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Feather corticosterone content in predatory birds in relation to body condition and hepatic metal concentration

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Running head: Steroids in raptor feathers

ABSTRACT

This study investigated the feasibility of measuring corticosterone in feathers from cryo-archived raptor specimens, in order to provide a retrospective assessment of the activity of the stress axis in relation to contaminant burden. Feather samples were taken from sparrowhawk *Accipiter nisus*, kestrel *Falco tinnunculus*, buzzard *Buteo buteo*, barn owl *Tyto alba*, and tawny owl *Strix aluco* and the variation in feather CORT concentrations with respect to species, age, sex, feather position, and body condition was assessed. In sparrowhawks only, variation in feather CORT content was compared with hepatic metal concentrations. For individuals, CORT concentration (pg mm^{-1}) in adjacent primary flight feathers (P5 and P6), and left and right wing primaries (P5), was statistically indistinguishable. The lowest concentrations of CORT were found in sparrowhawk feathers and CORT concentrations did not vary systematically with age or sex for any species. Significant relationships between feather CORT content and condition were observed in only tawny owl and kestrel. In sparrowhawks, feather CORT concentration was found to be positively related to the hepatic concentrations of five metals (Cd, Mn, Co, Cu, Mo) and the metalloid As. There was also a negative relationship between measures of condition and total hepatic metal concentration in males. The results suggest that some factors affecting CORT uptake by feathers remain to be resolved but feather CORT content from archived specimens has the potential to provide a simple effects biomarker for exposure to environmental contaminants.

1. Introduction

The vertebrate stress response is a highly conserved suite of neuroendocrine, metabolic and behavioural adjustments that are evoked in response to destabilizing or threatening challenges (Koolhaas et al., 2011; Romero, 2004). In free-living vertebrates these stressors may include competition, poor nutritional state, and harsh environmental conditions (Hirschenhauser et al., 2000; Kitaysky et al., 2007; Romero et al., 2000; Wingfield, 2013). The hypothalamic–pituitary-adrenal (HPA) axis is a pivotal element of the stress response and, when activated, the resultant hormonal cascade terminates in the release of corticosteroids into the bloodstream. Although the stress response is a fundamentally beneficial adaptive mechanism, there are conflicting maladaptive consequences associated with prolonged or frequent activation of the stress axis (e.g. Sapolsky et al., 2000). Consequently, there is considerable interest in assessing the status and activity of the stress axis in animals, as both an index and predictor of fitness and performance.

Corticosterone (CORT) is the primary stress-induced corticosteroid in the avian endocrine system and direct measurement of CORT concentrations in the blood is the preferred method of determining the activity of the stress axis. However, recent studies have shown that CORT accumulates within feathers and that feather CORT concentration reflects plasma levels of CORT, a relationship that is most apparent when plasma CORT concentrations are elevated for a sustained period above baseline (Bortolotti et al., 2008; Fairhurst et al., 2013). Some ambiguity surrounds the interpretation of feather CORT concentrations in relation to blood CORT levels, particularly with respect to the mechanism by which CORT is deposited within the feather (Lattin et al., 2011). Nonetheless, measurement of CORT in feathers offers an

alternative when blood samples are not available or when an integrated measure of CORT levels over a longer period is required to complement the "snapshot" of a blood CORT measurement and many studies have exploited this (see below).

Feathers are relatively easily collected and require no special conditions for storage and transport for CORT to remain viable. Furthermore, there is no evidence that the CORT content of feathers declines with time after collection making possible the analysis of steroid hormone concentrations in feather samples from long-term archives and museum collections (Bortolotti et al., 2009; Kennedy et al, 2013). A significant caveat in the use of feathers as a source of steroid hormone data is that the uptake of CORT by feathers is, as noted above, considered to occur during the growth phase of the feather only, and therefore the period during which CORT is accumulated may be at some remove from the time at which somatic or contaminant data may be collected. This issue is central to the use of material deposited in a cryo-archive for which no control can be exerted over the date of collection of carcasses for inclusion in the archive. However, contaminant burdens are acquired over a prolonged period of time and for the purposes of the present study, which lays the groundwork for more detailed investigation of links between feather CORT and tissue contaminant levels, this was not considered to be problematic.

Not every study seeking to identify functional correlates for feather CORT concentrations has successfully done so (e.g. Bourgeon et al., 2014; Carbajal et al., 2014; Sepp et al., 2014) but these are in the minority. Concentrations of CORT in feathers have been shown to vary in response to life history events (Bortolotti et al., 2008; Crossin et al., 2013), sibling competition (Yosef et al., 2013), health status (Harriman et al., 2014; Meitern et al., 2013; Mougeot et al.,

2010; Sild et al., 2014), overwinter survival (Koren et al., 2012), food limitation (Will et al., 2014), reproductive investment (Fairhurst et al., 2012a; Kouwenberg et al., 2013), and with environmental conditions (Carrete et al., 2013; Fairhurst et al., 2011; Fairhurst et al., 2012b; Legagneux et al., 2013). Feather CORT concentrations have also been shown to be related to problem solving and learning (Bókony et al., 2014), and to feather pigmentation (Fairhurst et al., 2014; Kennedy et al., 2013; Lendvai et al., 2013; Martínez-Padilla et al., 2013). However, as far as we are aware there have been only a few studies that report feather CORT concentration in relation to pollutant exposure (Cruz-Martinez et al., 2015; Harms et al., 2010; Lattin et al., 2014).

The purpose of this study was to assess the feasibility of using feather CORT concentration to provide a retrospective assessment of the activity of the stress axis in predatory birds, acquired specifically for contaminant monitoring purposes. As such, it is the first investigation into the variability of feather CORT in raptor feathers and some of the factors that might account for this variability. Various monitoring schemes across Western Europe measure contaminant concentrations in raptors and owls, using these apex predators as sentinels of environmental pollution or to directly assess the risk to individual species that are of conservation concern (Gomez-Ramirez et al., 2014). In the United Kingdom this work is conducted by the Predatory Bird Monitoring Scheme (PBMS; Walker et al., 2008). Most schemes, including the PBMS, measure exposure of birds to particular contaminants but lack information on collateral effects. In this context, feather CORT content may provide a simple effects biomarker that can be related to exposure to one or more environmental contaminants (e.g. Nordstad et al., 2012).

In order to identify contaminant effects on the HPA axis it is necessary to understand how other factors may influence feather CORT concentrations. Potential sources of variation in feather CORT content in predatory birds were investigated by examining CORT concentrations in feathers taken post mortem from five species of raptors (barn owl, tawny owl, Eurasian sparrowhawk, common kestrel, common buzzard) that had been found dead at various locations in Britain and submitted by members of the public to the PBMS. Specifically, we examined (i) the reproducibility of feather CORT concentrations between different feathers from a single individual and (ii) whether feather CORT varied systematically with species, age, sex, and body condition. Condition can change in response to environmental factors and changes in condition that either cause, or are a consequence of, activity in the HPA axis (e.g. Braasch et al., 2011; Harms et al., 2010; Sockman and Schwabl, 2001) may occur during a period when feather growth has ceased, and therefore hormone uptake by the feather is not occurring. We nonetheless included condition as a variable, assuming that for a substantial proportion of individuals CORT accumulation in feathers would coincide with the period during which the bird was exposed to the factor(s) resulting in a persistent change in condition, and if not, that the determinants of condition at time of death were carried over from the period in which feather hormone concentration was accumulated (Legagneux et al., 2013; Harms et al., 2015). In addition to these somatic variables, hepatic metal and metalloid concentrations were available for the group of sparrowhawks included in this study. Many pollutants, including metals, are known to affect adrenal function in birds, resulting in alterations to both stress-induced (Baos et al., 2006; Moore et al., 2014; Wayland et al., 2002) and baseline blood CORT concentrations (Franceschini et al., 2009; Herring et al., 2012; Pollock and Machin, 2009). We were therefore able to (iii) investigate whether feather CORT varied in relation to tissue metal concentrations for this species.

2. Materials and methods

2.1 Source of material and collection of somatic data

Birds were randomly selected from the PBMS archive which contains selected tissues from the carcasses of predatory birds that have died from a variety of causes (Newton et al., 1999; Walker et al., 2008). Whole wings from a total of 83 individuals were stored at -20°C until analysed. CORT concentrations were determined in feathers from five species (barn owl, *Tyto alba*, n = 21; tawny owl, *Strix aluco*, n = 17; Eurasian sparrowhawk, *Accipiter nisus*, n = 30; common kestrel, *Falco tinnunculus*, n = 7; and common buzzard, *Buteo buteo*, n = 8). Primary flight feathers were used for all hormone analyses. Sex, body mass, pectoral muscle mass, sternum (keel) length, diagonal (distance from the distal point of the sternum to the anterior point of the coracoid) and wing length were recorded during post-mortem examinations carried out on all birds received by the PBMS. Birds were classified as juvenile (birds that hatched in the same or previous calendar in which they died) or adult on the basis of their plumage.

