Effect of transportation and shackling on plasma concentrations of corticosterone and heterophil to lymphocyte ratios in market weight male turkeys in a commercial operation

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ABSTRACT There is limited information on the effects of stress and/or physiological manipulation on either plasma concentrations of corticosterone (**CORT**) and/or heterophil: lymphocyte (**H : L**) ratios in turkeys. The present studies examine the effects of catching/transportation/lairage in a holding shed and shackling on plasma concentrations of CORT and H : L ratios in male market weight turkeys. Plasma concentrations of CORT were increased after transportation and lairage but not further elevated by shackling, irrespective of its duration up to 240 s. In one study, there were increased H : L ratios following catching/placing birds into transportation cages/transportation/lairage. In one study, H : L ratios declined following shackling. It is concluded that while moving turkeys from the farm to immediately before the shackling line is stressful, shackling for up to 4 min was not perceived as more stressful in turkeys. There were also differences between farms/houses for both plasma concentrations of CORT and H : L ratios.

Key words: shackling, corticosterone, transportation, stress, turkeys

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INTRODUCTION

The welfare of poultry raised under commercial conditions continues to be important to producers, consumers, regulators, activists, and others with poultry welfare research reviewed (e.g., Mench, 1998; Mench and Duncan, 1998; Appleby et al., 2004; Bessei, 2006; Berg and Raj, 2015; Thaxton et al., 2016). The "*Five Freedoms*" are built on the concepts developed in the Brambell (1965) Report and are still viewed as the "*gold standard*" for animal welfare (American Humane, 2016). One of the 5 freedoms for animal welfare is "*freedom from fear and distress by ensuring conditions and treatment which avoid mental suffering*" (Farm Animal Welfare Council, 1979). Moreover, the World Organisation for Animal Health adopted a series of 10 recommendations or principles for the welfare of livestock and poultry (OIE, 2012; Fraser et al., 2013). Among these is the following: "*the handling of animals ….. should not cause injury, panic, lasting fear or avoidable stress*" (OIE, 2012; Fraser et al., 2013). There is a particular concern for the welfare of livestock and poultry at the time of killing (Farm Animal Welfare Committee, 2019). The present studies evaluate 2 indices of stress, namely plasma concentrations of corticosterone (CORT) and the heterophil: lymphocyte (**H : L**) ratio in turkeys between prior to transportation from the grower house to the processing plant to immediately before shackling and during shackling. This is consistent with the need for "*evidence-based decision and policy-making*" (Farm Animal Welfare Committee, 2018). Both plasma concentrations of CORT and the H : L ratio have been widely employed in poultry as indices of stress (reviewed e.g., Scanes, 2016) and together are at present considered the best available methods to assess stress in poultry. This is the first paper to report the effects of either shackling or transportation on plasma concentrations of CORT and H : L ratios in turkeys. There were only 2 papers published with covering both CORT and H : L ratios in turkeys based on a systematic data base search using the Web of Science covering all published studies up to 9 March 2012 (Goessling et al., 2015).

What is not known is whether the practices employed in transferring turkeys from farm to processing and during shackling are stressful. Experimental transportation of turkeys has been reported to be followed by increased expression of putative indicators of stress, namely, pro-inflammatory genes: lysozyme, interleukin-(**IL-**) 1β and IL-6, together with the cellular stress marker gene, heat-shock protein 70 (**HSP-70**), in peripheral blood cell turkeys (Wein et al., 2017).

There is evidence that stressors increase plasma concentrations of CORT in poultry. In broiler chickens, plasma concentrations of CORT are elevated

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when birds experience prolonged acute stresses such as shackling (Kannan and Mench, 1997; Kannan et al., 1997) or manual restraint (placing adult laying hens on their side for 4, 6, and 8 min) in (Korte et al., 1997). There is evidence that the duration of the stressor can influence the physiological response. Plasma concentrations of CORT progressively increase with duration of manual restraint in adult laying hens (Korte et al., 1997). Similarly, there were progressively greater increases in plasma concentrations of CORT when broiler chickens were placed in mock shackles in an inverted position (Bedanova et al., 2007). In turkeys, plasma concentrations of CORT are elevated following adrenocorticotropic hormone (**ACTH**) administration (Davis and Siopes, 1987; C.G. Scanes and colleagues unpublished observations) and stressors such as low environmental temperatures (El Halawani et al., 1973), withholding feed and water for 12 h (Bartz et al., 2018), fasting for 18 h combined with either cold stress or heat stress and crowding (Bartz et al., 2018), protein deficient diets (Carsia and McIlroy, 1998), and herding (C.G. Scanes and colleagues, unpublished observations).

In chickens, H : L ratios are increased by stresses including killed *Escherichia coli* (Gross and Siegel, 1983), elevated environmental temperatures (30°C) (Shumorucha et al., 2010), fasting (Gross and Siegel, 1983; Gross, 1990), feed restriction (Zulkifli, 2000; Azis, 2012), feed contaminated with the mycotoxin, deoxynivalenol (Ghareeb et al., 2011; 2014), manual restraining (Wein et al., 2017), social disruption (mixing 2 groups of birds) (Gross, 1990), social stress (Gross and Siegel, 1983), and shackling (Bedanova et al., 2007). In contrast, H : L ratios are decreased after the administration of malathion (Gross and Siegel, 1983). The increases in H : L ratios appear to be a *sequela* of activation of the HPA axis. Evidence for this includes H : L ratios being increased after CORT administration (Gross and Siegel, 1983; Gross, 1990; Mehaisen et al., 2017; Weimer et al., 2018) and the reduced H : L ratios after administration of adrenal cortical blockers (1,1dichloro-2,2-bis/p-chlorophenyl ethane or metyrapone) (Gross, 1990). There is evidence, albeit limited, that stressors also increase H : L ratios in turkeys. Activation of the turkey HPA axis by either administration of ACTH or fasting 48 h increases the H : L ratio (Gross, 1990). *E. coli* respiratory challenge increased H : L ratios (Huff et al., 2004). Exposure to elevated environmental temperatures (35°C) for 8 h resulted in a marked increase in the H : L ratio in female turkeys in transportation crates (Vermette et al., 2017). In contrast, exposure to cold did not influence H : L ratios in male turkeys in transportation crates (Henrikson et al., 2018). It is presumed that the HPA axis will be similar in turkeys to that of chickens. However, that is not necessarily the case. The present studies examine the effects of production related stresses on plasma concentrations of CORT and H : L ratios in male turkeys.

