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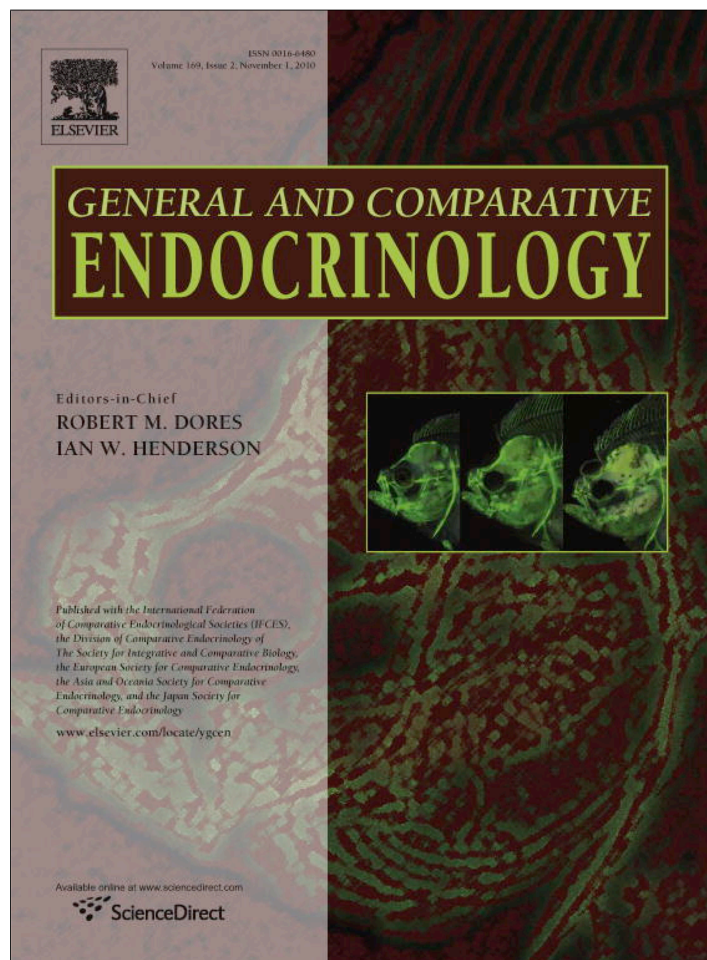

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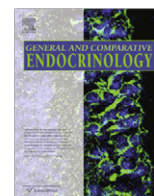

Short Communication

Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration

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ABSTRACT

Avian endocrinology is a productive field that could benefit from increased application of non-invasive techniques. Although assay protocols vary, most studies that measure hormone metabolites in avian feces struggle with an artificial effect of sample mass on steroid metabolite concentration. Hormone metabolite concentrations measured in small samples are consistently higher than concentrations in larger samples, and this appears to be due to multiple methodological problems. We systematically tested several causal hypotheses for the mass effect. Based on results from these tests, we modified and validated our assay protocol to effectively eliminate the mass effect. Future studies should implement the following procedures when measuring hormone metabolites from small fecal samples (particularly of birds and reptiles): (1) remove urates from the fecal sample as completely as possible; (2) lyophilize the sample prior to extraction; (3) maximize accuracy of small mass measurements; (4) increase the volume of ethanol in the extraction to 15 ml per 0.05–0.1 g of dried feces; and (5) eliminate ethanol from all samples prior to radioimmunoassay by drying down extract solutions and rehydrating in buffer. By applying these precautions we successfully eliminated the mass effect from fecal samples ranging in mass from 0.001 to 0.1 g using a radioimmunoassay commonly employed for studies of fecal glucocorticoid metabolites. These corrections also resulted in a more than 3-fold increase in effect size in glucocorticoid concentrations from a controlled test of the effects of 1 h motorcycle exposure on northern spotted owls. These methods have important implications not only for avian studies, but for any study measuring hormone metabolites from small fecal samples.

