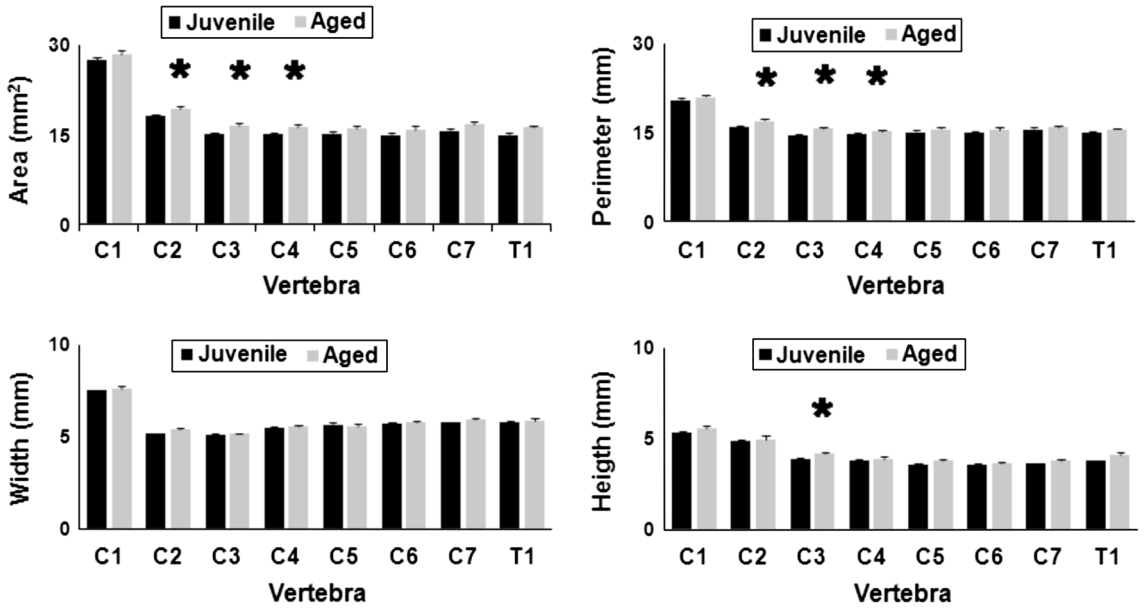


Fig. 3 Morphometric parameters of the skeleton mask in juvenile and aged rats. *Graphs* show similarities in area, perimeter, width and height between juvenile and aged rats. *Asterisks over bars* indicate a significant difference (*$p < 0.05$) from the corresponding aged counterpart

Table 2 Centroid size (*t* Student test) and shape (Procrustes ANOVA) differences between juvenile and aged groups

Vertebra	Size		Shape	
	t	*p*	*F*	*p*
C1	0.26	0.6175	1.37	0.1333
C2	0.11	0.7417	1.42	0.1091
C3	1.88	0.188	1.19	0.2571
C4	3.55	0.0767	1.45	0.0986
C5	0.04	0.8538	1.41	0.1129
C6	4.3	0.0537	0.55	0.9431
C7	2.5	0.132	0.64	0.8812
T1	2.73	0.1182	1.79	0.0209*

* Significant value ($p < 0.05$)

neither size nor shape is changing with age during the range of ontogenetic time considered in our study.

Discussion

Results obtained in our work favour the understanding of the dynamics of morphological variation during late adulthood in a mammal animal model. This represents a contribution to the biology of ageing, a developing area of our time, devoted to study processes that affect many human populations, where life expectancy has clearly increased (Nelson and Weiss 1999).

Regarding osseous system, some degenerative problems related to ageing have been of primarily interest for human species. Osteoporosis and osteopenia are two of these disorders, which imply bone mass loss, deterioration of its microstructure, and increased fracture risk (Kalu 1999). According to our results, there are no regressive changes in the vertebral canal morphology during advanced stages of rat's postnatal life. This is in line with other studies that described that in the vertebrae of non-ovariectomised female rats, there was no significant loss in bone mass due to osteoporosis or osteopenia as occurs in women (Jee and Yao 2001). A word of caution should be expressed, consequently, when using animal models in the study of problems that affect other mammals, since metabolic and developmental differences could generate information that is not homologue.

Spinal injuries have been related to bone degeneration (Maïmoun et al. 2011), a situation that as expected during normal ageing process was not detected in our healthy aged rats. Moreover, as rat's spinal cord showed an enlargement (Fontana et al. 2009; Portiansky et al. 2011) during ageing, our hypothesis was aimed at determining whether the growth of the spinal cord induced some remodelling of the vertebrae that host it.