2.2 Feather extraction procedure

CORT was extracted from feathers as per Bortolotti et al. (2008). The calamus was removed and the remainder of the feather was measured, weighed and cut into < 5mm^2 pieces. The pieces were extracted with 10 ml (or 20 ml for buzzard feathers) of methanol (Chromasolv, Sigma-Aldrich) in a capped glass scintillation vial that was placed in a sonicating water bath for 30 minutes at room temperature and then in a shaking incubator at 40°C overnight. The

solvent extract was separated from the feather fragments by passing through a plug of glass wool in a filter funnel, then evaporated at 40°C under a gentle stream of air and reconstituted in 1.0 ml ethyl acetate (HPLC grade, Sigma Aldrich). Extracts were stored at -20°C until analysis. Procedural losses were assessed by including feather samples spiked with c. 11,000 DPM of ^{3}H-CORT in each batch of extractions. The recovery of ^{3}H-CORT added to the initial feather extract was 67.3 ± 1.9 % (mean ± SEM, n = 34). Samples were not subsequently corrected for procedural losses.

2.3 Corticosterone radioimmunoassay

Feather corticosterone (CORT) levels were determined using the radioimmunoassay (RIA) protocol described by Pottinger and Carrick (2001) for cortisol but substituting [1,2,6,7-^{3}H]-corticosterone (2.4 TBq mmol^{-1}; GE Healthcare) as tracer with an anti-corticosterone antibody (Abcam ab1022, 1:2000 dilution; cross-reactivity: 11-dehydrocorticosterone 0.67%, Deoxycorticosterone 1.5%, 18-OH-DOC <0.01%, Cortisone <0.01%, Cortisol <0.01% and Aldosterone 0.2%). Feather hormone concentrations were expressed as pg mm^{-1} of feather (Bortolotti et al., 2008, 2009). Two 100 µl aliquots of a pool of feather extracts were included with each RIA to assess inter- and intra-assay variation. The coefficients of variation were 7.7% and 22.0% respectively. To determine whether matrix effects might interfere with the performance of the RIA, aliquots of feather extracts from three different individuals containing high levels of CORT were serially diluted with ethyl acetate. The CORT concentrations in the serial dilutions of feather extracts were compared with serial dilutions of CORT standards (ranging from 6.25 to 800 pg) in ethyl acetate. The concentrations of CORT estimated in serial dilutions of feather extracts consistently deviated from the dilution profile

of the CORT standards when 200 μl aliquots of extract were assayed (Fig. S1, supplementary data). However, extract volumes of 100 μl or less exhibited no systematic interference and therefore this volume of extract was used routinely. An additional assessment of assay interference was conducted by adding 100 μl aliquots of a pool of five feather extracts to tubes containing each sequential dilution of standard CORT (800 – 6.25 pg tube^{-1}) in quadruplicate. The extracts plus standard CORT were assayed against a CORT standard curve and deviation from the expected result was noted. Assay of standard amounts of CORT added to aliquots of feather extracts resulted in an underestimation of the higher concentrations of the standards (800 and 400 pg; 30.7% and 62.1% detected respectively). However for concentrations of steroid between 6.25 and 200 pg tube^{-1}, which was the concentration range for the majority of feather extracts, estimation of the added standards was more accurate (84 ± 5%, n = 12).

2.4 Feather CORT concentrations within individuals and across species

To assess variation in feather CORT levels within individuals, the primary P5 feather was taken from the left and right wings of a sub-set of sparrowhawks (n = 10), barn owls (n = 5) and tawny owls (n = 5) and analysed as above. In addition, the P6 feather of the right wing in 15 of these individuals was sampled to compare CORT concentrations in adjacent feathers of the same wing. The effect of feather length on steroid concentrations was assessed in primary P5 – P8 feathers from the left wing. Between-species comparisons were conducted using P5 feathers taken from barn owl (n = 21), tawny owl (n = 16), Eurasian sparrowhawk (n = 30), common kestrel (n = 7), and common buzzard (n = 8).

2.5 Body condition and feather steroid concentrations

The relationship between body condition and feather hormone content was investigated by utilizing three different measures of condition. These were the residual variation in (i) body mass and (ii) pectoral muscle mass, after correcting for variation in size by using wing and sternum length as measures of skeletal size (Gosler et al., 1998; Gosler and Harper, 2000), and (iii) pectoral muscle mass as a proportion of body mass [pectoral muscle index; PMI = 100*total pectoral muscle mass / (body mass – (mass of crop contents + gizzard contents))]. The residual values for body mass and pectoral muscle mass, and PMI, were taken to reflect variation in reserves of protein and fat and to provide a proxy index of available protein and lipid respectively.

2.6 Hepatic metal content and feather corticosterone concentrations

For fifteen of the sparrowhawks used in the present study the hepatic concentrations of sixteen potentially toxic and essential or trace metals, and metalloids (see Table S3), were determined (Walker et al., 2012; inductively coupled plasma mass spectrometry ICPMS, in acid-digested liver samples). Hepatic metal concentrations were compared with feather CORT concentrations to determine whether any relationships between metal concentration and feather steroid content were evident.

2.7 Statistical analysis

Paired T-tests were used to conduct within-species comparisons of P5 feather CORT content in left and right wings and CORT content in P5 and P6 feathers within right wings. One-way ANOVA was used to compare CORT concentrations between species and with respect to age and sex. Post-hoc pairwise multiple comparisons of means were carried out using Holm-Sidak method (Sigmaplot for Windows, v. 12; Systat Software). Non-normal data were log-transformed prior to analysis and a Gamma error distribution was assumed. A generalised linear model (GLM, R Core Team, 2013) was used to define the relationships between both body mass and pectoral muscle mass and three structural metrics: sternum length (the maximum distance between the anterior and posterior edges of the keel), sternum diagonal (the maximum distance between the posterior edge of the keel and uppermost edge of the coracoid bone) and wing length. Condition was defined as the difference between observed body mass and that predicted by the model (the residual). Where insufficient data were available to discriminate between the sexes, an overall analysis combining both sexes was conducted. The relationship between condition and feather hormone content, and that between hepatic metal content and feather CORT was evaluated by linear regression (Sigmaplot; Minitab 16, Minitab Inc.).

3. Results

3.1 Feather CORT concentrations in relation to feather position and species

In all species there was no significant difference in CORT content (Fig. 1) between primary flight feathers (P5) from matched left and right wings ($P = 0.4 - 0.8$; paired t-test; Fig. S2b), or between adjacent feathers (P6 and P5) from the right wing ($P = 0.1 - 0.6$; paired t-test; Fig. S2a). Sparrowhawk P5 CORT content was lower than that of barn owl or tawny owl (Fig. 1; ANOVA, $F_{2,17} = 15.9$, $P < 0.001$). Further analysis of P5 feathers from five species confirmed between-species variation in CORT content (Fig. 2; ANOVA, $F_{4,78} = 4.6$, $P = 0.002$) with lowest CORT levels in sparrowhawk feathers. The difference in CORT content between tawny owl and barn owl evident in the positional comparisons (Fig. 1) was not apparent in this larger sample.

3.2 Feather CORT concentrations in relation to age, sex and date of delivery to PBMS

There was no significant effect of age (juvenile/adult; $F_{1,71} = 0.4$, $P = 0.5$) or sex ($F_{1,72} = 0.04$, $P = 0.8$) on feather CORT concentrations. However, there was a significant species x sex interaction ($P = 0.049$) which resolved as higher feather CORT concentrations in male tawny owls (3.7 ± 0.4 pg mm^{-1}; n = 12) than in females (2.0 ± 0.2 pg mm^{-1}; n = 4). In sparrowhawks, there was a significant negative relationship between feather CORT concentration and the month the specimen was received by the PBMS ($r^2 = 0.18$, $P = 0.02$, n = 30) with feather CORT concentrations showing a tendency to decline from January to December (Fig. S3a). Conversely, in the same species feather CORT showed a significant positive relationship with

year manifested as a tendency for higher feather CORT levels in birds received by the archive most recently ($r^2 = 0.19$, $P = 0.02$; Fig. S3b). No relationship was evident between any somatic measurement and either month or year for sparrowhawk, and no significant date-related trends were apparent for any variable among the other species examined.

3.3 Feather CORT concentrations in relation to feather length and mass

There was no relationship evident between feather length and CORT content (as pg CORT mm^{-1}) in sparrowhawk (n = 52, $r^2 = 0.07$, $P = 0.06$; Fig. 3a) or tawny owl (n = 24, $r^2 = 0.004$, $P = 0.8$; Fig. 3c). However, for barn owl, variation in feather length explained 30% of variation in feather CORT content (n = 25, $r^2 = 0.31$, $P = 0.004$; Fig. 3b). Similarly, CORT content in sparrowhawk and tawny owl feathers did not show a significant relationship with feather mass (p > 0.1; Fig. S4a, c), whereas for barn owls the regression was significant ($r^2 = 0.19$, $P = 0.03$; Fig. S4b).