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1. Introduction

Comparative avian endocrinology has flourished thanks to a diversity of readily observed species and excellent field work conducted using blood. Factors such as low blood volume associated with small size and stress associated with capture and blood withdrawal, suggests that avian endocrinology could also greatly benefit from non-invasive methods using feces. However the application of fecal hormone methods to avian species has been constrained by a persistent effect of low sample mass on measures of steroid metabolite concentration (Cyr and Romero, 2008; Goymann et al., 2006; Millspaugh and Washburn, 2004; Tempel and Gutierrez, 2004; Washburn et al., 2004). Hormones appear to be disproportionately elevated in low mass fecal samples, common in birds and other small vertebrates. Although, in some cases the mass effect may be at least partially spurious (due to correlations that depend on the way in which hormone concentrations are calculated; Goymann, 2005), in other cases it is due to methodological problems. Here we identify and remedy methodological causes of the mass effect, thereby significantly reducing measurement error and allowing inclusion of small samples in final analysis.

Tempel and Gutierrez (2004) proposed eliminating the subset of samples that have masses below a set threshold of, for example, 0.01 g. This approach can ameliorate the problem, but does not eliminate the mass effect. Eliminating samples of less than 0.01 g can substantially reduce sample size for many avian species. It could also introduce significant bias if, for example, stressed animals defecate more frequently, eat less and/or produce smaller fecal samples.

We hypothesized five methodological contributors to the mass effect observed in studies of avian fecal metabolites: (1) failure to remove hormone-inert urates; (2) variation in fecal moisture content due to differential sample dehydration; (3) reduction in extraction efficiency at low extract solvent volume to feces mass ratios; (4) asymmetrical contribution of measurement error in the final calculation of hormone concentration; and (5) interference of ethanol with radioimmunoassay accuracy. We addressed each potential contributor in a series of validation experiments on pooled feces and then tested whether the refined protocol could eliminate the mass effect from samples collected in the field.

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1.1. Urate content

Avian feces, urine and urates (the pasty white nitrogenous waste produced by birds and reptiles) are all voided simultaneously through a single opening, the cloaca. Radiolabel infusion studies showed that urine and feces both contain steroid hormone metabolites, whereas urates do not (Wasser and Hunt, 2005). Urine tends to soak into the ground before it can be collected, making it unfeasible to reliably collect in most field studies. Since hormone-inert urates vary in amount, they can inflate sample mass without increasing hormone concentration. Feces should thus be separated from urine and urates prior to hormone extraction, with fecal hormone concentrations expressed solely on a per gram fecal mass basis (Wasser and Hunt 2005), versus per gram excretion mass that also includes the urine and urate mixture (e.g. Kellam et al., 2004). For some species (such as the northern spotted owl) such separation is fairly easy at the time of collection because a protective membrane encapsulates feces to protect the reproductive tract from fecal bacteria. For other species separation may not be possible due to mixing of urine, urates and feces in the cloaca (Klasing, 2005).

1.2. Moisture content

Moisture content varies among avian fecal samples due to size-dependent dehydration; smaller samples have a larger surface to volume area causing them to lose disproportionately more moisture than larger samples over time. This disproportionate reduction in sample mass can inflate hormone concentrations when expressed per gram wet weight. Freeze-drying samples prior to extraction and expressing hormone concentrations per gram dry weight eliminates this variation. It also helps control for diet-related effects resulting from associated changes in sample water content (Wasser et al. 1993).

1.3. Asymmetrical effect of measurement error

Error associated with mass measures tends to be relatively larger for small samples (i.e. a 0.001 error in sample weight measurement represents 20% of a 0.005 g sample versus 1% of 0.1 g sample). The quantitative impact of measurement error in calculations of hormone concentration is stronger when it reduces rather than increases sample mass; impacts of error in the denominator become exponentially exaggerated as the denominator approaches zero in a 1 year relation. The simulation shown in Fig. 1, illustrates that measurement error is likely to exacerbate the mass effect problem because under-estimating sample mass has a disproportionate impact on calculated concentration. In this simulation the 'true' value of the denominator ranged from 0.01 to 0.03 and the 'true' value of the numerator was always assumed to be 10 times the denominator. The 'measured' values of the denominator and numerator were subject to error by adding uniformly distributed values to each. If error is small relative to the lowest value of the denominator, then the measured fractions were fairly close to the 'true' value of the fraction. However, if the error is large relative to the lowest value of the denominator, then the measured fractions tend to deviate strongly above the 'true' value for the denominators with the smallest measurements (Fig. 1). As mass measurement accuracy decreases the size of samples for which hormone concentration can be accurately calculated increases.

