Eggshell speckling in a passerine bird reveals chronic long-term decline in soil calcium

Short title: ***Eggshell and soil calcium***

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SUMMARY

Capsule: Long-term changes in eggshell speckling correlated temporally and spatially with declining soil calcium in an English Great Tit population.

Aims: Eggshell speckling in the Great Tit correlates with eggshell thickness and calcium availability. We investigated whether eggshell speckling changed in a Great Tit population during a period when soil calcium declined, and estimated changes in eggshell thickness.

Methods: Eggshell pigmentation of 2,313 Great Tit clutches was recorded in 1988—2007 across a geological gradient in Wytham Woods, UK. Soil survey conducted in 1974 and 1991 was repeated in 2008. Temporal and spatial changes in eggshell speckling and soil calcium were examined using GLMM. We also estimated the change in eggshell thickness over this period.

Results: Soil calcium availability declined 1974—2008 especially in calcium-rich limestone areas. A systematic change in eggshell speckling, approximating to a 6.5% reduction in eggshell thickness, occurred between 1988 and 2007. The change was greatest in parts of Wytham that experienced the greatest decline in soil calcium. Hatching success was unaffected.

Conclusion: Soil calcium availability has declined in Wytham Woods, affecting Great Tit eggshell characteristics. This is the first evidence that declining soil calcium affects birds nesting in calcium-rich areas. Practical applications of these findings are considered.

Calcium is an essential micronutrient for breeding birds during egg formation (Reynolds & Perrins 2010). To obtain calcium, small woodland birds specifically target calcium-rich foods such as snails (Graveland 1995, 1996), the distributions of which are strongly influenced by the local availability of soil calcium (Jubb *et al.*, 2006). Consequently, birds breeding in areas with poor soil calcium may lay eggs with reduced eggshell-thickness (Mand *et al.*, 2000), which determines eggshell-strength and permeability (Ar *et al.* 1974, 1979), and/or may be restricted in the number and quality of offspring they produce. This is because a limited supply of calcium may need to be apportioned inadequately between nestlings, which may suffer from skeletal deformities as a consequence (Graveland *et al.* 1994, Eeva & Lehikoinen 1995, Graveland & Drent 1997, Tilgar *et al*. 2002).

Like many small passerine birds[a], Great Tits *Parus major* lay white eggs speckled with reddish spots. This speckling relates in complex ways to calcium availability and shell thickness since it consists of both heritable and non-heritable components (Gosler *et al.* 2000, 2005, Higham & Gosler 2006, Garcia-Navas *et al.* 2011, Mägi *et al.* 2012, Brulez *et al.* 2014). Nevertheless, the spots specifically mark thin areas of shell, and are typically more intense and concentrated towards the blunt pole or 'crown' (i.e. less evenly distributed over the shell surface) in eggs of birds nesting on low-calcium soils (Gosler *et al*., 2005, illustrated in Fig 2 herein). This 'pigment-spread' has been resolved as a non-heritable (i.e. largely environmentally determined) component of the speckling pigmentation, which can be influenced by the experimental provision of a calcium-rich food supplement (Garcia-Navas *et al.* 2011, Hargitai *et al.* 2013), and which is strongly, and positively, related to shell thickness and strength (Gosler *et al*., 2005, Gosler *et al.* 2011, and results herein) as well as hatching success (see Sanz & García-Navas 2009).

Hargitai *et al.* (2016a, b) have recently reported a number of environmental effects (including metal pollutants) on egg characteristics including eggshell pigmentation in the Great Tit. While an alternative hypothesis of a signalling function for eggshell speckling in the Great Tit have been unsupported (Cherry & Gosler 2010, Stoddard *et al.* 2012), Hargitai *et al.* (2013, 2016a, b) have provided further support for its structural function. The link between dietary calcium for eggshell formation in the Great Tit and local soil calcium availability is made explicit through the nesting female's consumption during the egg-laying period of shell from small snails whose abundance and distribution are correlated with soil calcium availability (Graveland 1995, Gosler 2006, Jubb 2006). In this paper we demonstrate a long-term decline in pigment-spread in a Great Tit population, correlated both temporally and spatially with a measured decline in soil calcium availability, and present evidence to suggest that this also reflects a long-term reduction in eggshell thickness in this population.

METHODS

Study site

The study was conducted as part of the long-term population study of the Great Tit in Wytham Woods, near Oxford UK (51° 47’N, 1° 19’W). The study site consists of the north slope of Wytham Hill, which is produced by a geological transition from clays of the Thames floodplain at its base (Oxford clay), through sands to limestone (Lower and Upper Corallian beds respectively) on the hill-top. This transition has resulted in a steep gradient in soil calcium, from the hill-top, where calcium should never be limiting, to the Thames floodplain, where its availability (based on 1974 soil data) is some 400 times less and may be in short supply (Farmer, 1995, Gosler *et al*., 2005, Jubb *et al*., 2006).

Soil sampling

Wytham’s soil chemistry, including calcium, was first surveyed in May to August 1974 by the Commonwealth Forestry Institute, University of Oxford (Dawkins & Field, 1978). A partial re-survey, covering a sub-set of 50 of the 163 original plots, was undertaken in 1991 (Farmer 1995). This resurvey followed the same protocols as the original, i.e. samples were collected using augers from three depths (1: 0–10 cm; 2: 11–20 cm; 3: 21-30 cm) at four locations within 10m of each grid point. Soils from the four locations were then pooled within depth categories to give three samples per plot. In a few cases the soil was shallower than 30cm so that samples could not be collected from the deepest level in these cases. A further re-survey of these 50 re-sampled sites was undertaken in March 2008 following the same protocols (again some plots could not be sampled at depth). In each analysis, a number of samples collected in the previous surveys were re-analysed in the laboratory as controls. These gave good agreement with the earlier analyses[b]. For logistical reasons (stated above) comparison across the three years 1974, 1991 and 2008, could only be made for all three depths from 31 plots. Thus considering all depths, we have 150 re-samples between 1974 and 1991 (Farmer 1995), and 93 between 1974 or 1991 and 2008. As a further test of the agreement of current analysis with those conducted earlier, we constructed a General Linear Model with the current analysis data as the dependent variable, and depth layer and year of collection as factors. As before, we found a very high degree of agreement between current and earlier calcium evaluations, and neither depth nor year of collection affected the results[c]. We therefore have a high degree of confidence in the comparative analyses presented here.

Egg sampling

The general methods of the Wytham Great Tit population study, started in 1947, have been described many times (Perrins 1979, Gosler, 1993, Gosler *et al*., 2005, Wilkin *et al*. 2006). Most of the population breeds in nestboxes (*n* = *c.*1000), which are visited by ornithologists weekly from early April to record the date of the first egg (assuming one egg is laid each day), clutch-size and egg-mass. From 1988 to 2007, the pigment patterns of every Great Tit clutch (2,313 clutches) was recorded by a single observer (AGG) according to a standardized protocol in which the intensity, distribution and size of pigment spots were recorded, and subsequently resolved into two principal components (Gosler *et al*. 2000, 2005, Gosler 2006) by way of a Principal Components Analysis. Variation in the first principal component (pc1 termed 'pigment darkness') is partly heritable (Gosler *et al*., 2000) whilst that of the second (pc2 termed 'pigment spread') is not (Gosler *et al*. 2005), i.e. implying that variation in it is more strongly related to environmental effects such as soil calcium availability than to genetic influence. Soil calcium was related to each nestbox as the maximum (from the 1974 Ca values) of the nearest 3–5 soil samples, and normalized as its Log_{10} transformation (Gosler *et al*., 2005). Adult birds were trapped, whenever possible, about a week after the eggs hatched; they were individually identified by numbered metal BTO rings. Nestlings were ringed and weighed on day 15 after hatch (hatchday=1).

