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Further Development of Non-Invasive Stress Measures

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Introduction

Within animal welfare research, interest has turned to non-invasive methods of measuring corticosterone concentrations in the animal. Currently the most recognized method to measure corticosterone is plasma corticosterone concentration which involves blood sampling the animals. Blood sampling in itself is invasive because it involves handling an animal and drawing blood from the animal which can cause a stress response. If a blood sample is not taken within a short period of time, the corticosterone concentration measured within the plasma could be elevated due to a stress response caused by the procedure itself, rather than measuring the animal's general physiological state. For most species glucocorticoids are released in approximately 2 minutes (Broom and Johnson, 1993). If corticosterone can be measured non-invasively, the risk of a confounded result is decreased because handling and blood sampling the animal is not required. In the laying hen it is possible to measure corticosterone non-invasively through the egg or the excreta (Rettenbacher *et al.*, 2005). The egg can also be divided into its two components, the yolk and the albumen, and corticosterone can be measured in both. In excreta, corticosterone is measured as its metabolites. While research has been done on the relationship between plasma corticosterone and each of the sources of a non-invasive sample, however, there have been no experiments that measured all 3 non-invasive samples with a corresponding plasma sample.

Rettenbacher *et al.* (2005) reported that, after administering radio-labeled corticosterone and Adrenocorticotropic Hormone (ACTH) to laying hens, peak levels of radio-labeled corticosterone were measured in the albumen and outer-most layer of the yolk the following day. Downing and Bryden (2008) reported a significant positive correlation between plasma and albumen corticosterone in hens given 0, 5, and 10 mg of corticosterone in peanut oil via subcutaneous injection, eggs were collected on the 2 days following injection. Further, they reported significantly higher albumen corticosterone in hens that were handled, heat-stressed, or for 3 days after being moved to a new cage. Housing system has also been shown to cause differences in egg corticosterone. Sas *et al.* (2006) reported higher egg corticosterone in free-range versus cage housed laying hens. However, Royo *et al.* (2008) reported no differences in albumen, yolk, or whole egg corticosterone between hens housed in single production cages versus hens group-housed in furnished cages. They did not, however, measure plasma corticosterone concentrations and compare these with the albumen, yolk, or whole egg corticosterone concentrations.
Faecal corticosterone concentrations have also been compared with plasma corticosterone concentrations. Dehnhard *et al.* (2003) reported a similar, but less pronounced, pattern of corticosterone metabolite secretion in faeces to plasma corticosterone concentrations after ACTH administration. The response in faeces occurred 2-6 hours after the response noted in plasma.

Fraisse and Cockrem (2006) were unable to measure any difference in faecal corticosterone between strains of white egg layers and brown egg layers, while they reported differences in plasma corticosterone in response to a stressor and behavioural indicators of fear. Likewise, upon collection of faeces from free range and conventional cage farms, Christensen (2009) was unable to measure differences in faecal corticosterone. Due to effects of age, sex, diurnal and seasonal variations in adrenocortical activity, it is important to validate the use of faecal corticosterone metabolites (Touma and Palme, 2005).

The objective of this experiment is to investigate the relationship between plasma and egg corticosterone concentration and faecal corticosterone metabolites. In order to have variation in plasma corticosterone concentrations, hens in two stages of lay will be studied: Davis *et al.* (2000) reported differences in plasma corticosterone in hens in peak versus late lay (significantly higher plasma corticosterone in hens during peak lay (7.27 and 6.30 ng/mL; 26 and 34 weeks of age, respectively) vs. hens later in the laying cycle (4.48 ng/mL; 43 weeks of age). Further, behavioural tests have shown that hens on the outer ends of the rows are less fearful than hens housed in the middle of rows (Engel, 2008). This is expected to be due to increased human activity focused around the ends of the shed. Therefore, hens housed at the ends of the shed are exposed to more activity and are less fearful than those on the inside of the shed. Fear can invoke a stress response and therefore, an increase in basal plasma corticosterone could be present in hens housed in the middle of the shed, rather than the end of the shed. Therefore, within each shed, 5 of the sampled cages will be in the middle of the shed and 5 of the sampled cages will be at the end of the shed. Thus a comparison can

also be made between the middle and end of the shed. This would be the first study to use both faecal and egg (albumen and yolk) corticosterone measurements in comparison with plasma corticosterone.

Objectives

The objective of this experiment was to investigate the relationship between plasma, egg albumen, egg yolk and faecal corticosterone concentrations in peak and late production laying hens.