1.4. Volume of extract solvent

Fecal hormones from avian scat are commonly extracted in 2–5 ml of ethanol or methanol (Schwarzenberger et al., 1991; Wasser et al., 2000), regardless of sample mass. However, Wasser et al. (2010) recently demonstrated that ethanol extraction becomes less effective as the volume to mass ratio falls below 15 ml ethanol per 0.1 g feces. In the past, using too little extraction solvent per unit

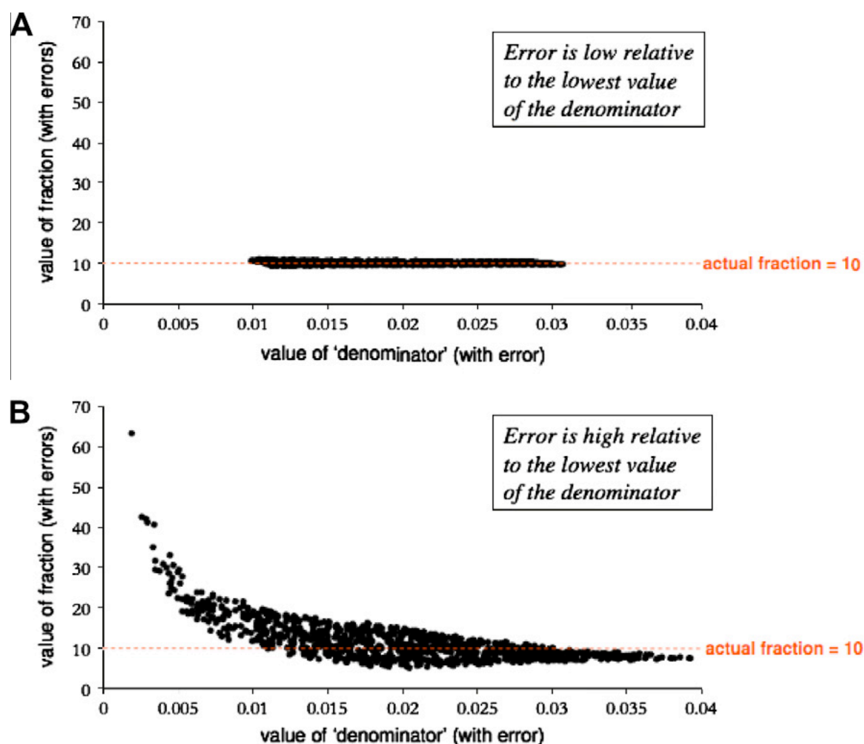

Fig. 1. Simulation showing the mass error effect. (A) The measured fractions were fairly close to the 'true' value of the fraction if measurement error was very small relative to the lowest value of the denominator. However, (B) the measured fractions tend to deviate strongly above the 'true' value for the denominators when the error is large relative to the lowest value of the denominator.

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feces may have contributed to the mass effect by disproportionately lowering hormone concentrations for large samples.

1.5. Interference of ethanol with radioimmunoassay accuracy

Ethanol can interfere with the accuracy of competitive binding radioimmunoassay, artificially inflating hormone concentrations. While this effect can be eliminated in many cases by diluting the ethanol in assay buffer (Wasser and Hunt, 2005; Wasser et al., 2000), sample mass is likely to vary considerably between samples in avian fecal steroid metabolite studies, while volume of extract solution remains constant. Small samples could thus suffer from greater assay interference if sample mass buffers antibody from interference impacts of ethanol. The net result would be more inflation in measured hormone concentrations for small samples than for large.