Gosler *et al*. (2005) showed that pigment spread is positively correlated with eggshell thickness, egg-shape (defined as the breadth: length ratio), and Log_{10} soil calcium in this population. To estimate the dependence of pigment-spread on these three predictors (i.e. to assess whether pigment spread might be taken as a surrogate for shell thickness and calcium availability) we used data from 36 unincubated eggs from six clutches collected under license in 2004 for the purpose (see Gosler *et al*., 2005). From these data we calculated the change in deviance of the Minimum Adequate Model (MAM) from a Generalized Linear Mixed Model (GLMM) using Restricted Maximum Likelihood (REML in Genstat Release 8), with pigment-spread as the dependent variable, nest I.D. as a random variable, and shell-thickness at the shoulder (Gosler *et al*., 2005), Log_{10} soil calcium, and egg-shape as fixed effects (see also Gosler *et al*., 2005). The linear equation relating eggshell-thickness to pigment-spread was derived from the same model, with eggshell thickness as the dependent variable, and used to estimate the change in shell thickness corresponding to any observed change in pigment spread over the 20-year study period.

Relating eggshell and soil-calcium changes

To examine the change in soil calcium over time, we constructed a General Linear Mixed Model with soil calcium as the dependent variable, and all available factors as predictors. These included: sampling plot as a random factor together with its interaction with year (1974, 1991 or 2008), and year and depth level as fixed effects. Only plots examined in both 1991 and 2008 were included in this model.

To determine the interaction of effects between soil calcium and year on eggshell pigment spread, we used a GLMM (REML in Genstat Release 8) with pigment spread as a dependent variable, female I.D. (*n* = 2,024) as random factor, female age and Year × $\log_{10}$ soil calcium interaction as fixed effects (MAM).

To test further for a correlated response over time of eggshell pigment spread (PC2) across plots to changing soil calcium availability, we calculated the correlation across plots between changes in eggshell pigment spread between 1991 and 2007, and the change in calcium availability in those plots between 1991 and 2008. In so doing, we have also taken into consideration the finding that breeding data were most strongly correlated with soil calcium at a scale of around 300m from each nestbox in this population (Wilkin *et al.* 2009). This indicates the scale at which individuals acquire calcium and therefore also the scale at which changes in soil calcium would be expected to correlate with associated changes in pigment spread if the two processes were related. To test this prediction, circles of 150m and 300m radius were drawn around each of the 50 soil plots sampled in both 1991 and 2008 (see Fig. 1). Data collected from all nestboxes within one year of soil sampling (1991 and 2007) within a 150m or 300m radius were averaged for the plot concerned. This gave n = 20 units for comparison at 150m and 28 at 300m (Fig. 1). At each scale (150m and 300m), and for each plot, the difference was then calculated between the mean pigment spread (PC2) of clutches laid in 1991 and of those laid in 2007, and the correlation between that difference and the difference in observed calcium at the plot between 1991 and 2008 was calculated across plots (i.e. d.f. = 18 at 150m and 26 at 300m). Our previous studies (Wilkin *et al.* 2009) make a strong directional prediction: that there should be a significant positive correlation at 300m but a weaker (or no) response at 150m. We therefore report the correlation coefficients with one-tailed probability at 150 and 300m range from the plots.

Note that although the nestbox sampling plots, based on soil survey locations, overlap (i.e. a nestbox may be included in more than one soil-sampling plot) this does not constitute pseudoreplication in this analysis since the sampling units (and degrees of freedom for the analyses) are based on the individual soil sampling plots and not on the nestboxes. Comparisons of means were made over a long period of time during which a total change in the population would have occurred. Therefore, whilst it is possible that changes in both soil calcium and eggshell pigmentation could reflect an unknown third factor, any correlation between them is not the result of Type I error and the most parsimonious explanation is that they are functionally linked.

Changing response of clutch-size to changing calcium availability

Although eggshell pigmentation data are available for the last 20 years for this population, a longer series of observations is available on clutch-size (since 1965 for our specific study area). Wilkin *et al*. (2009) found a significant positive effect of local soil calcium on clutch size. However, they did not explore whether the size of the effect had changed over time, as might be expected from a significant change in environmental availability of calcium. To examine this, we used a GLMM (REML in Genstat Release 8) with clutch size as dependent variable and female I.D. (n = 4265) as a random factor. Lay-date, tessellated territory size restricted to 2ha (Wilkin *et al*. 2006) and female age were included as fixed effects in the model as these are known to be strong predictors of clutch-size, and the interaction Log_{10} soil calcium × year was also included as a fixed effect to test for a change in the size of the effect over time.

RESULTS

The dependence of eggshell-pigment spread on shell thickness

Data from 36 eggs used previously to examine the relationship between pigmentation and shell-thickness (Figure 3 in Gosler *et al*., 2005), revealed that 90.5% of the variation in pigment-spread is due to shell-thickness (70.9%, Wald_1=26.88, P<0.001) and egg-shape (19.6%, Wald_1=-7.06, P=0.008), both of which are strongly influenced by calcium availability (Gosler *et al*., 2005); a further 6.5% is explained directly by soil-calcium availability (Wald_1=6.81, P=0.009). Pigment spread therefore potentially offers an effective, non-destructive, surrogate measure of eggshell thickness. Furthermore, the lack of heritable variation in this measure (Gosler *et al*., 2005) means that any significant change in pigment-spread over time can be confidently attributed to factors associated with calcium availability. Reduced major axis regression of shell thickness (ET) on pigment-spread (PC2) gave the following calibration: $ET_{(mm)}= 0.069858$ [±0.000954 s.e.] + 0.004671 [±0.001066 s.e.] × PC2, $F_{1,31}=19.2$, $P<0.0001$.

Changes in eggshell pigment spread over time

We found long-term changes in pigment-spread in the Wytham Great Tit population. Over the 20 years of this study, pigment-spread declined strikingly (Fig. 2) and, based on the calibration given above, the overall change over this period is equivalent, on average to a 6.7% reduction in eggshell thickness. Between 1988 and 2006 (the lowest year - see Fig 2) this is a change from a mean estimated shell thickness from 0.0725 mm to 0.0675 mm: a change of 6.89%.

Changes in calcium availability of Wytham soils

The partial resurvey of Wytham soils in 1991 revealed that although highly correlated with 1974 values (across three samples per plot: $r_{148} = 0.891$, $P<0.0001$), calcium availability had declined, and especially in the most calcium-rich soils on limestone (Correlation: calcium-change and 1974 value: $r_{148} = -0.868$, $P<0.0001$, Fig. 3), (Farmer, 1995). Indeed, the least-calcic soils showed a weak increase in available calcium (Fig. 3). The reduction in soil calcium availability between 1974 and 1991 was strongly correlated with increased acidification of the soil, i.e. a decline in soil pH (correlation between % change in calcium and % change in pH across all samples: r = 0.364, P $P<0.0001$; the mean change in pH -0.87 or -13.77%).