3.4 Body size and condition, pectoral muscle index, and feather steroid concentration

For all species P5 feather length and feather mass both increased significantly with body mass (n = 75, $r^2 = 0.6$, $P < 0.001$; $r^2 = 0.8$, $P < 0.001$ respectively) and the mass of feather per unit length also increased with body mass (n = 75, $r^2 = 0.8$, $P < 0.001$). Buzzard feathers (n = 8) were excluded from these regressions because, although their length scaled with body mass in a manner consistent with the other species, their mass did not, meaning that mass per unit length for buzzard feathers was much lower than predicted by the feather mass/length relationship for the other four species. Length-normalised feather CORT (pg mm^{-1}) was not

related to mean body mass (r^2 = 0.24, P = 0.15) and no relationship was evident between mass-normalised feather CORT concentration (pg mg^{-1}) and body mass (r^2 = 0.14, P = 0.3). Three different estimates of body condition (body mass and pectoral muscle mass residuals, pectoral muscle index) were investigated for their contribution to intra-species variation in feather CORT concentration. Where the relationship between skeletal size (model terms: sternum length, diagonal, wing length) and body mass and/or pectoral muscle mass was statistically significant (Table S1), residual values, describing the extent to which the body mass or pectoral muscle mass of each individual deviated from that predicted by the model, were regressed against feather CORT concentration (as pg mm^{-1}) to determine whether this measure of condition was related to feather CORT content (Table S2). Feather CORT concentration was significantly related to condition in only the kestrel and tawny owl and only when body condition was defined as either PMI (Fig. 4a & 4b; Table S2) or pectoral muscle residual (Table S2). In these cases condition explained 64% and 35% of the variation in CORT content respectively. There were no significant relationships between body condition and feather CORT in barn owls, sparrowhawks or buzzards (Fig. 4c and 4d).

3.5 Feather steroid content and hepatic metal concentration.

Data were available for the hepatic concentrations of sixteen metals and metalloids in each of fifteen sparrowhawks for which feather steroid measurements were conducted. Feather CORT concentration displayed significant positive relationships with six metals (Mn, Co, Cu, Mo, Cd) and As (Fig. 5; $r^2 \geq$ 0.28, P≤0.05 in all cases; Table S3). There was a considerable degree of co-correlation between those metals that were significantly associated with feather CORT concentrations. For example, variation in hepatic Mn concentration accounted for 72% of the

variation in Co concentrations. In males only, the total hepatic metal content of the sparrowhawks was inversely and significantly related to the body condition index (body mass/sternum keel length; Fig. S4a) and pectoral muscle weight (Fig. S4b).

4. Discussion

In this study, we investigated variation in feather corticosterone content among different raptor species within individuals (between feathers), between individuals (with age, sex and body condition), between species, and in relation to hepatic concentrations of toxic and trace metals and metalloids.

4.1 Between-feather variation in corticosterone content

Good agreement was observed between feather CORT content in adjacent primary flight feathers and for feathers matched by position in left and right wings from sparrowhawk, barn owl and tawny owl. Lattin et al. (2011) have also reported close agreement in CORT concentrations between adjacent feathers (secondary 1 and 2) from individual starlings. Compelling reasons for quantifying feather steroid hormone content as a time-dependent unit (mass of hormone per unit length; pg mm^{-1}) rather than mass-dependent unit (pg mg^{-1}) have been presented elsewhere (Bortolotti et al., 2009; Bortolotti, 2010). The primary rationale for this approach is to avoid artifacts arising from variation in mass between feathers, potentially resulting in mass dilution of the deposited/sequestered steroid. Steroid deposition in feathers has been assumed to be independent of mass and not constrained by

the incorporation of the steroid into specific elements of the feather. Instead, the steroid has been suggested to accumulate within the structural matrix of the feather in direct proportion to its concentration in the blood, irrespective of the mass of feather at the point of incorporation. However, the data presented here (Figs. 3 and S4) suggest that expressing feather steroid hormone content per unit length does not fully compensate for differences in feather length and feather mass for every species: CORT concentration per unit length varied with both length and mass of the feather in barn owls. This observation does not undermine the value of feather steroid concentration as an investigative tool but suggests that the principles underlying the deposition process require further investigation (see also Lattin et al., 2011). For within-species comparisons, care should be taken to compare feathers matched for size.

4.2 Intra-specific variation in feather corticosterone concentrations

The lack of consistent effects of age and sex on feather CORT content may be a consequence of a relatively small data set within which it was not possible to control for variables which would be expected to modify hormone profiles, such as time of year. Previous studies have reported similar observations. Feather CORT concentrations were unrelated to sex in house sparrows (*Passer domesticus*; Koren et al., 2012), red-legged partridge (*Alectoris rufa;* Bortolotti et al., 2008) or the broiler (*Gallus gallus domesticus*; Carbajal et al., 2014). The limited data available suggests that feather CORT concentrations discriminate or diverge most effectively when high, stress- or implant-induced, levels of CORT are present in the blood (Fairhurst et al., 2013; Lattin et al., 2011). In sparrowhawks only, feather CORT concentration

did show significant trends in relation to month (declining during the year) and year (rising with successive years) the causes of which remain to be investigated.

When feather CORT content was regressed against condition as represented by the body mass residual no significant relationships were evident for any species. However, for only tawny owl and kestrel, feather CORT concentration was significantly and inversely related to both the pectoral muscle mass residual and the PMI. These results are consistent with other reports of a negative relationship between feather CORT concentration and measures of condition (e.g. Harms et al., 2010). Feather CORT in tawny owl and kestrel was related to pectoral muscle indices, not to a condition index based on overall body mass. As a single discrete tissue, the pectoral muscle may be resistant to, or protected to some extent from, post-mortem changes that might undermine the accuracy of whole-body mass as a retrospective measure of the size of the bird while alive. Or, as an important source of stored protein and lipid the pectoral muscle may be more sensitive to energy-mobilizing stressors. Ultimately, a range of factors may contribute to loss of body condition and these may not all be related to, or result in, elevated blood CORT concentrations. Any underlying relationship between feather CORT and condition may also be confounded by a temporal mismatch between the period of feather growth (during which CORT was deposited) and time of death (when body condition was recorded). Acknowledging these sources of variation still fails to explain why a CORT-condition relationship is evident for only two out of five of the species examined here.

4.3 Species differences in feather corticosterone concentration

There are few feather steroid data for raptors or owls. Feather CORT concentrations for buzzards in the present study were similar to those reported for buzzard nestlings (Martínez-Padilla et al., 2013). In the present study, significant differences in feather CORT content between species were most evident between buzzard and sparrowhawk (both Accipitriformes species) whereas feather CORT concentrations did not differ significantly between barn owls, kestrels and tawny owls. This is consistent with reports that baseline and stress-induced levels of plasma CORT are similar in barn owls and kestrels (Almasi et al., 2012, 2013; Meijer and Schwabl, 1989; Müller et al., 2009; Stier et al., 2009; Strasser and Heath, 2011).

4.4 Variation in feather corticosterone concentration with exposure to toxic and trace metals

Feather CORT concentrations in sparrowhawks were significantly and positively associated with hepatic concentrations of six of the sixteen metals and metalloids for which data were available. Of these, two are considered pollutants and potential toxins (Cd, As) while four are essential or trace elements (Mn, Co, Mo, Cu) although Cu is potentially toxic at high concentrations. It is unclear whether these associations were causal, reflecting a functional relationship between individual or total hepatic metal content and the stress axis across time, or indirect, arising from variation in a third factor related to both variables. Metals are known to modulate the activity of the stress axis in birds but there is no clear pattern of cause and effect (Pb: Baos et al., 2006; Hg: Franceschini et al., 2009; Heath and Frederick, 2005; Herring et al., 2012; Cd, Hg, Se: Pollock and Machin, 2009; Wada et al., 2009; Cd, Se: Wayland et al., 2002, 2003). Overall, the consistency of the positive relationships reported here between hepatic Mn, Co, Cu, Mo, Cd, As and feather CORT is surprising when set against the

inconsistent nature of the published data on metal concentrations and CORT levels and, in particular, the relatively low levels of the toxic metals in these liver samples. Cadmium is highly toxic (Li et al., 2013) but hepatic Cd concentrations in the present study (mean 0.3 μg/g dw) are considerably below the limit of <3 μg/g dw that is considered representative of background exposure (Larison et al., 2000; Scheuhammer, 1987). Arsenic is also toxic to vertebrates (Khan et al., 2014) but hepatic levels in the present study (mean 0.1 μg/g dw) are well within background levels (<5 μg/g dw; Eisler, 1988). It therefore seems unlikely that "conventional" toxic action is the factor that links feather steroid content with hepatic metal concentration unless feather CORT levels reflect an adverse impact of total hepatic metal loading. Certainly, hepatic trace element concentrations below the threshold for toxicity do not preclude the occurrence of sub-lethal effects (Lucia et al., 2012) and in this context it is relevant that there was a significant negative relationship between total hepatic metal content and both pectoral muscle weight and body condition index (Fig. S5) suggesting a possible adverse effect of the combined metal burden on performance. It is possible that variation in feather CORT associated with hepatic metal loading reflects disruption of steroid metabolism by metals (Dyer, 2007) something which might realistically occur at metal concentrations below those considered to be toxic. It is also possible that links between hepatic metal concentrations and CORT are driven by behavioural, metabolic, or nutritional correlates of variation in CORT that can influence the accumulation and/or turnover of metals. The co-correlation between hepatic concentrations of several of the metal species measured here suggests that their accumulation and/or retention in the liver may be linked, for example by interaction with a single class of binding site such as the metal-binding protein metallothionein (Andrews et al., 1996; Babula et al., 2012), which can be induced by glucocorticoids (Haq et al., 2003; Jacob et al., 1999). Notwithstanding the limited size of the

data set, the results are sufficiently robust to suggest further investigation of the links between metal loading and feather CORT concentration are justified, particularly regarding the possibility of a functional relationship between the two.