2. Methods

2.1. Fecal pool

Scat was collected from two captive spotted owls at the Woodland Park Zoo in Seattle, Washington over the course of two weeks in the fall of 2007 in accordance with University of Washington IUCAC protocol # 2850-04. Prior to sample collection the floor of the owls' enclosure was covered with plastic. Scat was collected at roughly the same time everyday and immediately frozen until sufficient fecal mass had been accumulated to provide a pool large enough to create the needed subsample replicates. Samples were stored in ¼ oz polypropylene jars with unlined screw caps (similar to SKS Bottle & Packaging # 0611-01). Urates were removed as much as possible prior to combining samples and thoroughly homogenizing them in the sample pool. Separation of urates from feces was accomplished with wooden stir sticks at the time of collection and more thoroughly later in the laboratory with the use of metal prods and spatulas. It was not always possible to completely remove all urates. Urine was not collected although some may have soaked into feces.

2.2. Moisture content

Fifty subsamples of the pooled feces, ranging from 0.002 to 0.18 g wet weight were each weighed in a vial of known mass, and stored in ¼ oz polypropylene jars at −20°F for one month to simulate storage time in the field during which evaporative moisture loss could occur. Owing to their small mass, disproportionate moisture loss may also have occurred during weighing. Samples were reweighed after one month. The loss in mass was calculated as a percentage of initial sample mass and regressed against initial sample mass after normalizing the distribution by LOG transformation.

2.3. Scale accuracy

A 0.001 g calibration weight was weighed 50 times on two different scales that varied in precision from 0.1 mg readability and repeatability (Denver Instruments APX-200 scale) to 0.01 mg readability and 0.015 repeatability (Mettler Toledo AT261 Delta range scale). The effects of the precision-related weight variations on final hormone concentrations of the same samples were then compared.

2.4. Ethanol interference

The fecal pool was freeze-dried and divided into 80 subsamples in 10 mass categories that ranged from 0.01 to 0.1 g. Forty subsamples were weighed on the Denver scale and 40 additional subsamples were weighed on the Mettler scale. Each subsample was extracted with 15 ml of 70% ethanol prior to standard fecal corticosterone assay.

2.5. Extraction

Although extraction protocol varied among different tests (see below), all samples were subjected to 20 min of shaking with extract solution on a pulsing vortexer at room temperature.

2.6. Radioimmunoassay

All samples were assayed for fecal glucocorticoid metabolites using an I^{125} corticosterone kit from MP Biomedicals, Costa Mesa, California (MP Biomedicals # 07-120103) following accompanied instructions, but halving the volume of all reagents (Wasser et al., 2000). This kit was previously validated for spotted owl feces (Wasser et al., 1997; Wasser and Hunt, 2005). Samples were run in two assays. For the first assay, the 70% ethanol extract solution was diluted 1:4 with assay buffer (Möstl et al. 2005; Wasser et al. 2000; Wasser and Hunt 2005) according to standard practice. For the second assay the extract solution was transferred to test tubes, then dried down and resuspended in buffer to remove all ethanol before adding assay reagents. Interassay variation was 5.4%.

2.7. Analysis

Shapiro–Wilk W tests were used to test distribution of data. Linear regression was performed to test for relationships between sample mass and fecal corticosteroid metabolite concentration for samples weighed on both the Denver and Mettler scales.

2.8. Testing modified protocol with samples from the wild

Samples were collected from free-living northern spotted owls in Shasta-Trinity National Forest, CA between the months of April and July 2007 and 2008 and processed with the prescribed methods: cleared of urates, lyophilized, extracted in 15 ml 70% ethanol, dried and resuspended in buffer prior to RIA. Unfortunately, only the less accurate Denver scale was available for weighing these samples, requiring us to exclude samples <0.02 g dry mass from the analysis. Samples of more than 0.05 g dry mass were homogenized and sub-sampled at approximately 0.05 g. The mass effect among these samples was then compared to the mass effect among samples collected from the same population of spotted owls in the same months of 2005 and 2006 (before validations were conducted to refine extraction and assay methodology). In 2005 samples were cleared of urates but extracted wet in 2 ml of 90% methanol. In 2006 samples were cleared of urates, lyophilized and extracted with 2 ml 90% ethanol. Each sample from 2005 and 2006 was assayed two ways: either by diluting the ethanol in assay buffer, or by drying the ethanol and resuspending the extract in assay buffer. For purposes of analysis all samples of less than 0.02 g were also removed from the 2005 and 2006 subsets.