The 2008 partial resurvey reported yet a further fall in available calcium (Figs. 4 & 5) since 1991, a period which largely embraces that during which eggshell pigmentation was recorded. The GLM constructed to test the significance of changes found strong effects of sampling plot ($F_{30,168}=2.78$ $P<0.0001$) and year ($F_{2,168}=8.42$ $P<0.001$) on available calcium, and a highly significant interaction between plot and year ($F_{60,168}=2.31$ $P<0.0001$) on calcium content, indicating that plots differed strongly in the degree of change that they experienced. The sample depth was not significant ($F_{2,168}=0.36$ $P=0.696$). Note that across the 31 plots (×3 samples per plot) for which we have three observations over 34 years, the change observed between 1974 and 1991 was uncorrelated with that observed between 1991 and 2008 ($r_{91} = 0.110$, $P=0.312$). However, plots that lost calcium between the first two sampling periods tended either not to have changed or to have lost further calcium during the second sampling period, and no plots increased in calcium availability during both sampling periods (Fig. 6).

Relating eggshell pigmentation/thickness to calcium availability

If the temporal change in eggshell pigmentation observed resulted from the reduction in calcium availability, the year effect should be greatest on the limestone soils and least on the clays. This is indeed the case. The GLMM assessing the overall year effect on pigment spread in the population (across 1,925 breeding attempts where both the female's identity and soil calcium were known and entered as a random effect) showed a strong effect of female age, with older females showing less spread in pigmentation (-0.1358 ± 0.03311, $\text{Wald}_1 = 16.83$ $P < 0.001$) and year (-0.05932 ± 0.003464, $\text{Wald}_1 = 293.27$, $P = 0.0001$) and a strong interaction between year and soil-calcium (-0.01937 ± 0.005778, $\text{Wald}_1 = 11.23$ $P < 0.001$), with an increasing strength of relationship (negative effect) with increasing soil calcium value. This is shown by Figure 7, which indicates a convergence between shell-pigment spread in different regions, suggesting that the effect of differences in geology is becoming less distinguishable.

The importance of local soil calcium in determining eggshell pigmentation is emphasized by the correlated response across survey plots over time between the degree of change in eggshell pigmentation and the degree of change in soil calcium. As predicted, whilst we found no significant correlation between changes in pigment spread and soil calcium at a radius of 150m from each nestbox ($r_{18} = 0.218$, $P = 0.178$ [one-tailed]), the correlation at 300m was both significant and positive ($r_{26} = 0.371$, $P = 0.026$ [one-tailed]). This result indicates that the greater the change in soil calcium at a plot, the greater the response in pigment spread, but, consistent with earlier studies, this was only true for birds nesting at a distance of about 300m from the focal soil plot: the distance at which females are believed to obtain calcium. Note that the difference in statistical significance between ranges was not due to the difference in sample size because the correlation at 150m was still non-significant when using the 300m d.f. of 26 ($r_{26} = 0.218$, $P = 0.133$ [one-tailed]). The relationship at 300m is shown in Figure 8, which also shows that all plots declined in both calcium availability and pigment spread.

Response of Clutch-size to changing calcium availability

For clutch-size, we found strong effects of factors already known significantly to predict this, namely lay-date ($\text{Wald}_1 = 838.24$, $P<0.001$ – negative effect), tessellated territory size restricted to 2ha (Wilkin *et al*. 2006 – positive effect) ($\text{Wald}_1 = 16.68$, $P<0.001$), year ($\text{Wald}_{40} = 24.68$, $P<0.001$) and female age ($Wald_1$= 30.69, P<0.001 – positive effect), but we also found a strong interaction between Log_{10} soil calcium and year ($Wald_{40}$= 1.85, P<0.001), indicating through an increasing effect size, that calcium availability has become increasingly important as a predictor of clutch-size (positive relationship) since 1965 (Fig. 9).

Finally, it should be noted also that, across years, pigment darkness (which is independent of spread, is partially heritable, and which indicates the 'depth' of shell thinning) showed a general increase (r_{17} = 0.611, P = 0.007), which, while consistent with the possibility that shells became more deeply pitted at pigment spots over time (see Fig. 3. in Gosler *et al*., 2005), we cannot determine whether the temporal variation in this trait was due to changes in the environmental or genetic components (or both) of the phenotypic variation.

DISCUSSION

Unlike large 'capital breeder' species, such as Domestic Fowl *Gallus gallus*, which can store large quantities of calcium in their skeletons that can be liberated for eggshell formation (Whitehead 2004), small passerine birds such as the Great Tit are 'income breeders' with respect to calcium, since their skeletons are too small to store much calcium for eggshell formation. Calcium availability during egg laying should therefore be expected to influence eggshell thickness and consequently eggshell speckling, and so changes in calcium availability over time should be reflected also in the speckling of Great Tit eggs.

We found a striking decline in eggshell pigment spread (Fig. 2), consistent with a 6.7% reduction in eggshell thickness, over the twenty years of the study. The lack of heritability (e.g. mother-daughter correlation) in this character (unlike pigment darkness which shows strong mother-daughter correlation) suggests that its systematic change over time did not reflect an underlying change in genetic or maternal effects, e.g. resulting from selection (Gosler *et al*. 2005). It follows therefore that the changes reflect environmental influences on the eggshell, and, from previous studies in Wytham and elsewhere, the most likely influence is calcium availability. This argument is strongly supported by the fact that some 96% of the variation in pigment spread can be accounted for directly by eggshell thickness, which is related to calcium availability, or by calcium-related factors (Gosler *et al*. 2005). Furthermore, when taken together with the observed reduction over years in soil calcium within sampling plots, and the interaction of effects across sampling plots, which corresponds with the interaction of effects on eggshell pigmentation, the evidence that this is the cause becomes compelling.

The cause of the decline in soil calcium availability in Wytham Woods is unknown, although it is consistent with widespread soil acidification in England resulting from acid deposition from air pollution (Johnston *et al.* 1986, Blake *et al.* 1999). Globally, soil acidification resulting from Sulphur and other emissions from power stations is a significant environmental concern (Galloway 1995). While we cannot prove that calcium-leaching due to acid deposition is the cause of the reduced soil-calcium availability observed in Wytham Woods, it is difficult to conceive of an alternative factor that could reduce calcium availability in such shallow, limestone-underlain, soils within the relatively stable environment of this site, where the only agricultural activity within 1 km of the limestone woodlands is sheep grazing. Around the northern margin of Wytham Woods, on the clay soils of the Thames flood plain, arable farming has sometimes been undertaken. The 1974 soil analysis for these areas shows increased potassium levels at the edge of the wood, up to three times that found 300m into the wood on the same clay soils. The buffering effect of this, which may have resulted from fertilizer drift into the wood from adjacent farmland, might account for the small but significant increase in calcium availability between 1974 and 1991 in those clay areas.