Methodology

Animals and Housing

Laying hens (n=154) were used of the HyLine Brown variety and housed in cages within two commercial poultry sheds. The ages of the hens were 34 and 47 weeks of age. Within each shed (age group), 10 cages were randomly selected. Two cages from each aisle within the shed were allocated with one being toward the middle (M) of the shed and the other toward the end (E) of the shed. The cage was the experimental unit. Hens were exposed to 14.5 and 16 hours of light for 34 and 47 weeks of age, respectively. They were fed a balanced diet and feed was topped up 4 times daily. Prior to sampling, cages labelled 1 to 10 based on the randomized blood sampling order. Hens were monitored daily for any signs of disease, injury, or mortality.

Sample Collection

Sampling order was randomised for each day. The pre-determined random order for each day was used for egg and faecal collection. Blood sampling occurred on the first day with corresponding 24 hour pooled faecal and egg samples. Sampling then ceased for 1 day to remove any effects of blood sampling. On Day 2 no sampling occurred to allow the animals to recover from blood sampling and to ensure that samples were representing basal corticosterone concentrations. Eggs and Faeces were then collected and pooled for each cage over 24 hours for the following 2 days.

Blood Collection

Cages were blood sampled in a pre-determined random order. All the hens from each cage were blood sampled on the first day of sampling. Samples were collected by two trained staff. Two handlers each removed a hen from the cage and held it for the sample to be taken. Care was taken to get all samples within 2 minutes of each hen being removed from her cage (maximum 8 minutes sampling time for the whole cage) to prevent increases in plasma corticosterone due to physical handling. Approximately 1.5 mL of blood was taken via the wing vein using a 3 mL heparinized syringe with a 23 gauge needle. Slight pressure was then placed on the puncture sight to prevent any bleeding. The hen was then placed in a crate for holding and monitoring until the entire cage had been sampled to prevent any hen being sampled twice. Hens were then checked and placed back in their home cage. Whole blood samples were transported on ice back to the lab where they were centrifuged and the plasma poured into 1.5 mL tubes. The samples were stored at -20ºC until analysed for corticosterone concentration.

Faecal Collection

Faecal samples were collected and pooled for each cage over 3 – 24 hour periods. A piece of cardboard coated in aluminium foil and matching the dimensions of the cage was placed under each cage and removed 24 hours later. The faeces were placed in aluminium pans and weighed before being placed in a drying oven at 60ºC for 48 hours. Once dried, the samples were weighed, ground and stored in a freezer bag at -20ºC until they were analysed for corticosterone concentration.

Egg Collection

Eggs were collected to correspond with the faecal samples. The eggs laid the same morning the 24 hour pooled faecal sample was collected were also collected as they would have been forming over the same period of time the sample of faeces was being produced. Eggs were numbered via their corresponding cage, placed in trays and transported back to the lab where whole egg, albumen, and yolk weights were measured. The albumen and the yolk for each egg were individually stored in 40 and 10 mL conical tubes, respectively, and placed in a -20ºC freezer for storage until analysed for corticosterone concentration.

Sample Analysis

All samples were analysed for corticosterone using a Corticosterone HS Enzyme Immunoassay (EIA) (IDS Ltd., Boldon, UK). Egg albumen samples and egg yolk samples were pooled for each cage on each day. Plasma samples were pooled for each cage.

Sample Preparation

No sample preparation was necessary for the plasma.

Faecal Samples

0.1 g of sample was extracted with 1 mL of 80% methanol. The samples were then vortexed for 30 minutes and centrifuged. The supernatant was taken and dried down. The samples were then resuspended in Phosphate Buffered Saline (PBS) and run through the EIA.

Egg Yolk Samples

0.5 g of sample was taken and 1 mL distilled water was added and vortexed until mixed. The mixture was extracted with 3 mL hexane:diether (30:70 ratio) and vortexed and left to settle before snap freezing with an ethanol/dry ice bath. The supernatant was collected and dried down. 1 mL of ethanol was added to the samples which were then were frozen at -20ºC overnight. The samples were centrifuged the next day, and the supernatant taken and dried down once more. The samples were then resuspended in PBS and run through the EIA.

Egg Albumen Samples

5 g of sample were taken and 5 mL of distilled water was added. These were mixed, and 0.5 g of the mixture was taken for extraction with 4 mL of diether, shaken for 10 mins then frozen at -80ºC, after which the supernatant was collected and dried down. The samples were resuspended in PBS and run through the EIA. Corticosterone recovery was run for egg albumen and applied to the results.