In order to quantify the impact of noise created by the mass effect on signal strength from a controlled experiment, we used linear regression to compare the treatment effect found among males in May of 2006 with the treatment effect found among males in May of 2008 (Hayward et al. in submission). In this case treatment consisted of 1 h of experimentally applied motorcycle exposure. Fecal samples collected at least 2 h post treatment (or at an equivalent time on control sites) were analyzed for GCs using methods described above. Only males sampled in May were included in this analysis to control for gender and breeding season effects; owl responsiveness is greatest early in the breeding season (May) and

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particularly in males (Wasser et al., 1997; Hayward, unpublished data). Sample size was nine males from 2006 and nine males from 2008 (total number of males sampled in May of 2008 was 14; five were dropped from analysis at random to facilitate comparison of effect size with 2006).

3. Results

3.1. Feces pool

3.1.1. Moisture loss

As predicted, the proportion of mass lost during one month of storage in the freezer was inversely proportional to the size of the fecal sample (R^2 adjusted = 0.71, $P < 0.001$).

3.1.2. Scale accuracy

Measurements of the 0.001 g calibration weight taken with the Mettler scale conformed to a normal distribution ($W = 0.99$; $P = 0.80$), but measurements from the Denver scale did not ($W = 0.93$; $P = 0.004$). The Denver scale systematically underestimated the mass of the calibration weight (mean = 8.63 e^{-4}), while the Mettler scale showed no measurement bias (mean = 1.00 e^{-3}). Standard deviation was an order of magnitude higher for the Denver scale than for the Mettler (4.43 e^{-4} vs. 4.30 e^{-5}, respectively).

3.1.3. Ethanol interference

Hormone concentrations measured in the first assay (ethanol diluted in assay buffer) were not normally distributed, showing significant skew toward lower concentrations, even after LOG transformation (Denver W = 0.61, $P < 0.001$; Mettler $W = 0.62$, $P < 0.0001$). Linear regression demonstrated significant correlation between LOG fecal glucocorticoid concentration and LOG mass for both sample subsets (Denver R^2 adjusted = 0.68, $P < 0.001$; Mettler R^2 adjusted = 0.94, $P < 0.001$; Fig. 2A and B).

By contrast, glucocorticoid metabolite concentrations were normally distributed in the samples in which ethanol was evaporated prior to assay, for both the Denver ($W = 0.98$; $P = 0.63$) and Mettler-weighed subsets ($W = 0.96$; $P = 0.20$). A significant mass effect persisted in the subset of samples weighed on the less accurate Denver scale (R^2 adjusted = 0.19; $P = 0.003$ Fig. 2C). However, the mass effect was successfully eliminated for samples weighed on the more accurate Mettler scale (R^2 adjusted = −0.01; $P = 0.52$ Fig. 2D).

3.1.4. Samples collected in the field

There was a strong mass effect among samples collected from the field and processed using traditional extraction and assay methods in 2005 and 2006 (R^2 adjusted = 0.34, 0.39, respectively; $P < 0.0001$ for both; Table 1). When extracts were dried and resuspended in buffer before radioimmunoassay the mass effect was diminished but not eliminated (R^2 adjusted = 0.09, 0.10, respectively; $P < 0.0001$ for both; Table 1). By comparison, the mass effect was effectively eliminated in the 2007 and 2008 samples processed using the new methodology (R^2 adjusted = −0.01, −0.004; $P = 0.86$, 0.53, respectively; Table 1).