Although all rainwater is acidic (typically pH 5.3) because of the acidifying effect of CO_2 in the atmosphere, it is generally expected that limestone soils are buffered against the acidifying effects of rainfall since the pH of $CaCO_3$ in water is 9.9. It is reasonable, therefore, to look for a significant pollution source within the vicinity of Wytham. During the period of our study, the English Midlands received some of the most acidic rainfall in the UK, due to sulphur deposition resulting from industrial and power-station SO_2 emissions. Between 1987 and 2004, sulphur emissions decreased by 78% in the UK, with a consequent 50% reduction in wet deposition (UKEA 2008). However, emissions from Didcot ‘A’ Power Station, just 16km south of Wytham (and visible from Wytham Hill), remained high (UKEA 2008). Didcot ‘A’ Power Station had been one of the most significant point sources emitting sulphur in the UK (Barrett 2004), and as a consequence ceased operations on 22 March 2013; demolition commenced on 27 July 2014[d]. Continued monitoring at Wytham would indicate whether this decommissioning reverses the long-term trend of reduction in soil calcium availability.

There is no evidence that the changes observed in eggshell pigmentation over this period have influenced the fitness of eggs. The Hatching success of eggs within the north Wytham study area averaged 86% throughout the study period ranging 80–90% over years, and across years (all data) the within-clutch % hatching success rate was correlated neither with soil calcium nor with pigment spread (r=0.012, n.s. and -0.035, n.s. respectively). However, this should be expected from an optimized system in which pigmentation compensates for the reduction in shell thickness (Higham & Gosler 2006). Hence our data do not suggest that this acidification necessarily poses a long-term threat to the birds' breeding success or to the conservation status of this or other species. Nevertheless, we believe that these observations are important because although the effects of acid precipitation on eggshell-quality of birds on acid soils are well documented (Graveland 1998, Green 1998), they are unprecedented on 'supposedly' calcium-rich soils. The implications for snail survival and conservation may be more significant than those for the birds since we have also noted declining numbers of snails: the birds' primary source of calcium (Jubb *et al.*, 2006). These observations offer further evidence of decreasing calcium availability.

Our observations suggest that the weakly-acid precipitation still present in central England (Hayman, 2004), is leaching calcium from shallow calcic soils (Cadee, 1999) sufficiently to affect avian physiology. They also demonstrate that monitoring eggshell pigmentation might provide a simple means to detect this effect. This is the first time that eggshell pigmentation has been used to assay shell thickness, and provides the first evidence that soil acidification can affect birds nesting in calcic (rather than base-poor) areas. Following from this, and from the effective replication of earlier findings relating Great Tit eggshell pigmentation to environmental quality (Hargitai 2013, 2016 a,b), we believe that eggshell pigmentation can provide an important non-destructive method for environmental monitoring. An illustrated description of the method for scoring eggshell pigmentation was given in Gosler (2006), and preliminary trials using help from student volunteers, with both direct observation and determination from photographs, suggests that volunteer citizen scientists could readily acquire the skills necessary to assess eggshell pigmentation for these purposes with a little tuition.

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Endnotes

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[a] Studies of large passerine species such as the Mexican Jay *Aphelocoma ultramarina*, have not found evidence of a relationship between eggshell pigmentation and calcium availability (Berg et al. 2009). Such species might be able to buffer against environmental effects through the use of skeletal calcium, as occurs in non-passerine capital breeders such as the Domestic Fowl *Gallus gallus* (Whitehead 2004).

[b] Reanalysis (undertaken by Eurofins Laboratories Ltd.) of 37 control samples collected in 1974 (n=18) and 1991 (n=19) agreed strongly with analyses conducted by the ITE (now CEH) laboratories at Merlewood in the years of collection ($r_{35} = 0.963$, $P<0.0001$, regr. equation: Log_{10} 2008 ca = 0.170 + 0.926 Log_{10}previous Ca).

[c] A GLMM analysis of the control soil sample data, with 2008 reanalysis values and dependent variable, including location (random), year (fixed), depth (fixed) and previous evaluation (covariate) revealed only location and previous Ca evaluation to be significant predictors of the Ca levels determined in the 2008 reanalysis (year $F_{1,36} = 0.43$, $P=0.514$, depth $F_{2,36} = 0.66$, $P=0.523$, previous Ca $F_{1,36} = 21.58$, $P<0.0001$, location $F_{7,36} = 16.08$, $P<0.0001$, $r^2_{(adj)} = 93.8\%$). Excluding the confounding effect of location confirmed the strong agreement between original and recent laboratory determinations of calcium in the same samples (year $F_{1,43} = 0.21$, $P=0.649$, depth $F_{2,43} = 0.26$, $P=0.776$, previous Ca $F_{1,43} = 171.88$, $P<0.0001$, $r^2_{(adj)} = 78.6\%$).

[d] Even after decommissioning, the Didcot A power station continues to be a source of public interest and concern: https://en.wikipedia.org/wiki/Didcot_power_stations

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Figure Legends

Figure 1. Maps of Wytham Woods showing sampling circles of A) 150m and B) 300m radius, centred on soil resampling plots, used to relate changing eggshell pigmentation (1991–2007) to changing soil calcium availability (1991–2008). Also shown are all nestboxes captured within these circles (note that not all nestboxes were used by great tits in these years) and the North Wytham area used for this study enclosed by the rectangle. Eggshell pigmentation was only assessed in the northern study area indicated by the rectangle on each map. Note that while sampling plots based on soil survey locations overlap (i.e. nestboxes may 'repeat' in different plots) this does not influence the independence of egg data based on nestboxes, which are compared over a long period of time (see text).

Figure 2. Decline in mean (± se) pigment spread (pc2) of 2,313 great tit clutches (*n* = 55–202 per annum) in the 100-ha north-Wytham study population over 20 years of study. Estimated from the regression of shell thickness on pigment-spread (see text), this represents a decline in shell thickness of 6.53% between 1988 and 2007. The inset images are representative of the high spread (pc2) low darkness (pc1) eggs typically associated with high calcium soil areas of Wytham

(top right), and of low spread (pc2) high darkness (pc1) eggs typically associated with low calcium clay soil areas of Wytham (bottom left).

Figure 3. The change in calcium content (mg ca/100g soil) of Wytham soil samples between 1974 and 1991 in relation to the original 1974 value. The shaded area shows the likely boundaries to change (maximum increase based on that observed). Soil calcium bands of equal breadth on $\log_{10}$ scale (designated CALC 1–5) were used to summarise the interaction between pigment spread and year (see Fig 7) are indicated by vertical lines. Although the values from 1974 and 1991 are significantly correlated (see text), the graph shows substantial declines in available calcium in higher-calcium plots, such that above an initial value of 2100 mg.100g^{-1}, all samples showed a decline. Interestingly, some of the samples with very low calcium values in 1974, showed increases to 1991.

Figure 4. Observed calcium values (all depths) in 31 soil plots sampled in 1974, 1991 and 2008. The figure reveals a dramatic reduction in available calcium in the soil over this time.