5. Conclusions

The results of this study indicate that CORT can be measured in cryo-archived feathers sourced from predatory birds acquired for the purpose of environmental contaminant monitoring. However, uncertainties concerning the factors that influence the deposition of steroid hormones in feathers, and the mechanism by which this occurs, will require further investigation. In particular, there is ambiguity in the relationship between body condition and feather CORT content, and whether steroid deposition in feathers is a time-dependent or mass-dependent mechanism. In sparrowhawks, a significant positive relationship between variation in hepatic metal concentration and feather CORT concentration was evident suggesting that the measurement of steroid content in feathers from archived sparrowhawk specimens may be a viable approach to accessing information on the activity of the endocrine system in relation to metal contaminant exposure. However, the underlying causality of the relationship observed between hepatic metals and feather CORT is presently unknown and may be of regulatory or metabolic rather than toxicological significance. In addition to resolving these uncertainties we need to extend these preliminary findings to additional species, and to other contaminants. In particular, the simultaneous measurement of both contaminant concentrations and CORT in feathers that are shed serially, together with analysis of contaminant levels in tissues that bioaccumulate pollutants, would help

considerably in establishing the relationship between feather steroid content and its functional significance.

SUPPLEMENTARY INFORMATION

Fig. S1. Parallelism between feather extracts and the CORT RIA standard curve.

Fig. S2. The relationship between CORT concentrations in adjacent feathers and feathers from matched wings.

Fig. S3 The relationship between date of collection of the specimen and feather CORT concentration.

Fig. S4. The relationship between feather corticosterone content and feather mass in sparrowhawks, barn owls and tawny owls.

Fig. S5. The relationship between total hepatic metal content, body condition index (body mass/sternum keel length), and pectoral muscle weight in male and female sparrowhawks.

Table S1. Outcomes of GLM analysis of indices of skeletal size (sternum, diagonal, wing length) as predictors of body mass and pectoral muscle mass for five raptor species.

Table S2. P values for the regression of feather hormone content against the residuals derived from the body mass and pectoral muscle mass models and pectoral muscle index.

Table S3. The outcomes of linear regressions of feather CORT concentrations (pg mm^{-1}) on hepatic metal content in fifteen sparrowhawks.

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Figure captions

Figure 1. Comparison of corticosterone concentrations in primary flight feathers (P5) from the left and right wings (L and R) and for adjacent primary flight feathers (P5 and P6) within the right wing (R) in three species of raptor. Each bar is the mean + SEM, with n values indicated inside the bars. No significant differences were apparent between P5L and P5R or between P5R and P6R within species. Significant differences between P5 CORT content across species are indicated by dissimilar letters.

Figure 2. Mean feather concentrations of CORT in the P5 flight feathers of five raptor species. Each bar is the mean + SEM. Males (M) and females (F) are shown separately for each species and n is indicated by the number within the bar. No significant differences between sex within species were observed, letters above each species pair denote differences between species overall. Means sharing the same letter are not significantly different.

Figure 3. The relationship between feather length and corticosterone content for (a) sparrowhawk, (b) barn owl and (c) tawny owl. Data plotted are for primary flight feathers ●P5, △P6, ■P7, ○P8 from a total of 10 (sparrowhawk) or 5 (tawny owl, barn owl) birds. Multiple feathers from the same individual are included to give the range of lengths needed to derive a relationship. Best-fit regression lines and 95% confidence intervals are shown: (a) n = 52, r^2 = 0.07, p = 0.06; (b) n = 25, r^2 = 0.31, p = 0.004; (c) n = 24, r^2 = 0.004, p = 0.8.

Fig. 4. Pectoral muscle index [PMI = 100*total pectoral muscle mass / (body mass – (mass of crop contents + gizzard contents))] in relation to feather corticosterone content in (a) kestrel ● (r^2 = 0.64, P = 0.03), (b) tawny owl ▽ (r^2 = 0.35, P = 0.017), (C) male sparrowhawk ■ and barn owl □ (regressions NS), (d) female sparrowhawk ○ and buzzard ▲ (regressions NS).