One hour of motorcycle exposure did not appear to significantly increase GC levels for males sampled in May of 2006 when compared to controls ($n = 9$; R^2 adjusted = 0.12; $P = 0.19$). By contrast,

Table 1
Standard extraction and radioimmunoassay methods resulted in a strong mass effect for samples collected from the field in 2005 and 2006 (undried). This effect was reduced but still strongly significant once ethanol was dried off prior to radioimmunassay (dried) in the 2005–2006 samples. The mass effect was successfully eliminated when the revised methodology was applied to samples collected from the field in 2007 and 2008.

	N	R^2 adjusted	P
2005 Undried	218	0.34	<0.0001
2006 Undried	240	0.39	<0.0001
2005 Dried	218	0.09	<0.0001
2006 Dried	240	0.10	<0.0001
2007	77	−0.01	0.86
2008	151	−0.004	0.53

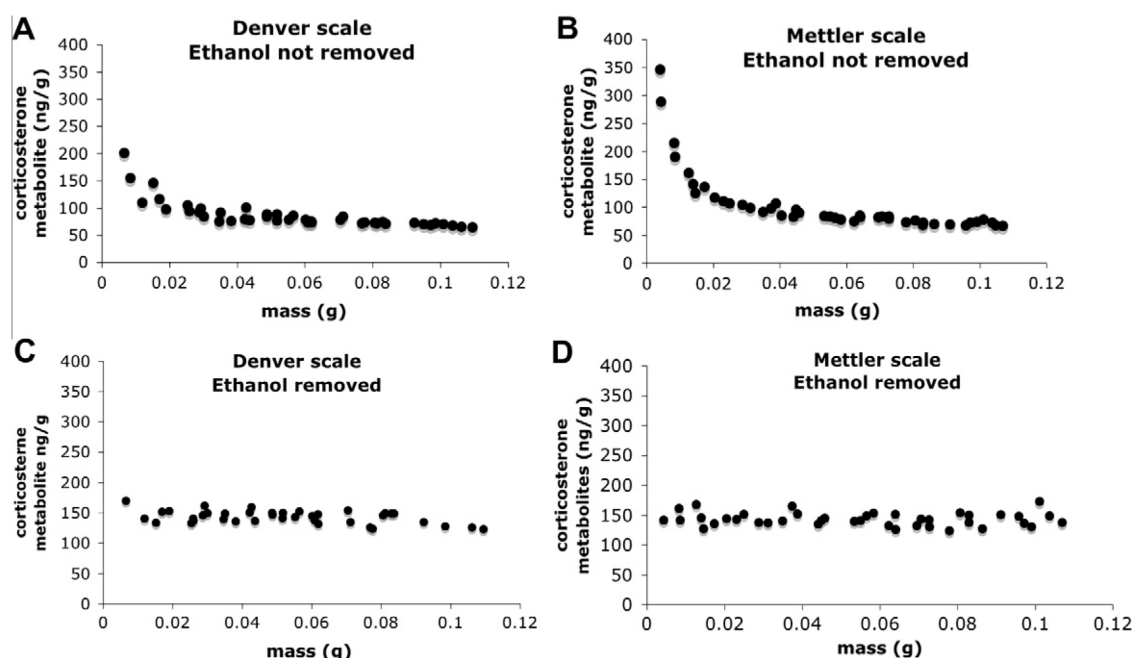

Fig. 2. Standard extraction and radioimmunoassay technique in many labs involves diluting ethanol extract solution with buffer, leaving small and variable amounts of ethanol in the assay. Ethanol interferes with the assay accuracy, creating a significant correlation between scat sample mass and measured hormone concentration (A, B). This mass effect was greatly reduced by removing the ethanol prior to radioimmunoassay (C, D). When using the less accurate Denver scale (C) a small mass effect persisted. However, when samples were weighed on the more accurate Mettler scale the mass effect was completely eliminated (D).