Figure 5. Changes in calcium content (mg/100g soil) of 93 soil samples from (31 plots × 3 depths) in relation to initial values: upper plot 1974–1991; lower plot 1991–2008. Note that the plots differ in their scales, but both plots show that the greatest declines in calcium content have occurred in the most calcic soils. In multivariate analyses, no significant differences were detected between depths in the direction or degree of change.

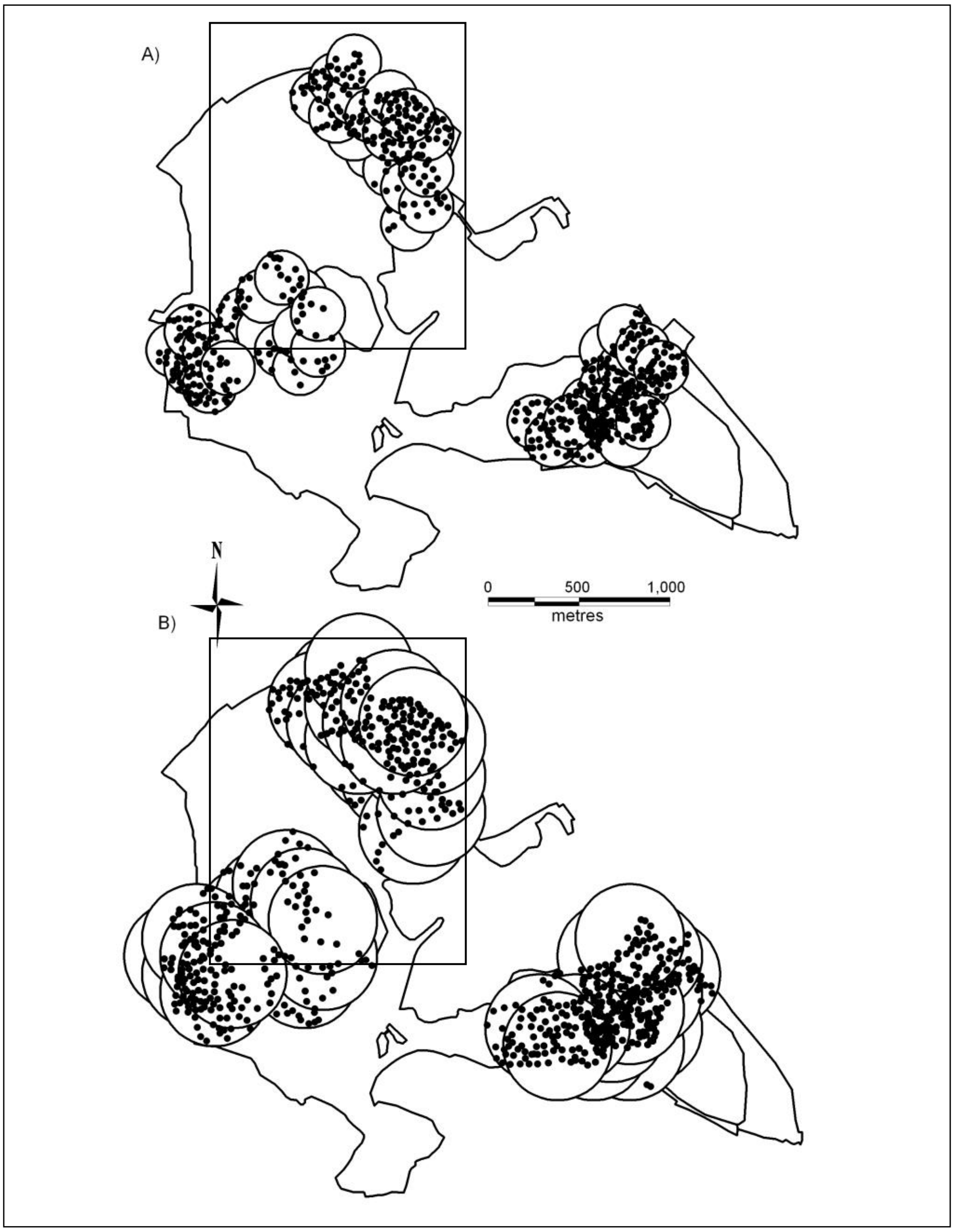
Figure 6. Changes in calcium content of 93 soil samples between 1991 and 2008 in relation to the change in the same plots and depths between 1974 and 1991. The graph shows: a) most plots changed little during either period; b) there was no correlation overall in change over time, and c) that plots whose calcium content increased between 1974 and 1991, all declined subsequently; fewer that declined between 1974 and 1991 subsequently increased.

Figure 7. Plot showing the interaction between declines in mean pigment spread (PC2) over years on the lowest (CALC 1) and highest (CALC 5) calcium soil areas (CALC class defined by $\log_{10}$ 1974 calcium content – see Fig. 3). Note that the variance of response in the low calcium areas tended to increase, whilst in the high calcium areas it remained constant.

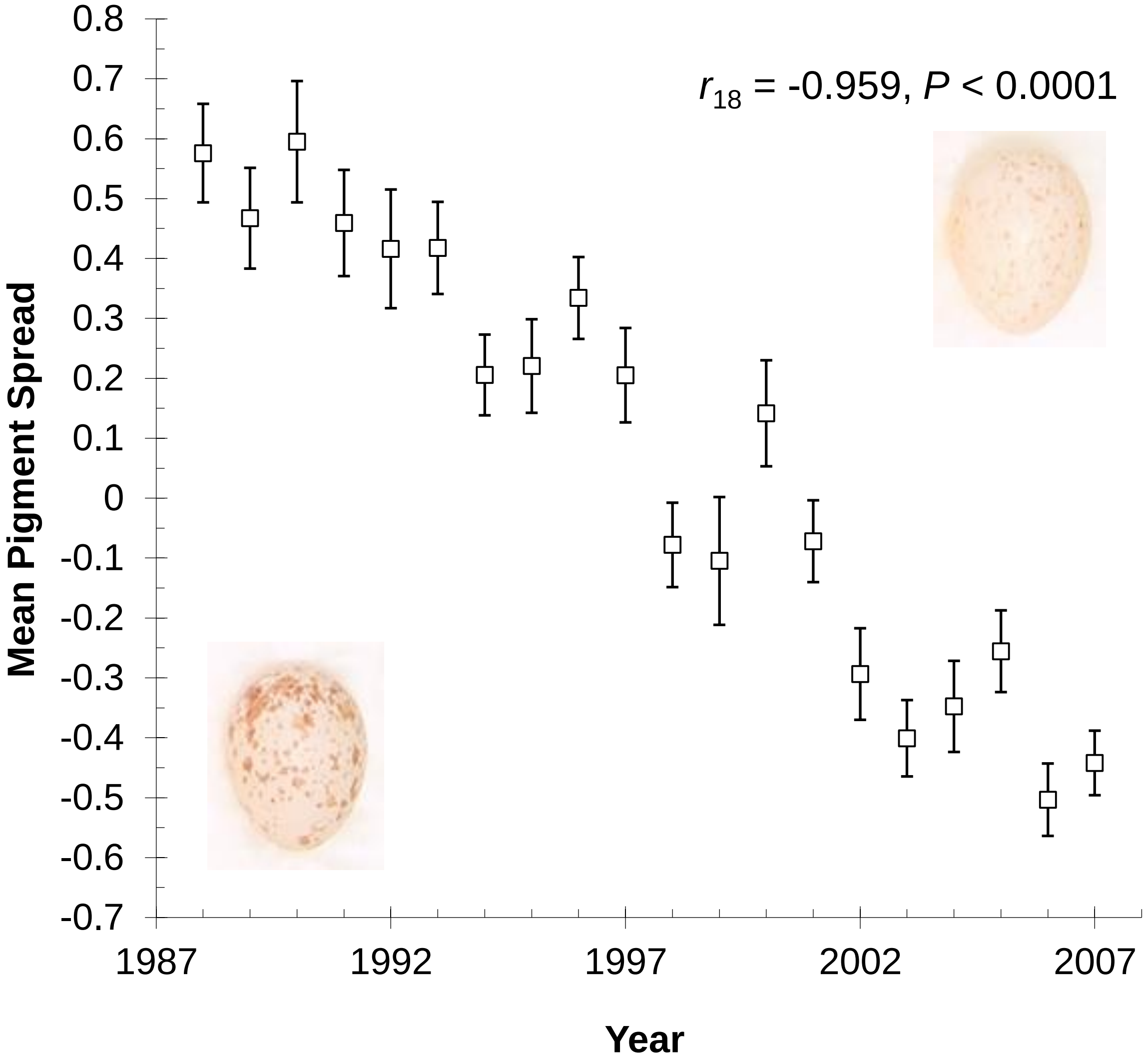
Figure 8. Across 28 soil sampling plots (with a sampling radius of 300m from the plot), declining soil calcium availability correlated with declining eggshell pigment spread (r_{26} = 0.371, P = 0.026 [one-tailed]).

