Online Resource 1. Sampling sites for fluoride analyses from the bones of eastern grey kangaroos at Portland and Cape Bridgewater. A – proximal right humerus (after longitudinal section); B – diaphysis of right humerus; C – right radius; D – right ulna; E – rib 7; F – vertebra (after sagittal section, medial view); G – mandible. The lines of section (i.e. where the bone was cut for the sample) are indicated by straight lines, and sample site denoted by F. Duplicate samples were collected from ribs 7, mandibles, vertebrae, ilia (all one sample each from left and right side of body); further duplicate samples were collected from the diaphyses of the femur and tibia (both samples from right side).
Online Resource 1 (cont.) A – proximal right femur (after longitudinal section); B – diaphysis of right femur; C – proximal right tibia (after longitudinal section); D – diaphysis of right tibia; E – distal right tibia (after longitudinal section); F – proximal right fibula; G – distal right fibula (after longitudinal section); H – pelvis; I – proximal right metatarsus 4 (Mt4) (after longitudinal section); J – diaphysis of right Mt4; K – distal right Mt4 (after longitudinal section); L – right calcaneus (after longitudinal section)
Sample selection and analysis

To test for possible bias associated with fluoride concentrations obtained from the main laboratory (Portland Aluminium), duplicate bone samples were collected from some sites and analysed in two laboratories. The majority of samples were analysed at the laboratory at Portland Aluminium, but duplicates of two sample sites per kangaroo were sent to an independent laboratory (ChemicalAnalysis Pty Ltd, Bulleen, Victoria). Sample sites were chosen to provide samples as symmetrical as possible, especially in regards to position within the bone and proportion of cancellous and cortical bone. This resulted in ten duplicate samples for each kangaroo (five vertebral body samples, femoral diaphysis, tibial diaphysis, ilium, mandible, rib 7). Out of these ten samples, two samples were randomly selected and sent to the independent laboratory for bone fluoride analysis. Out of the two duplicate samples analysed per kangaroo, one sample was then randomly selected for statistical analysis to simplify statistical analysis. Fluoride samples were analysed using fluoride ion selective electrodes, with both laboratories using the same (National Association of Testing Authorities, Australia (NATA)-approved) methodology.

Statistical Analysis

The comparison of fluoride analysis results between laboratories was performed using linear correlation, Passing Bablok regression and Bland Altman (Limits of Agreement) plots. Linear
correlation and regression analysis do not provide any information on the actual differences between the results (Bland and Altman 1986; Dohoo et al. 2003). The slope of the intercept and coefficient in regression analysis can be used to determine whether there is constant (intercept significantly different from zero) or proportional (slope significantly different from one) systematic error (Jensen and Kjelgaard-Hansen 2006). A constant error would suggest that results from one laboratory are consistently lower or higher than from the other laboratory; a proportional error would suggest that the differences between the two laboratories increase or decrease with increasing or decreasing fluoride concentration. Either type of error is a reflection of analytical bias (Jensen and Kjelgaard-Hansen 2006). Bland Altman plots demonstrate the difference of results between two methods or laboratories in relation to the mean result value of the two methods or laboratories. In addition, limits of agreement are calculated, based on 1.96 standard deviations above and below the mean difference in results of all samples examined (Bland and Altman 1986). Bland Altman plots and Passing Bablok regressions were calculated using MedCalc® Version 10.4.5.0 (Frank Schoonjans, 1993-2009).

**Results**

The mean fluoride concentrations (± SEM) for the 61 samples selected were 2550 ± 285 (Portland Aluminium) and 2135 ± 224 (independent laboratory) µg/g dry bone. Bone fluoride concentrations were log-transformed for subsequent analysis. There was a significant, positive linear correlation between the log-transformed dry bone fluoride concentrations of the two laboratories (Pearson’s rho = 0.968; p < 0.001). The Passing Bablok regression suggested no significant constant (intercept: -0.068; 95% CI of intercept: -0.29, 0.14; p > 0.10) or proportional error (coefficient of slope: 1.038; 95% CI of slope: 0.97, 1.10; p > 0.10). The Bland Altman plot showed that on average, results from the Portland Aluminium laboratory
Bone fluoride results from the Portland Aluminium laboratory tended to be higher than those from the independent laboratory, and the limits of agreement included a relatively large range of values, from -1030 to +1860 µg/g dry bone. Two laboratory methods are generally regarded as being in agreement if the differences in results falling within the limits of agreement are not clinically important (Bland and Altman 1986). While the differences observed here probably do have clinical importance, they may well be primarily a result of pre-analytical variation. Although the duplicate samples were collected so as to reduce
inherent differences in fluoride concentration, they were either collected from opposite sides (e.g. sample for Portland Aluminium from the left rib, duplicate sample from the right rib), or from immediately adjacent sites on the same bone (e.g. one sample from the left tibial diaphysis just proximal to the other sample). Some inherent sample variation may therefore have contributed to analytical variation. This could be avoided in the future by dividing already ashed samples into two equal portions and sending the two portions to separate laboratories for fluoride determination. There was, however, no systematic error detected in the results from the two laboratories, and hence no evidence of bias from either laboratory.

References