Results

Statistical Analysis

Statistical analysis was carried out using SPSS 18 (SPSS Inc., Chicago, IL, USA). Egg and faecal weights and all corticosterone results were analysed first using an ANOVA to examine effects of shed, location within the shed, and day of sampling. Corticosterone was also statistically analysed as total egg corticosterone (albumen + yolk). Albumen, faecal and total egg corticosterone concentrations were log transformed for normality prior to analyses. Significant effects were then compared using least square differences. Associations between measures of corticosterone were examined using correlation analyses and any of the non-invasive measures that were correlated with plasma corticosterone (at $P < 0.1$) were included in linear regression analysis to examine their relationship with plasma corticosterone.

Egg and Faecal Weights

Egg weights and faecal weights are reported in Appendix I.

Corticosterone (CORT) Concentrations

In the examination of shed effects are also age effects as the hens in Shed 13 were 47 weeks of age and the hens in Shed 15 were 34 weeks of age.

Shed

There was a significant effect of shed on yolk CORT with mean concentrations of 4.12 ± 0.14 ng/g and 3.61 ± 0.11 ng/g for Sheds 13 and 15, respectively ($P < 0.01$). There was also a trend for plasma CORT concentration to be higher in Shed 13 (47 weeks of age) than Shed 15 (34 weeks of age) with mean concentrations of 1.38 ± 0.14 ng/mL and 1.03 ± 0.14 ng/mL, respectively ($P < 0.10$).

Day of Sampling

Day of sampling had a significant effect on egg yolk and faecal CORT concentration (Table 1).

Table 1. Effects of Day of Sampling on Non-Invasive CORT concentrations (ng/g).

	Day of Sampling					
	1		3		4	
	Mean	SEM	Mean	SEM	Mean	SEM
Albumen	19.65	0.94	18.23	1.31	20.38	0.91
Yolk	4.20[a]	0.17	3.69[b]	0.15	3.70[b]	0.16
Faecal	28.15[b]	0.67	35.33[a]	1.39	29.37[b]	0.99
Total Egg (Albumen+Yolk)	32.35[b]	0.71	39.02[a]	1.46	33.06[b]	1.00

Note: Means within a row with different superscripts are significantly different ($P < 0.05$)

Location within the Shed

There was a trend for hens in the middle of the shed to have higher egg yolk CORT than hens on the end of the shed ($P < 0.10$). There were no other effects of location within the shed on CORT concentrations.

Shed by Day of Sampling Interaction

There was a significant Shed by Day interaction for faecal CORT concentration and a trend for the interaction in egg yolk CORT concentration ($P < 0.10$) (Table 2).

Shed by Location

There was a trend ($P < 0.10$) for faecal CORT concentration to be higher in the middle of Shed 13 than the end of Shed 13 and the middle and end of Shed 15 (Table 3).

Correlations and Regression

Few significant correlations were observed between plasma CORT and the non-invasive measures of CORT. There was a significant correlation between plasma CORT and mean yolk CORT sampled on days 1 and 3 ($r = 0.48$; $p = 0.03$). Regression analysis including plasma CORT, as well as TotalEgg_4 and Faecal_4 (both

correlated with plasma CORT at P<0.1), indicated that mean yolk CORT 1,3 accounted for 20% of the variation in plasma CORT (adjusted R^2 = 0.21, $F_{1, 19}$ = 6.01, p = 0.02 using the backward method). In general, the correlations between plasma CORT and other non-invasive measures of CORT were variable: in addition to being generally low, they varied in the direction of the relationship. Correlations between plasma CORT concentrations and non-invasive measures of CORT are shown in Appendix II.

Strong, significant positive correlations were observed between total egg (albumen + yolk) CORT concentration and faecal CORT concentration on each of the three sampling days (Table 4).

Table 2. The Effect of Shed*Day of Sampling on Non-Invasive Measures of CORT (ng/g)

				Albumen		Yolk		Faecal		Total Egg	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Shed	13	Day	1	20.27	1.51	4.69	0.20	29.41^{bc}	0.51	34.10	0.43
			3	19.05	2.39	3.97	0.21	39.15^{a}	1.50	43.13	1.56
			4	20.14	1.47	3.69	0.24	26.86^{c}	1.39	30.55	1.36
	15	Day	1	19.02	1.16	3.70	0.18	26.90^{c}	1.13	30.60	1.12
			3	17.42	1.16	3.42	0.19	31.50^{bc}	1.63	34.92	1.67
			4	20.63	1.16	3.70	0.22	31.88^{b}	0.89	35.58	0.97

Note: Means within a column with different superscripts are significant (P < 0.01)

Table 3. Effect of Shed*Location on Faecal Corticosterone CORT

				Faecal CORT (ng/g)	
				Mean	SEM
Shed	13	Location	E	30.22	1.47
			M	33.40	1.84
	15	Location	E	30.35	1.32
			M	29.84	1.00