Figure 9. Graph showing the increasing effect-size of (1974) soil calcium level on clutch-size in great tits breeding in the Wytham population from 1965 to 2005.

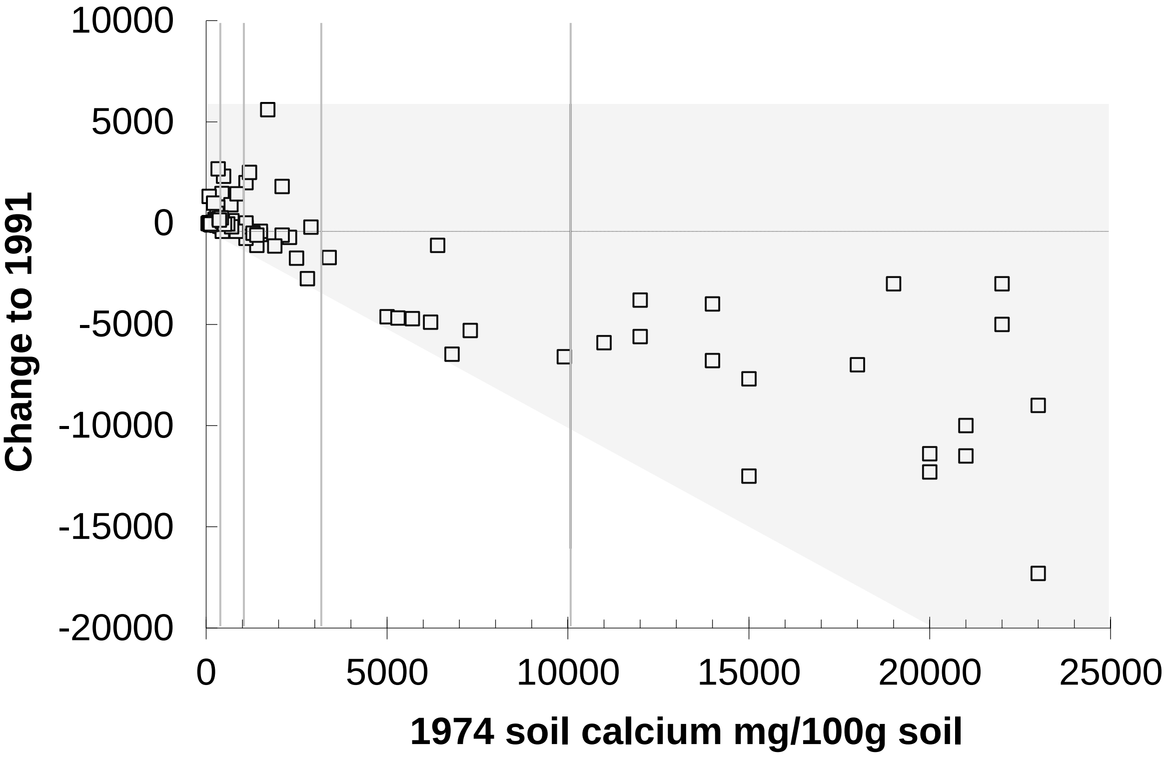
BS-2016-122 GOSLER FIG. 1