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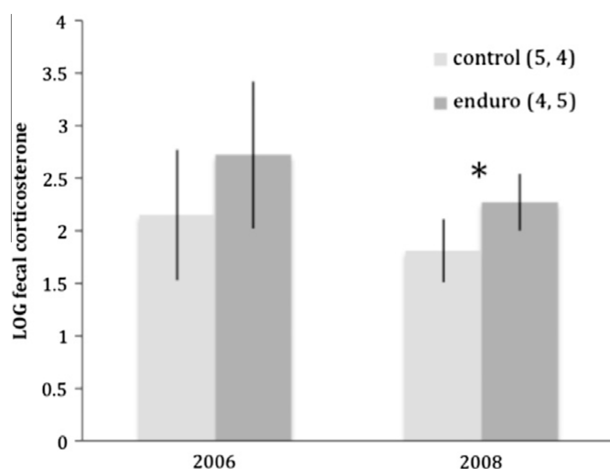

Fig. 3. Methodological problems increased the standard error among 2006 samples enough to mask a treatment effect in a controlled field experiment. An optimized assay protocol reduced standard error among samples collected in 2008 and decreased standard error enough to uncover significance with identical sample size in the same field experiment. Sample sizes are shown in parentheses.

effect size associated with identical treatment was more than three times higher among males sampled in May of 2008 (0.33 in 2008 vs. 0.14 in 2006) and significantly different from controls ($n = 9$; R^2 adjusted = 0.43; $P = 0.03$) Fig. 3.

4. Discussion

Multiple factors have likely contributed to the persistent mass effect demonstrated in most previous studies of avian fecal hormone metabolites. Our results suggest that by simultaneously controlling five potential contributors, the mass effect can be effectively eliminated from a set of samples ranging in mass from 0.001 to 0.01 g. This revised sample processing methodology will allow retention of small samples that otherwise would have to be eliminated from analysis, and also reduces the risk of bias associated with elimination of small samples. Applying these methods to experimental data in our study seemed to reduce measurement error in 2008 that masked a treatment effect on GCs in 2006.

Our results suggest the urine and urate mixture should be removed from the fecal mass prior to processing when feasible. We recognize that separation may be impossible for some species. The feces should then be freeze-dried prior to extraction to eliminate impacts of differential moisture loss. Care should be taken to minimize measurement error when weighing subsamples for extraction, preferably by using a higher precision balance than is typically available in most hormone laboratories (i.e. one with 0.01 mg readability and 0.015 repeatability or out to five decimal points). In order to optimize extraction, no more than 0.1 g dry feces should be added to 15 ml of ethanol solution. When smaller ratios of ethanol to feces are used, hormone concentrations appear lower, perhaps resulting from antibody cross-reactivity to compounds that do not readily dissolve in ethanol or to a reduction in feces surface area to solvent volume (Wasser et al. 2010). Finally, ethanol extracts should be dried and rehydrated in buffer prior to radioimmunoassay to eliminate ethanol-associated assay interference. Conducting all five of these procedures in unison should eliminate the strong effect of mass on hormone concentration from samples of avian feces ranging in mass from under 0.001 g to over 0.1 g.

If a high precision balance is unavailable, samples of very small mass (e.g. <0.2 g, but depending on scale) should still be eliminated from analysis. Eliminating the smallest samples did effectively eliminate the mass effect in our 2007 and 2008 field samples when other precautions were employed. In contrast, field samples from 2005 and 2006 (as well as samples from the fecal pool processed using traditional methods) exhibited a systemic mass effect that could not be eliminated by culling small samples.

Our work with pooled feces and spotted owl samples collected in the field shows that improved methodology can remove an artificial mass effect that, in some cases, masks significant treatment effects. However, it should be noted that quantity of food intake, rate of defecation and metabolism, and other factors may affect fecal hormone metabolite concentrations. These variables can change with disturbance, temperature, diet, and other environmental factors that should be controlled as much as possible in studies that employ fecal hormone measures (Goymann, 2005). However, some of these variables are also controlled by lyophilizing samples prior to extraction (Wasser et al. 1993).

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