Table 4. Correlations between Faecal and Total Egg CORT

			TotalEgg_1	TotalEgg_3	TotalEgg_4
Spearman's rho	Faecal_1	Correlation Coefficient	.947**	.668**	-.276
		Sig. (2-tailed)	.000	.001	.239
	Faecal_3	Correlation Coefficient	.782**	.997**	-.192
		Sig. (2-tailed)	.000	.000	.416
	Faecal_4	Correlation Coefficient	-.191	-.162	.979**
		Sig. (2-tailed)	.420	.494	.000

**. Correlation is significant at the 0.01 level (2-tailed).

Discussion

Plasma corticosterone (CORT) concentrations in the present study were similar to those reported by Mench *et al.* (1986), Lagadic *et al.* (1990), and Dehnhard *et al.* (2000) but were lower than those reported by Davis *et al.* (2000), El-Lethey *et al.* (2003), Dennis *et al.* (2008), and Downing and Bryden (2008). Many of the studies reporting higher concentrations of plasma CORT utilised white strains of laying hens rather than the brown strains as used in the present study (Davis *et al.*, 2000; El-Lethey *et al.*, 2003; Dennis *et al.*, 2008). Egg albumen CORT concentrations in the present study were higher than those reported by Downing and Bryden (2008), but similar to those reported by Singh *et al.* (2009). Egg yolk CORT concentrations in the present study were lower than those reported by Royo *et al.* (2009) and Singh *et al.* (2009). Faecal CORT concentrations were in the same range as those reported in laying hens by Dehnhard *et al.* (2000) and Dawkins *et al.* (2004).

The values of plasma corticosterone in the present study were much lower than and did not exhibit the peak and late production effects reported by Davis *et al.* (2000). A trend in the opposite direction was observed in the present study with the hens in late production (Shed 13) exhibiting slightly higher plasma CORT than the hens in peak production (Shed 15). However, Davis *et al.* (2000) studied hens of a white laying strain.

There was a trend for birds in the middle of the shed to have higher egg yolk CORT in the present study. There was also an interaction between shed and location with birds in the middle of the shed 13 (the hens in late production only) to have higher Faecal CORT and Engel (2008) found that birds of the same strain showed higher fear responses in similar locations to the present study.

There were few significant correlations within this study between plasma CORT concentrations and non-invasive measures of CORT. Mean yolk CORT sampled on days 1 and 3 was the best predictor of plasma CORT concentration. Interestingly, Singh *et al.* (2009) reported a positive relationship between egg yolk corticosterone concentration and heterophil-to-lymphocyte ratio.

In terms of correlations between the non-invasive measures, Total egg (albumen + yolk) and faecal CORT concentrations showed a significant positive correlation for each of the three sampling days, but these were not correlated with plasma CORT concentration.

It needs to be recognised that because of the small scale of this study there were a number of factors in this study limiting its ability to detect real relationships. It is therefore recommended that more extensive non-invasive sampling and more birds are required to comprehensibly study the relationships between plasma and non-invasive measures of CORT. Another limitation was that plasma was taken as a single sample while egg and faecal CORT concentrations are likely to reflect plasma CORT concentrations over a period of time. Because of the pulsatile secretion of plasma corticosterone, multiple plasma samples throughout a day are required to estimate basal plasma corticosterone concentrations.

Furthermore, investigators that have reported significant relationships studied more substantial variations in plasma corticosterone concentrations induced by imposing an acute stressor or an ACTH challenge (Dehnhard *et al.*, 2003; Rettenbacher *et al.*, 2005). It is possible non-invasive measures may be less predictive in measuring small differences between birds in basal CORT concentrations. For example, Nicol *et al.* (2006) reported no differences in faecal corticosterone concentration between treatments manipulating stocking density and flock size. Similarly, Christensen (2009) was unable to measure differences in faecal corticosterone between free-range and conventional cage farms. Those that have investigated the relationship between plasma and egg CORT have also had difficulty measuring any significant relationships. Royo *et al.* (2009) reported no significant difference between two housing systems. While a significant increase in egg albumen corticosterone concentration has been observed in response to a sharp drop in temperature in free-ranged laying hens, Sas *et al.* (2006) found that on all other days of sampling, there was no difference in egg albumen between hens housed in free-range versus cage systems.

Implications

It is clear that the relationship between plasma corticosterone concentrations and non-invasive measures of corticosterone requires further examination under a range of situations, both when birds are at 'rest' and under stress.