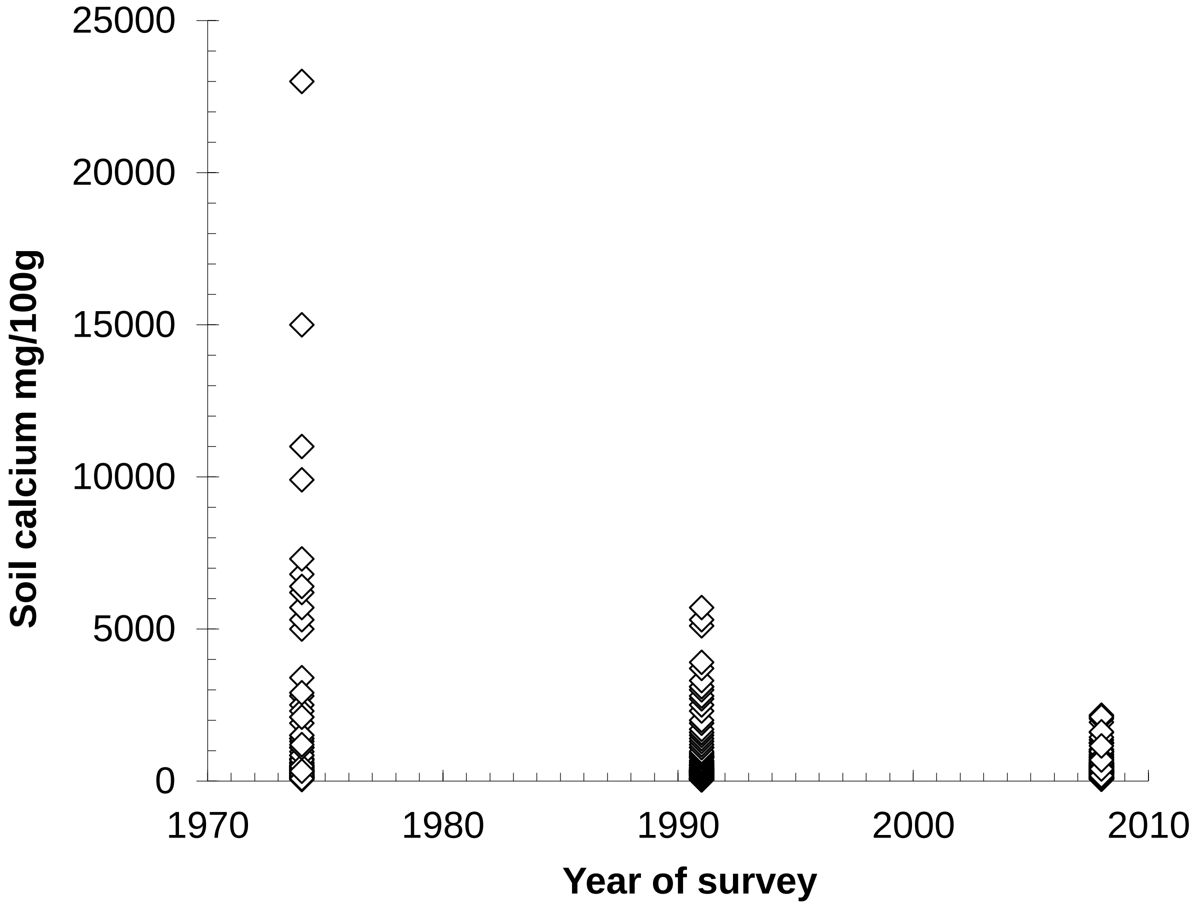
BS-2016-122 GOSLER FIG. 2



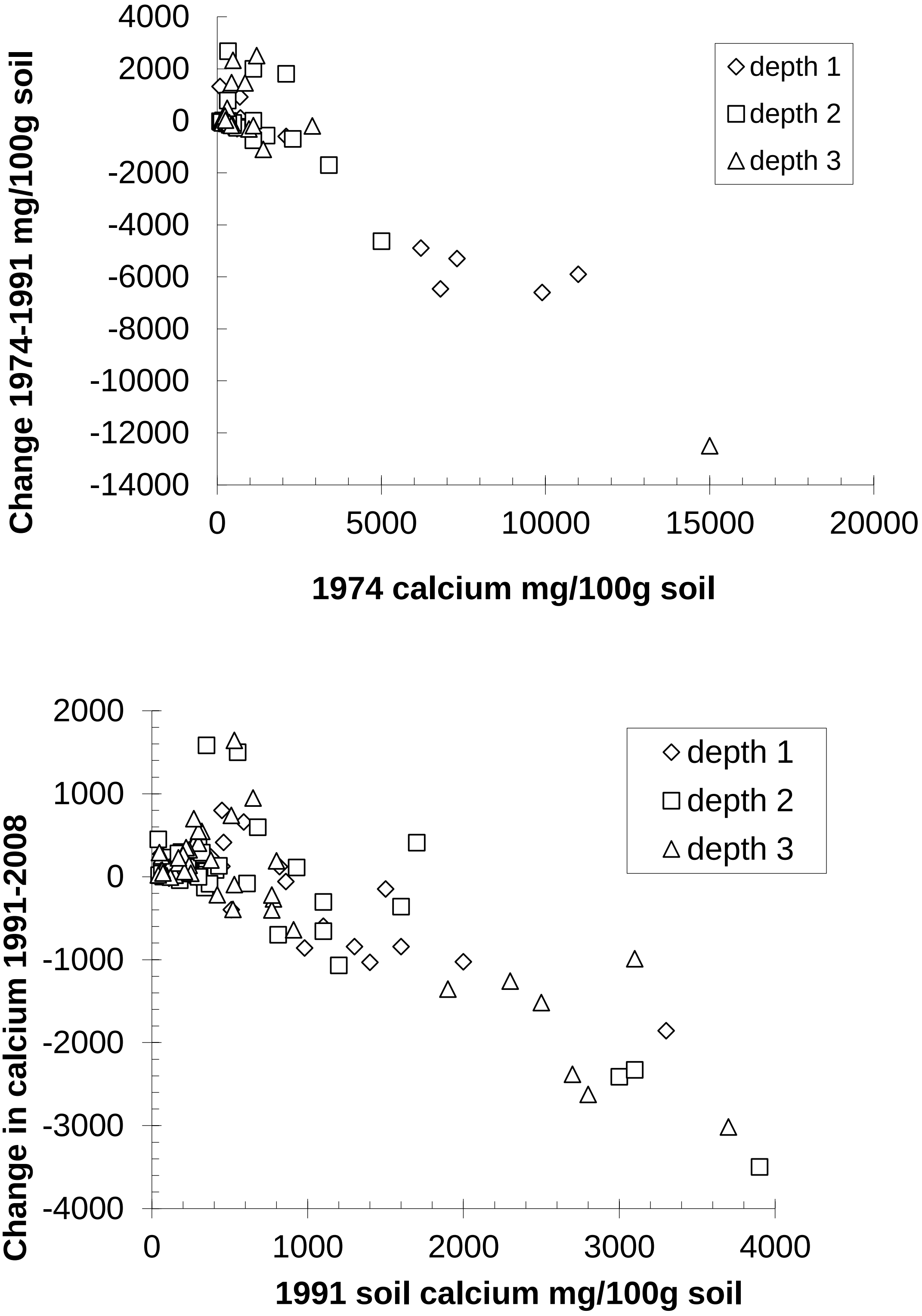
BS-2016-122 GOSLER FIG. 3



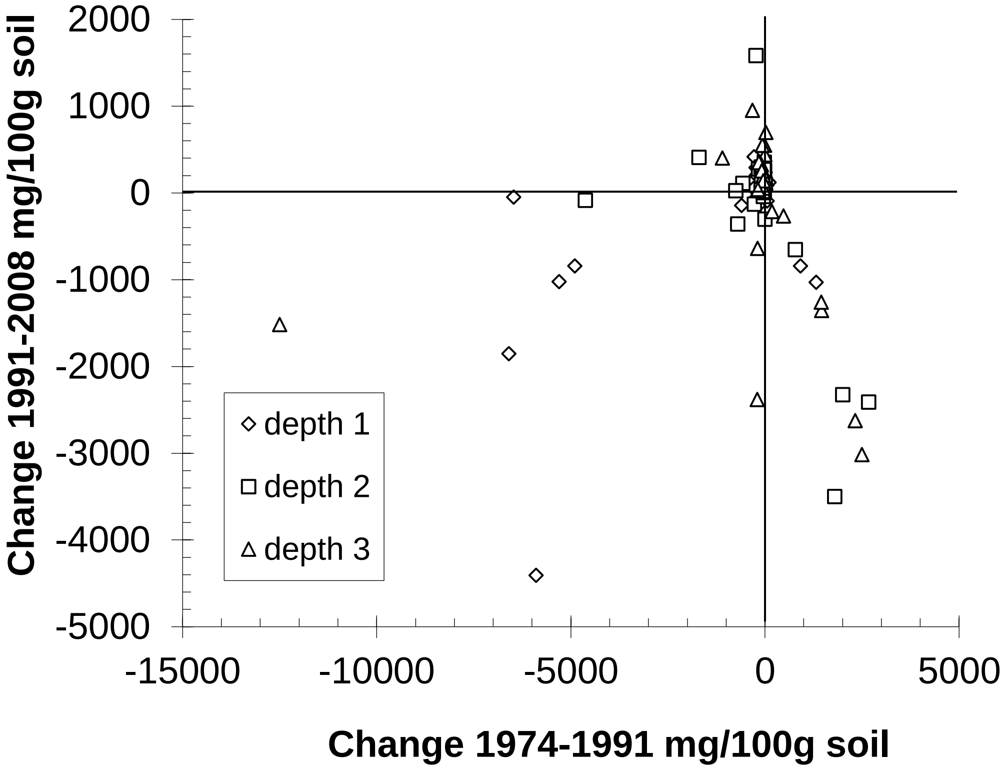
BS-2016-122 GOSLER FIG. 4