Fig. 5. The relationship between feather corticosterone concentration and liver metal concentration in fifteen sparrowhawks. The linear regression best-fit line is shown for each metal; (a) Mn: r^2 = 0.48, P = 0.004; (b) Co: r^2 = 0.31, P = 0.03; (c) Cu: r^2 = 0.3, P = 0.03; (d) Mo: r^2 = 0.32, P = 0.03; (e) Cd: r^2 = 0.31, P = 0.03; (f) As: r^2 = 0.26, P = 0.05. Results for male birds are denoted by filled circles, females by unfilled circles.
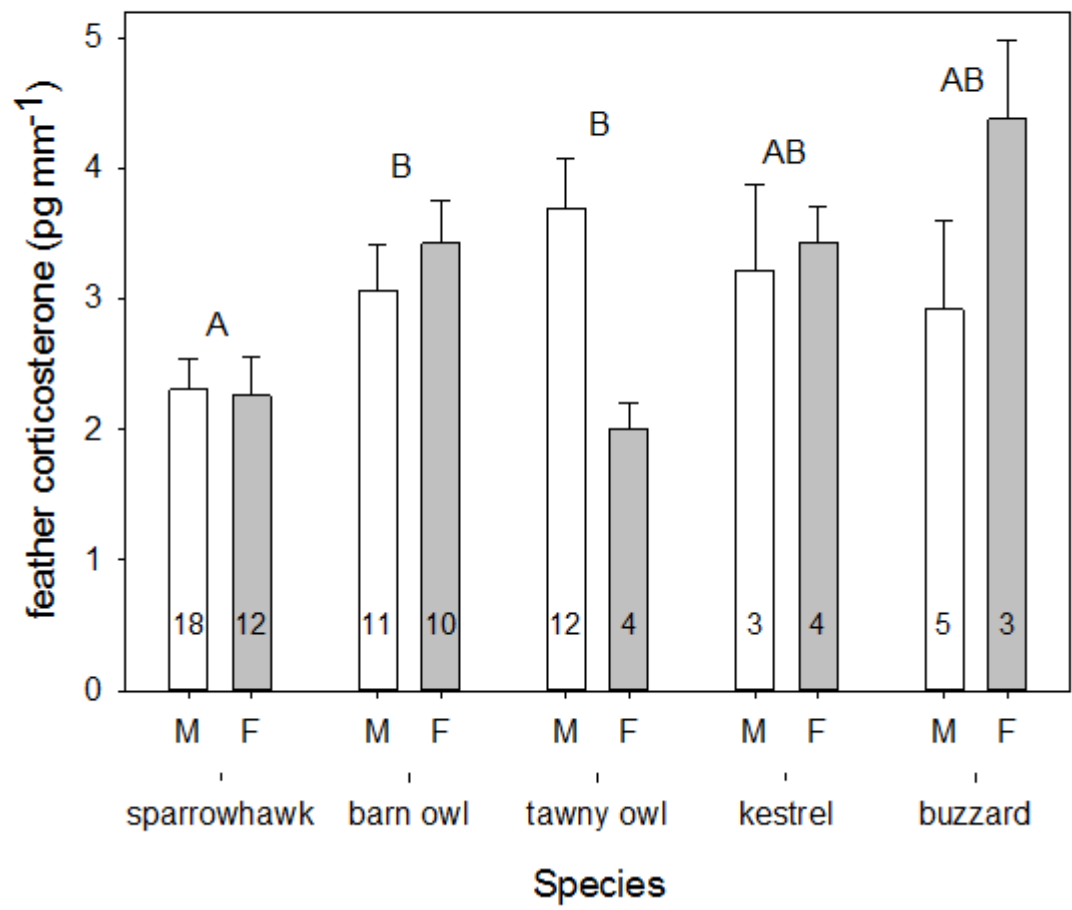
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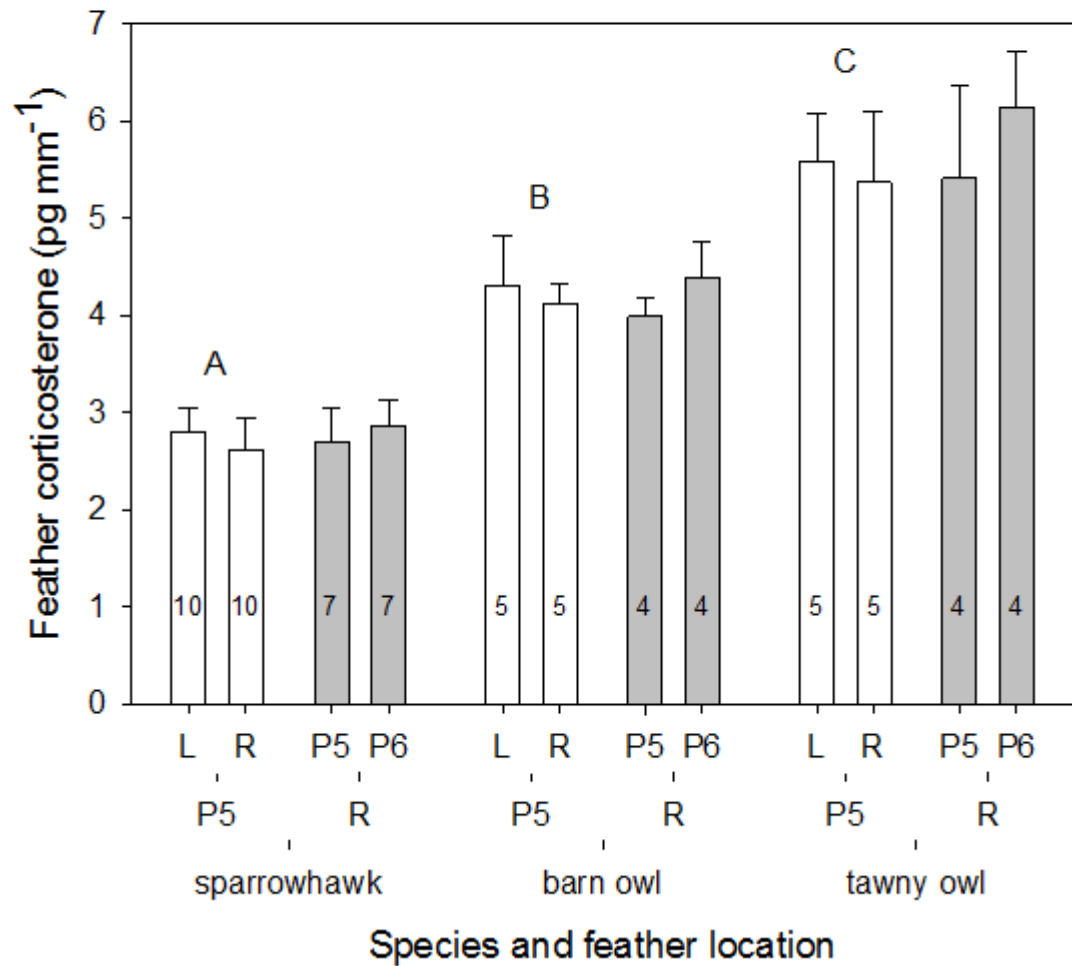
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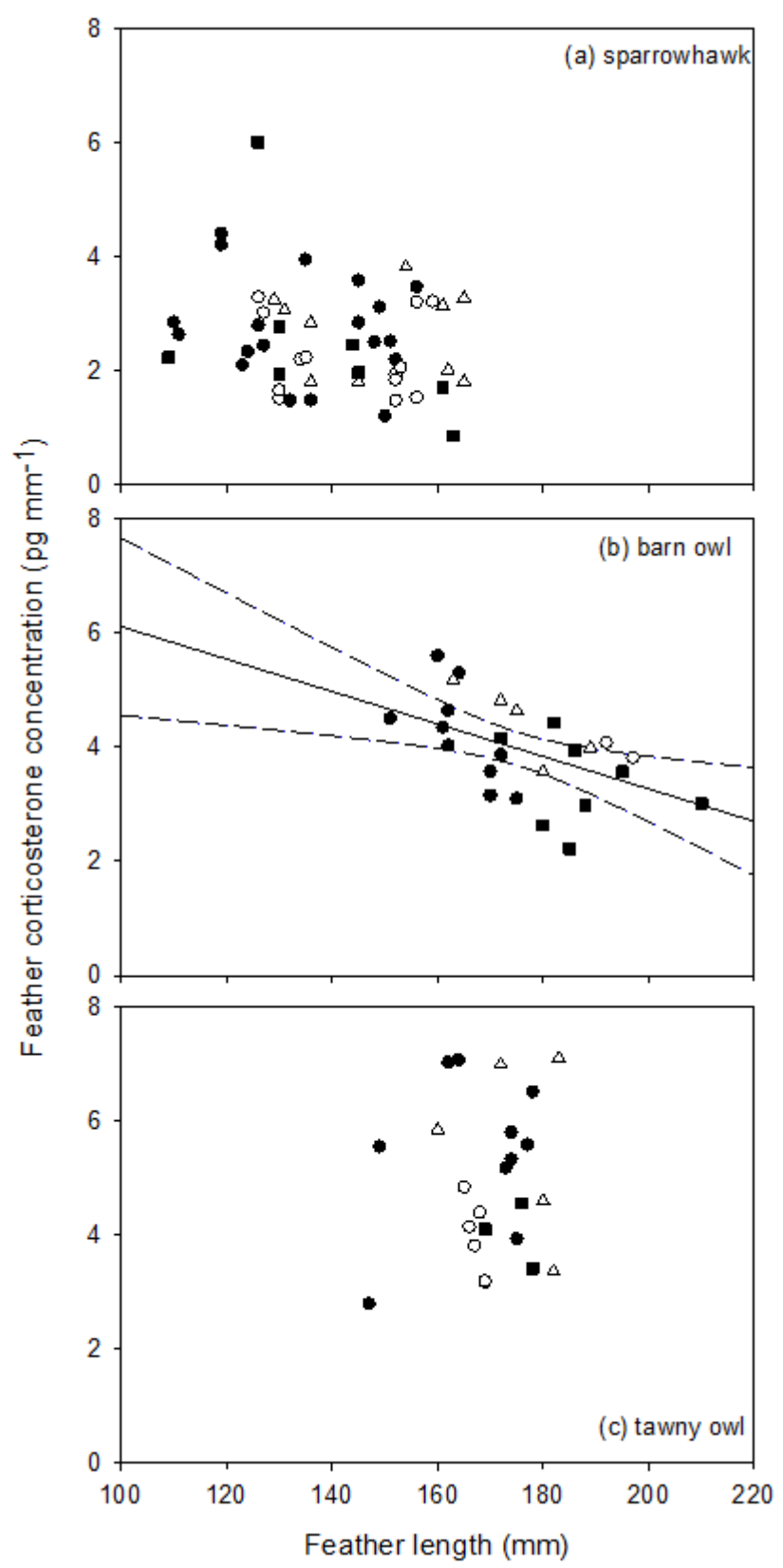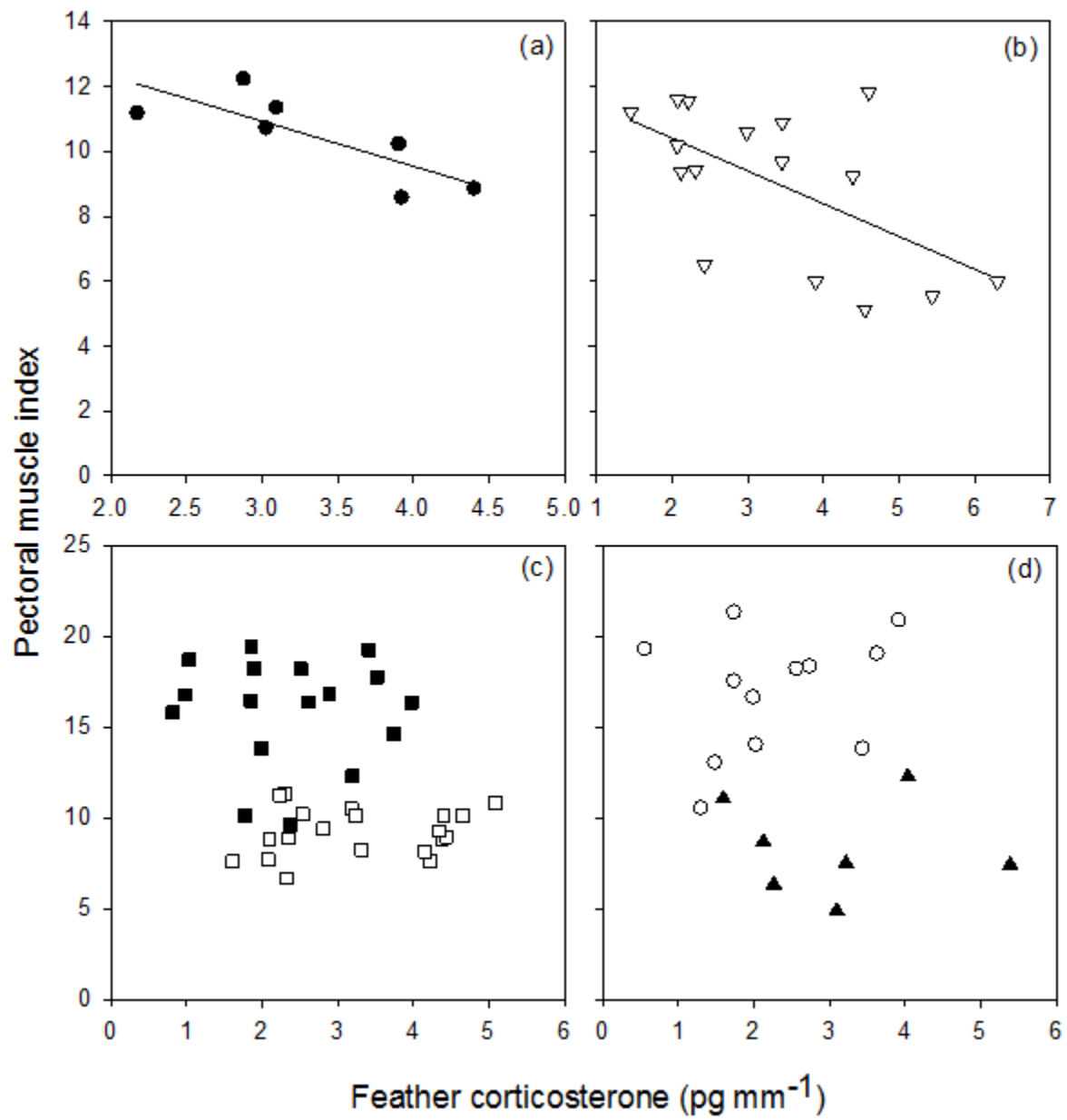
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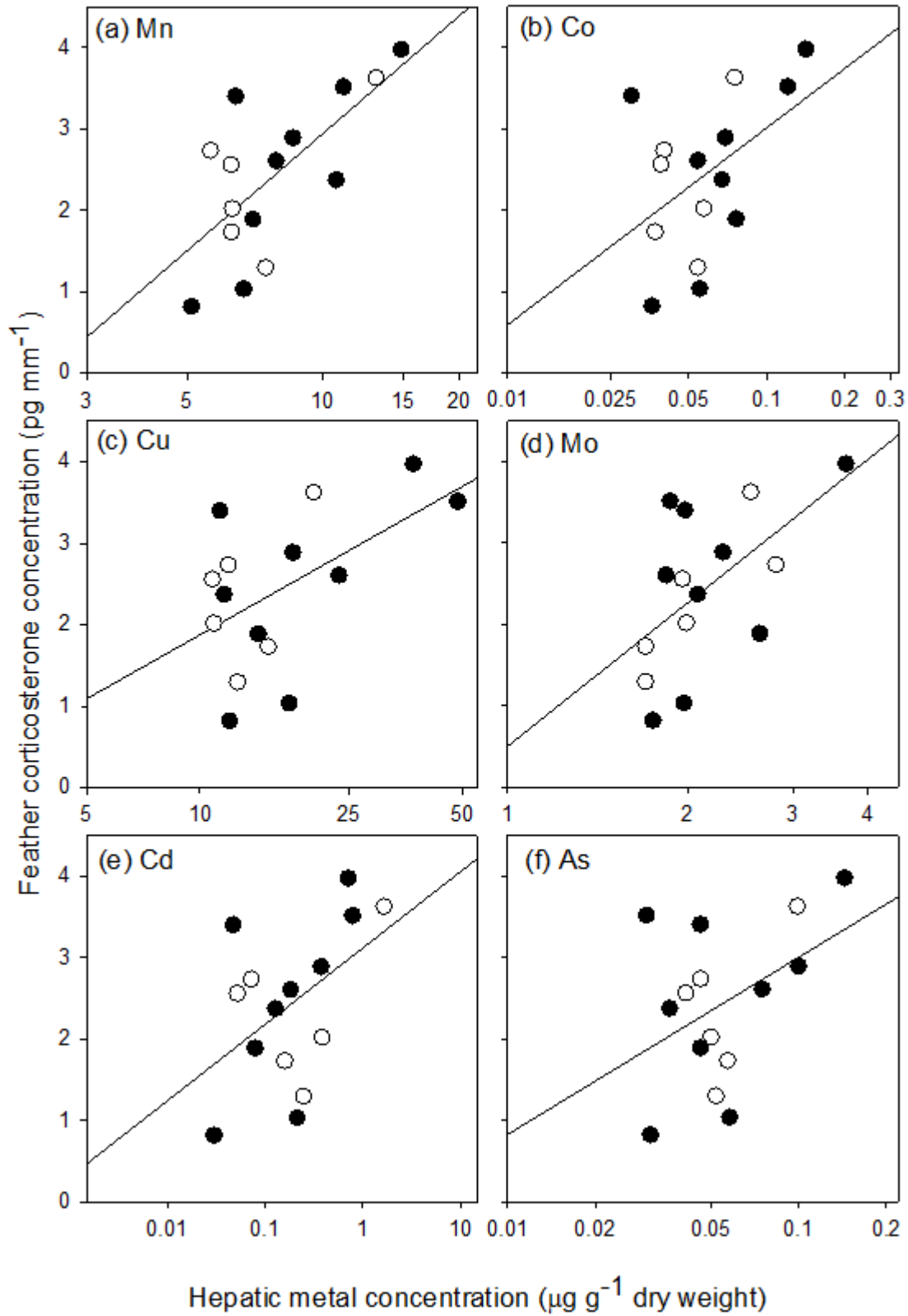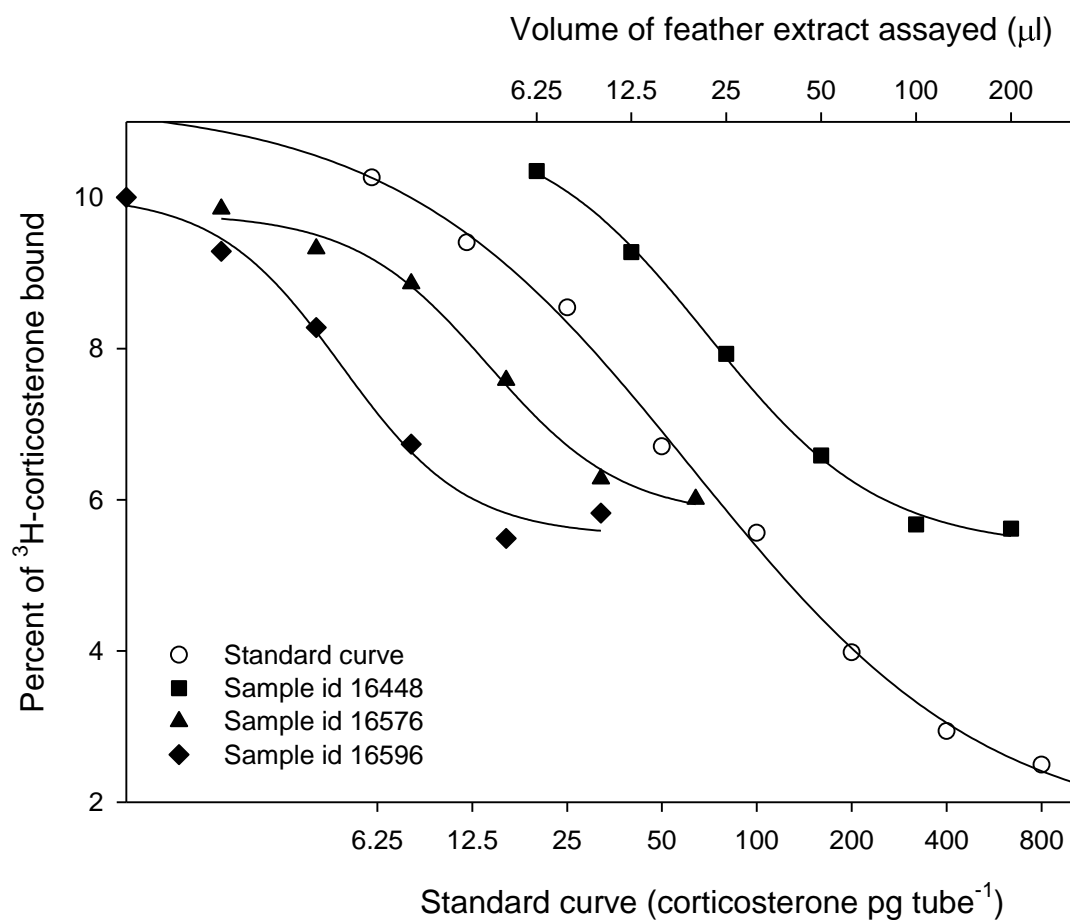
Figure 5.