Recommendations

Until we better understand the relationships between plasma corticosterone concentrations and non-invasive measures of corticosterone, studies utilising non-invasive measures of corticosterone need to be interpreted cautiously. Similarly, scientists using data on plasma corticosterone need to interpret the data cautiously particularly when based on single or infrequent samples, because of the pulsatile nature of corticosterone secretion.

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Appendix I - Egg and Faecal Sample Weights

There was a significant effect of age on whole egg, egg yolk, and faecal wet weights.

The effects of age on egg and faecal weights (g)

	Age (weeks)		SEM
	34	47	
Whole Egg	60.88[b]	63.50[a]	0.31
Albumen	38.01	38.16	0.25
Yolk	14.74[b]	16.39[a]	0.09
Faecal Wet	643.93[a]	572.02[b]	13.47
Faecal Dry	218.22	209.35	4.70

Rows with different superscripts are significantly different ($P < 0.01$)

There was also a significant effect of location within the shed on faecal weights.

The effect of location within the shed on faecal weights (g)

Variable	End (E)	Middle (M)	SEM
Faecal Wet	655.64[a]	560.31[b]	13.47
Faecal Dry	221.40[a]	206.17[b]	4.70

Rows with different superscripts are significantly different ($P < 0.05$)

Appendix II – Correlations of Plasma Corticosterone Concentration with Non-Invasive Corticosterone Concentrations

Correlations of Plasma Corticosterone with Non-Invasive Measures of Corticosterone

		Correlational Coefficient	Sig. (2-tailed)
Spearman's rho	Shed	-.416	.068
	Cage	-.226	.337
	Location	-.052	.828
	Plasma Cort	1.000	.
	Albumen_1	-.074	.758
	Albumen_3	.072	.762
	Albumen_4	-.284	.225
	Yolk_1	.388	.091
	Yolk_3	.238	.313
	Yolk_4	-.221	.349
	Faecal_1	-.017	.945
	Faecal_3	.170	.474
	Faecal_4	-.418	.067
	TotalEgg_1	.057	.811
	TotalEgg_3	.177	.454
	TotalEgg_4	-.403	.078
	TotalAlbumen	-.202	.394
	TotalYolk	.316	.175
	TotalFaecal	-.057	.811
	Yolk(1,3)	.481*	.032

*. Correlation is significant at the 0.05 level (2-tailed).

Plain English Compendium Summary

Project Title:	Further Development of Non-Invasive Stress Measures
Project No.:	09-30
Researcher:	Joanna Engel
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Objectives	
Background	Within animal welfare research, interest has turned to non-invasive methods of measuring corticosterone concentrations in the animal. Currently the most effective way to measure corticosterone in an animal is plasma corticosterone concentration, which involves blood sampling the animals. Blood sampling in itself is invasive because it involves handling an animal and drawing blood from the animal which can cause a stress response. If a blood sample is not taken within a short period of time, the corticosterone concentration measured within the plasma could be elevated due to a stress response caused by the procedure itself, rather than measuring the animal's general physiological state. If corticosterone can be measured non-invasively, the risk of a confounded result is decreased because handling and blood sampling the animal is not required. In the laying hen it is possible to measure corticosterone non-invasively through the egg or the excreta. We are unaware of any literature on the relationship between plasma, egg, and faecal corticosterone concentration in laying hens.
Research	Blood samples were taken from laying hens within 10 cages in 2 separate sheds on a commercial farm. Over the same 24 hours during which blood was taken, faeces were collected on a piece of aluminium foil-coated cardboard and the following morning, faeces and eggs were collected. After one recovery day, the collected of faeces and eggs was repeated over 2 more 24-hour periods. All samples were pooled for each cage and analysed for corticosterone concentration.
Outcomes	Plasma corticosterone was very poorly correlated with non-invasive measures of corticosterone and correlations were inconsistent within each of the non-invasive measures of corticosterone (albumen, yolk, and faeces). Yolk corticosterone concentration combined for the first and third sampling day provided the best prediction for plasma corticosterone concentration. Faecal and total egg (albumen + yolk) had a significant positive relationship on each of the three sampling days.
Implications	It is clear that the relationship between plasma corticosterone concentrations and non-invasive measures of corticosterone requires further examination under a range of situations, both when birds are at 'rest' and under stress. Until we better understand the relationships between plasma corticosterone concentrations and non-invasive measures of corticosterone, studies utilising non-invasive measures of corticosterone need to be interpreted cautiously. Similarly, scientists using data on plasma corticosterone need to interpret the data cautiously particularly when based on single or infrequent samples, because of the pulsatile nature of corticosterone secretion.
Publications	None