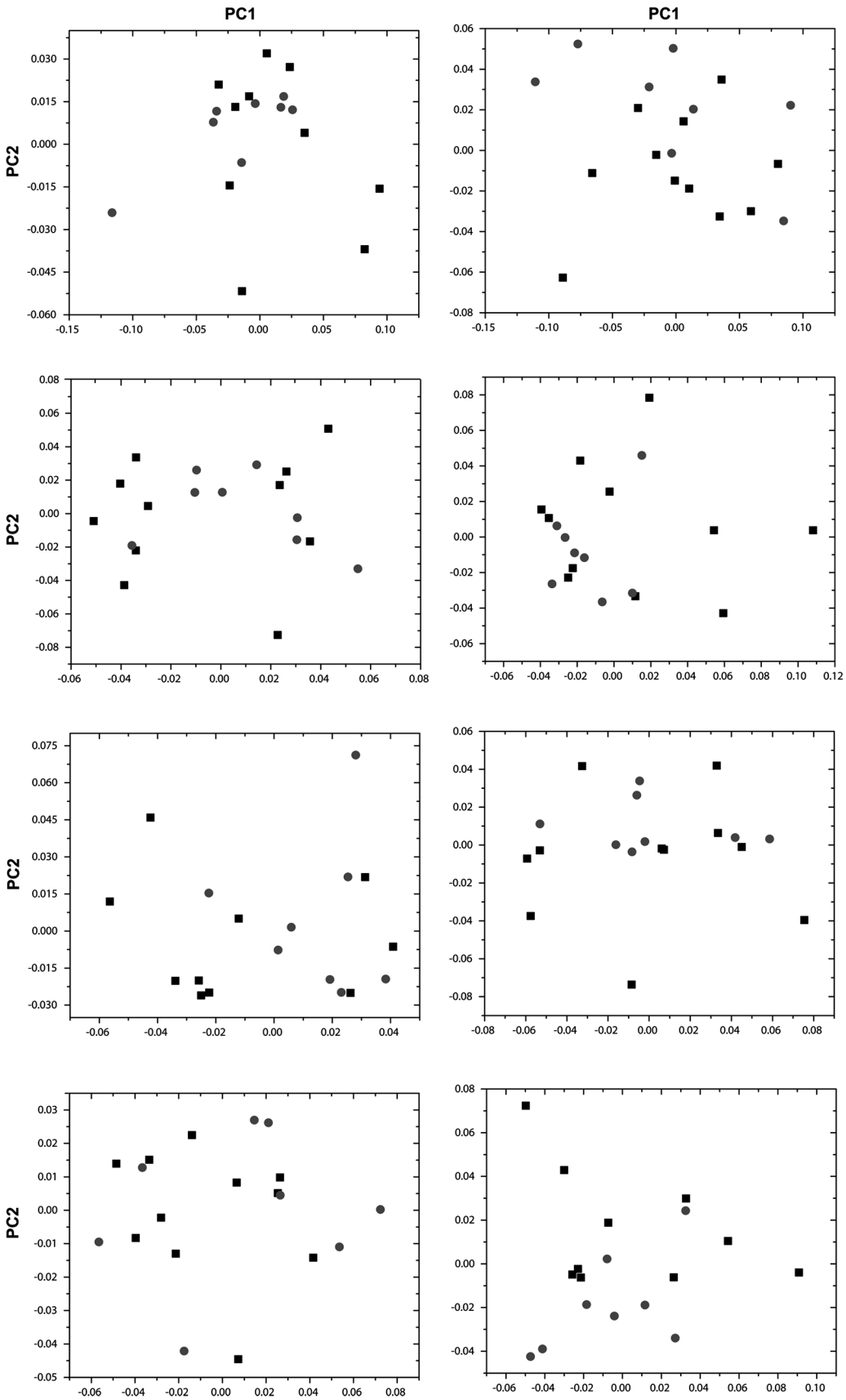
Considering our results from a general perspective, the structure of the cervical canal in rat's vertebrae showed a relative steadiness between juvenile and aged specimens. Geometric morphometric analyses demonstrated that shape and size of the canals did not vary due to ageing. In the same line, most of the manual and digital measurements displayed the same pattern, where the parameters were maintained. Therefore, a broad conclusion allows to speculate that in early adulthood, the vertebral canal is already prepared to host the growing spinal cord.

However, some regional differences were found for particular cervical vertebrae between both age groups. This is in accordance with the findings presented by Jee and Yao (2001) who described that the lumbar vertebral growth plates are open even in 21-month-old rats, meaning that localised modifications in the vertebral column of mammals could last until advanced ages.

A coincidence using manual and digital measurements was the variation in parameters at C3 between juvenile and aged rats. At the corresponding spinal cord segment, Portiansky et al. (2011) found that dimensions increased in size and enlarged up to 1.1 mm. Taking this into account, remodelling processes due to neural induction could not be completely discarded. Although during early embryogenesis the forming nervous system induces osteogenesis (Hall 2005), precise data concerning nervous tissue local regulation of bone remodelling during late postnatal life is not available. In the adult animal, insulin growth factor-1 (IGF-1) and members of the bone morphogenetic protein (BMP) family factors are produced in the neurogenetic areas (Peretto et al. 2004). These factors are relevant inducers of bone growth (Caetano-Lopes et al. 2007), and therefore, these would have a paracrine activity on the bone surrounding particular regions of the CNS.

Discrepancies among linear (manual and digital) and geometric morphometric techniques are expectable since it has been extensively demonstrated that the information obtained depends on the morphometric approach employed (Menendez and Pérez 2011). Our results reinforce the need to focus morphometric studies with complementary approaches, which give different information depending on the degree and pattern of variation of the structure under evaluation.

In conclusion, this work provides data regarding the ageing processes in the axial skeleton that could be related to spinal cord changes during this ontogenetic period. Although in general terms we can state that cervical vertebral canal remains stable, there are some regional


PC1
PC1
PC2
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PC2

◀**Fig. 4** First two PCs estimated from superimposed coordinates. Juvenile (*black square*) and aged (*grey circle*) rats dispersion along PC1 and PC2 is shown. From left to right and from top to bottom, *scatterplots* show the results for the successive vertebrae (C1–C7 and T1)

variations during adulthood that may be linked to the variation described for the spinal cord. This opens a question about possible interactions between normal aged spinal cord and bone that needs to be tested more extensively.

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