Figure S1. The effects of serial dilutions of three feather extracts (■,▲,◆; volume of original extract: 200 µl) on percent binding of ^{3}H-corticosterone. A standard curve is shown (O; 6.25 − 800 pg). A representative volume scale is provided for one of the extracts but the incremental change in volume is the same for all three.

Fig. S2. (a) A scatter plot of feather corticosterone concentrations in primary flight feathers P5 from the left and right wings of individual sparrowhawks (● n = 10), barn owls (▽ n = 5) and tawny owls (□ n = 5). The best-fit line is shown (linear regression, $P < 0.001$, $r^2 = 0.67$). (b) A scatter plot of feather corticosterone concentrations in adjacent primary flight feathers P5 and P6 from the right wings of individual sparrowhawks (● n = 7), barn owls (▽ n = 4) and tawny owls (□ n = 4). The best-fit line is shown (linear regression, $P < 0.001$, $r^2 = 0.69$).
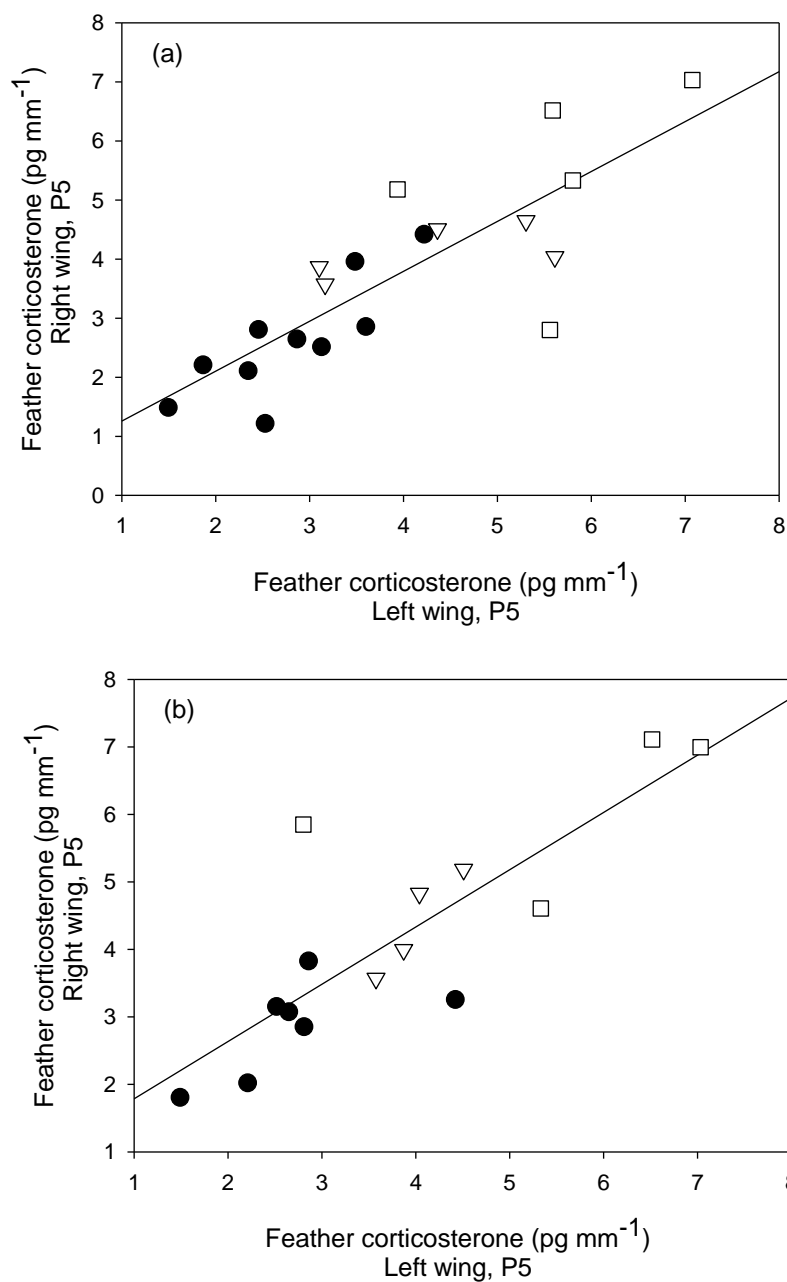
Fig. S3. The relationship between feather corticosterone concentration in sparrowhawks and (a) the month in which the specimen was received by the PBMS and (b) the year in which the specimen was received by the PBMS. Best-fit linear regression lines are shown: (a) $r^2 = 0.18$, $P = 0.02$; (b) $r^2 = 0.19$, $P = 0.02$. n = 30.
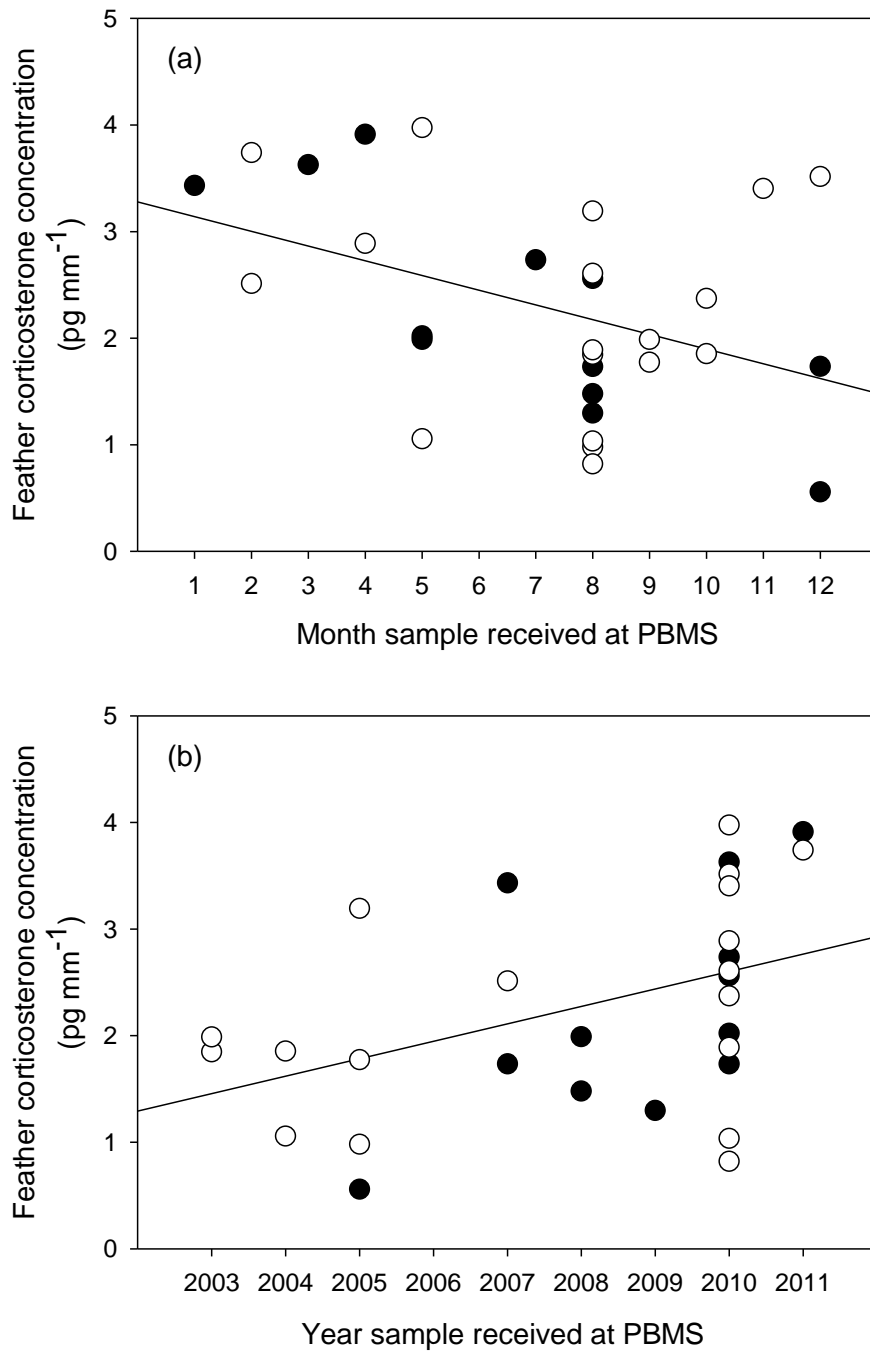
Fig. S4. The relationship between feather corticosterone content and feather mass in (a) sparrowhawks, $r^2 = 0.03$, P = 0.3; (b) barn owls $r^2 = 0.19$, P = 0.03; and (c) tawny owls $r^2 = 0.12$, P = 0.1. Position of feather is denoted by symbol: ● P5; △ P6; ■ P7; ○ P8. Linear regression line and 95% confidence intervals are shown.
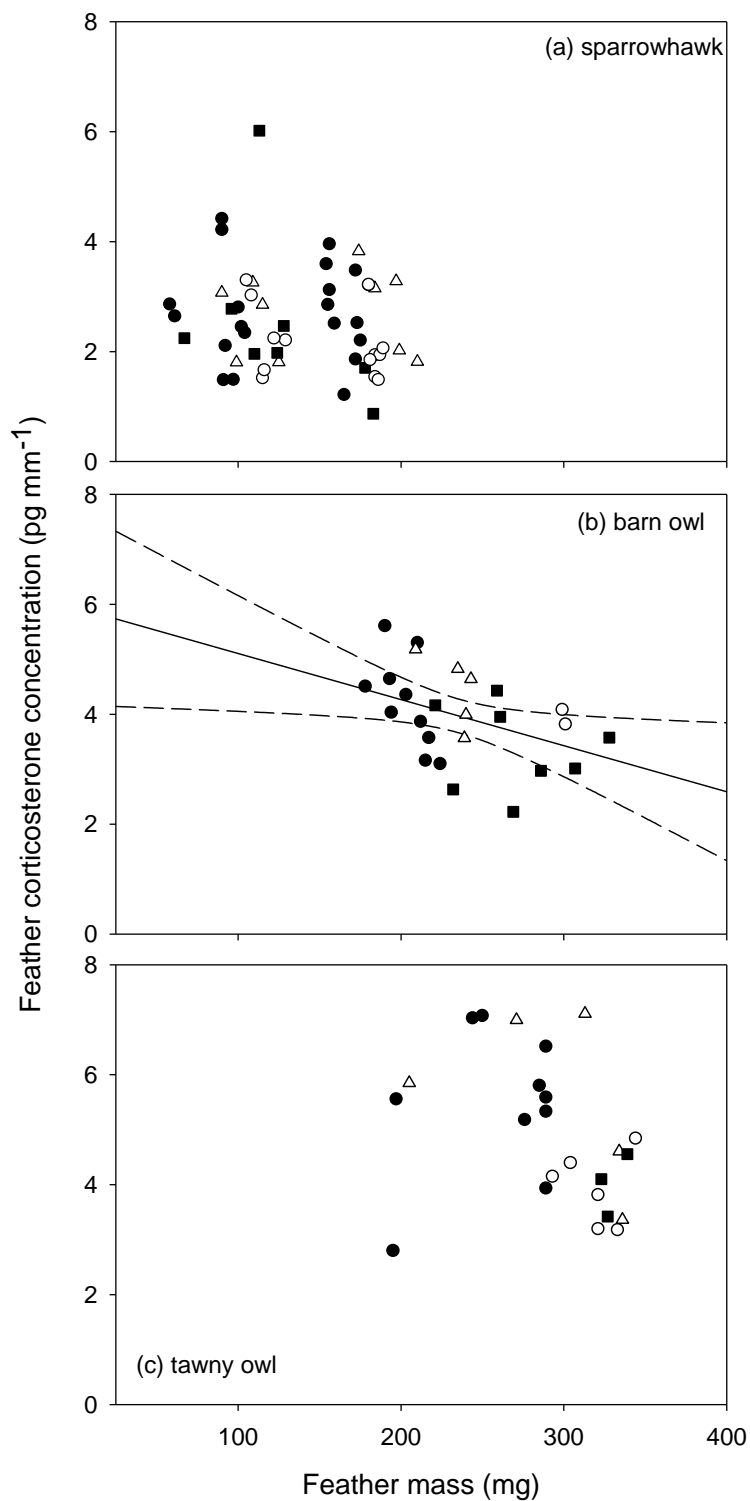
Fig. S5. The relationship between total hepatic metal content and (a) body condition index (body mass/sternum keel length) and (b) pectoral muscle weight in male (●) and female (○) sparrowhawks. In each case the regression was significant only for the male birds: (a) males $r^2 = 0.70$, P = 0.005; females $r^2 = 0.10$, P = 0.5 (b) males $r^2 = 0.68$, P = 0.006; females $r^2 = 0.19$, P = 0.4.
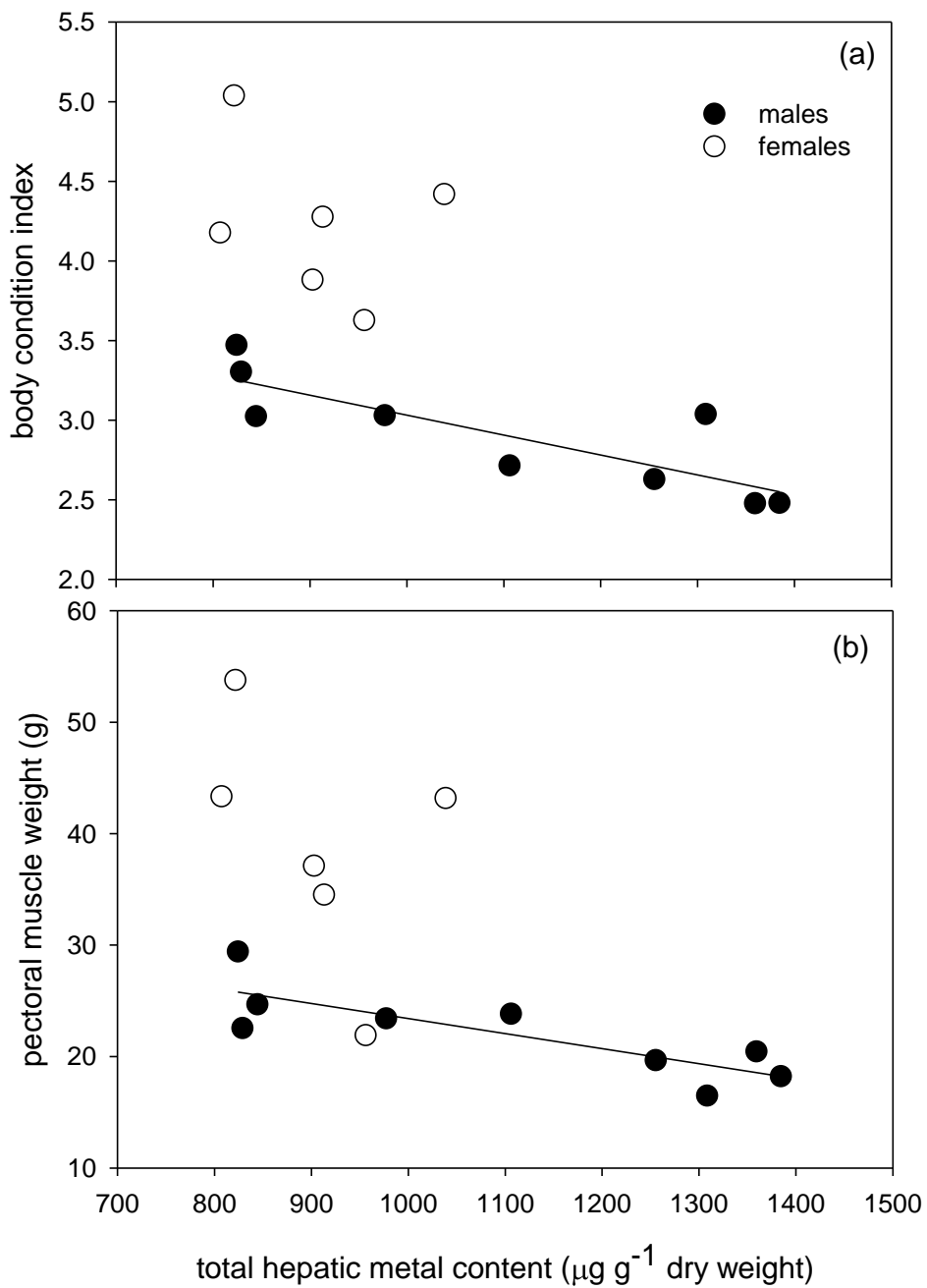
Table S1. Outcomes of GLM analysis of indices of skeletal size (sternum, diagonal, wing length) as predictors of body mass and pectoral muscle mass for five raptor species. Data were obtained from the PBMS archive. The significance of each model term, and overall r^2 values, are shown. The number of individuals within each group is shown in brackets.