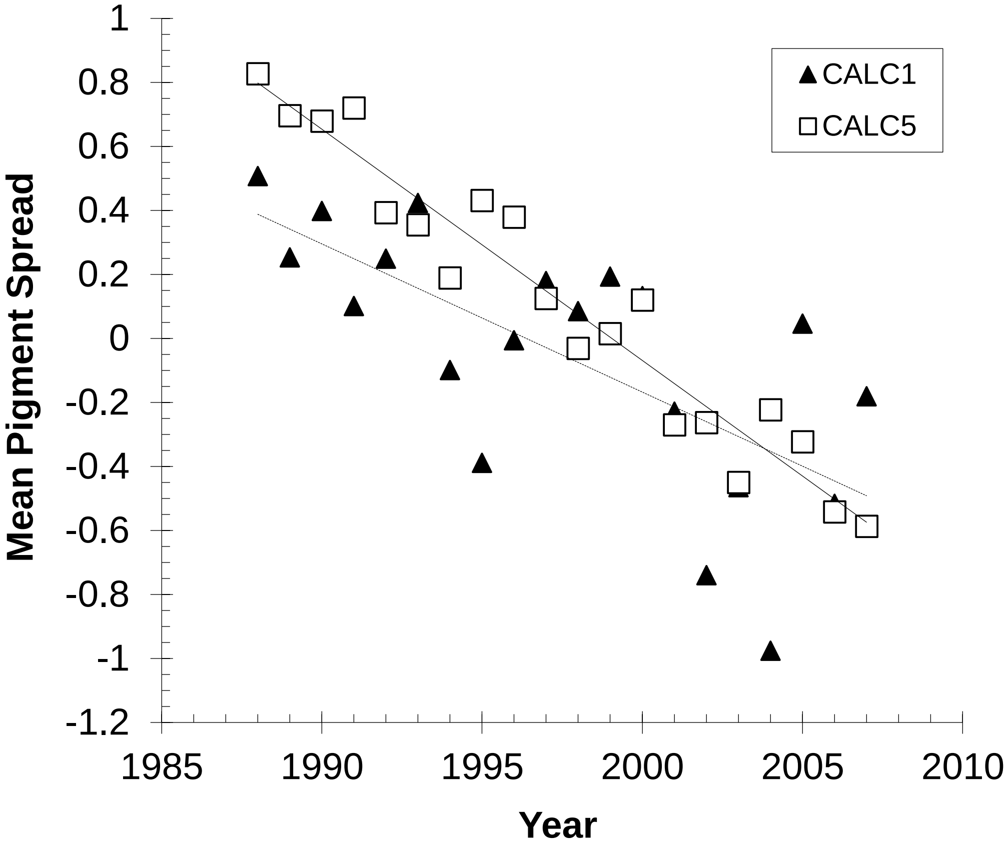
BS-2016-122 GOSLER FIG. 5



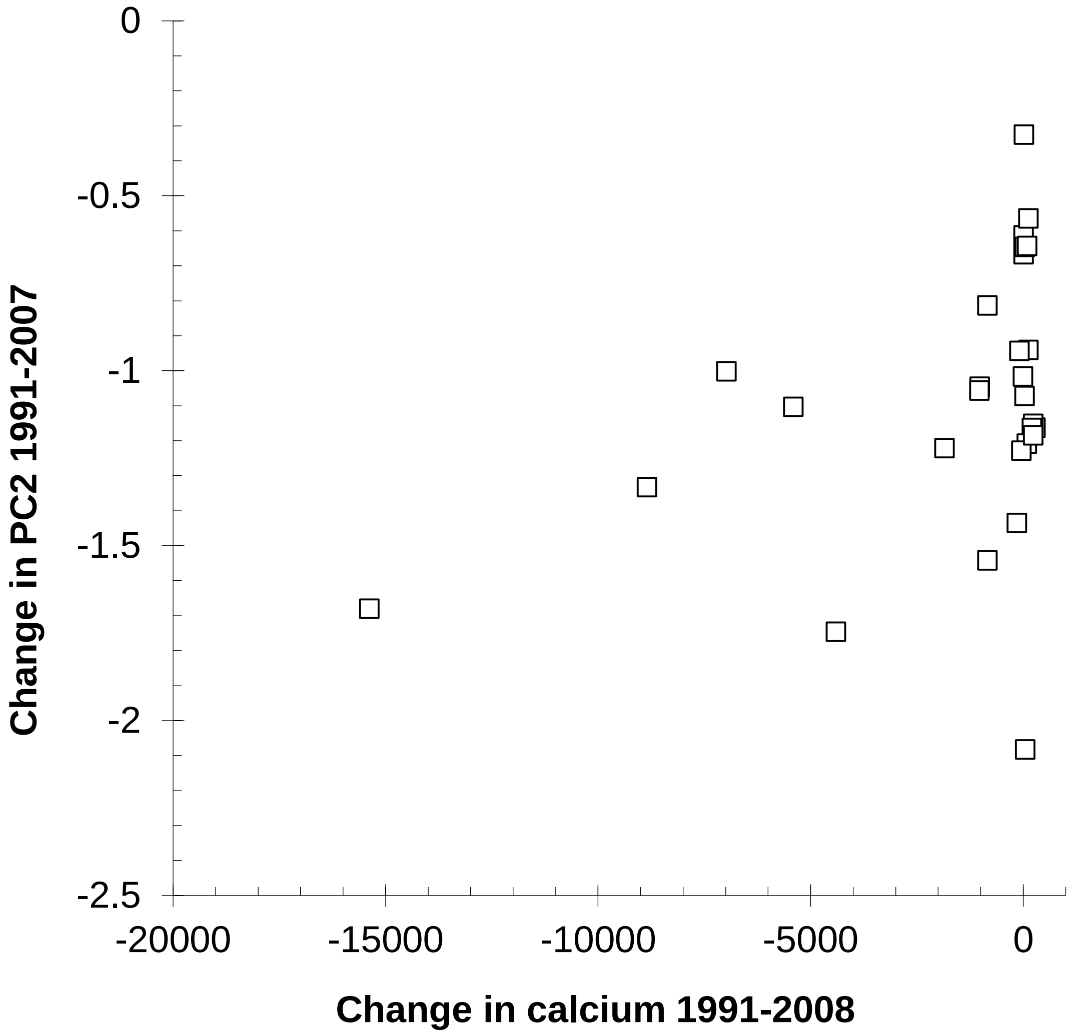
BS-2016-122 GOSLER FIG. 6



BS-2016-122 GOSLER FIG. 7



BS-2016-122 GOSLER FIG. 8



BS-2016-122 GOSLER FIG. 9