Model	Variables	Buzzard		Barn owl		Tawny owl		Kestrel		Sparrowhawk	
		Male (37)	Female (21)	Male (103)	Female (90)	Male (51)	Female (42)	Male (29)	Female (26)	Male (60)	Female (72)
Body weight	Sternum	0.20	0.17	0.067	**0.006**	0.514	**0.016**	0.616	**0.012**	0.136	**0.053**
	Diagonal	0.67	0.16	0.701	**0.023**	0.988	**0.037**	0.312	0.069	**0.009**	0.094
	Wing Length	0.57	**0.02**	**0.001**	0.176	0.513	**0.002**	0.467	0.415	0.116	0.100
	r^2	0.03	**0.52**	**0.15**	**0.20**	-0.03	**0.43**	0.01	**0.30**	**0.49**	0.04
Pectoral muscle	Sternum	0.063	0.389	0.541	0.229	0.428	0.100	0.382	**0.019**	0.485	0.090
	Diagonal	0.464	0.135	0.405	0.275	0.688	0.410	0.182	**0.004**	0.492	0.422
	Wing Length	0.389	0.291	**0.000**	**0.000**	0.918	0.315	0.437	0.602	0.683	0.215
	r^2	0.03	**0.19**	**0.27**	**0.24**	-0.05	**0.24**	0.19	**0.46**	-0.01	0.03

Table S2. P values for the regression of feather corticosterone content against residuals derived from the body mass and pectoral muscle mass models and pectoral muscle index.

Measure of condition	Buzzard M+F	Barn owl M+F	Barn owl F	Barn owl M	Tawny owl M+F	Kestrel M+F	Sparrowhawk M+F	Sparrowhawk F	Sparrowhawk M
Body weight	0.92	0.61	0.81	0.68	0.34	0.82	0.94	0.53	0.32
Pectoral muscle	0.87	0.43	0.83	0.31	**0.017**	**0.032**	0.67	0.39	0.87
Pec. musc. Index[1]	0.89	0.47	0.87	0.31	**0.017**	**0.033**	0.65	0.39	0.88
n	8	21	10	11	16	7	30	12	18

[1] pectoral muscle index = [total pectoral muscle mass / (body mass – (mass of crop contents + gizzard contents)) * 100]

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Table S3. The outcomes of linear regressions of feather corticosterone (CORT) concentration (pg mm^{-1}) on hepatic metal, non-metal and metalloid content in fifteen sparrowhawks. Significant results are highlighted.

Analyte	Class / type	CORT	
		r^2	P
Al	Toxic metal	0.03	0.9
As	Toxic metalloid	0.26	0.05
Cd	Toxic metal	0.31	0.03
Co	Essential metal	0.31	0.032
Cr	Trace metal	0	0.94
Cu	Essential metal	0.3	0.033
Fe	Essential metal	0.06	0.36
Hg	Toxic metal	0.13	0.19
Mn	Essential metal	0.48	0.004
Mo	Essential metal	0.31	0.03
Ni	Trace metal	0.12	0.24
Pb	Toxic metal	0.11	0.22
Sb	Toxic metalloid	0.34	0.21
Se	Essential non-metal	0.03	0.53
Sr	Trace metal	0.31	0.25
Zn	Essential metal	0.18	0.11