MATERIALS AND METHODS

Animals

Male turkeys were raised under commercial conditions in open sided houses in 4 farms in South West Missouri (Lockwood township, Dade County; Carthage township, Jasper County; Neosho township, Newton County and Miller township, Lawrence County) and 1 farm in South East Kansas (Columbus township, Cherokee County) until ∼20 kg. They were transported to a commercial processing plant (Carthage, MO) and processed in a conventional manner.

Blood Sampling

Blood samples were taken by board certified veterinarians by venipuncture from the jugular vein into heparinized syringes within 5 to 10 s of first handling the bird; this being argued to be insufficient time to activate the hypothalamo-pituitary- adrenocortical axis. Blood samples were obtained at the farm on the day of transportation (with morning and evening samples), in the transportation crates immediately prior to shackling and at after shackling on the processing plant shackle line. After centrifugation and separation, plasma samples were frozen on dry ice from transportation and stored at −20°C until assay. Blood were smeared on to histological slides and air dried. They were then transported for determination of H: L ratios.

Effect of Transportation and Shackling

Studies were conducted to examine effects of the putative stressors of transportation and shackling. Samples were obtained at farms prior to transportation with evening samples in study 1 and both morning and evening samples in study 2, in the transportation crates immediately prior to shackling (within 15 s of placing in shackles) and after 30, 90, 180, and 240 s of shackling on the processing plant shackle line.

Physiological Indices of Stress

Plasma concentrations of CORT were determined using an ELISA kit (Enzo Life Sciences, ADI-901-097, Farmingdale, NY); the assay being previously employed for poultry species (Huth and Archer, 2015; Archer, 2018). The inter-assay and intra-assay coefficient of variance were less than 5%.

The H : L ratio was determined on dried blood smears by cell counting (Huth and Archer, 2015; Archer, 2018). For each bird, a single slide was employed. The microscope field is set slightly in from the feathered end of the smear. The number of heterophil and lymphocytes is counted in the field and this is repeated as the field is moved down then over then up and so on. This is done

Table 1. Effects of processing procedures including transportation/lairage, shackling and time of shackling on plasma concentrations of corticosterone.

Time of sampling	Plasma corticosterone (mean in ng/mL ± (n =) SEM)		
	Study 1[a]	Study 2[b]	Overall (combining study 1 and 2)
On Farm—morning		6.4 ± (40) 0.86[a]	6.4 ± (40) 0.96[a]
On Farm—evening (fasted△)	10.1 ± (30) 1.34[a]	9.8 ± (40) 1.36[b]	9.9 ± (70) 0.97[b]
Immediately prior to shackling# (time 0)	14.8 ± (20) 1.03[b,c]	14.4 ± (40) 1.36[d]	14.5 ± (60) 1.54[c,d]
Shackled for 30 s	13.2 ± (28) 1.40[a,b]	12.8 ± (40) 1.26[c]	13.0 ± (68) 1.01[c]
Shackled for 90 s	16.5 ± (30) 1.39[b,c]	12.1 ± (40) 1.06[c]	14.0 ± (70) 1.84[c,d]
Shackled for 180 s	15.3 ± (30) 1.17[b,c]	12.6 ± (40) 1.18[c]	13.8 ± (70) 1.27[c,d]
Shackled for 240 s	17.9 ± (30) 1.48[c]	15.0 ± (40) 1.12[d]	16.2 ± (70) 1.14[d]

△ Fasted for 8 to 12 h
After catching/placing in transportation crates/transportation/lairage with birds still in transport cages
a, b, c, d Different superscript letters indicate difference (P < 0.05)

until the total number of cells counted (heterophils + lymphocytes) equals 100 cells. The ratio is then calculated. The incidence of wing flapping was measured for shackled birds by simple observation.

Statistics

One-way ANOVA and Tukey's range were employed to analyze the data. Data, per se and following log transformation, were analyzed with $P < 0.05$ in both analyses considered significant. Data in frequency distributions were analyzed by chi squared.

RESULTS

Effect of Catching/Transportation/Lairage and Shackling on Plasma Concentrations of CORT

Table 1 summarizes plasma concentrations of CORT from 2 studies of turkeys on farms (in their rearing houses), following catching, transportation, and lairage and after shackling at the processing plant. There was no difference between on farm evening sampling data for the 2 studies and data were also analyzed combining the 2 studies (Table 1, Figure 1). In study 2, plasma concentrations of CORT were low in the morning in turkeys at the grower facility but were increased ($P < 0.05$) in the evening (Table 1); feed having been withdrawn 8 to 10 h prior to the evening sampling. Plasma concentrations of CORT were increased ($P < 0.05$) after catching/placing in transportation cages/transportation/lairage in both study 1 (by 46.5%) and 2 (by 46.9%) together with the combination of study 1 and 2 (Table 1, Figure 1). Plasma concentrations of CORT did not differ ($P > 0.05$) between immediately prior to shackling and with shackling for 30, 90, 180, or 240 s. There was a difference ($P < 0.05$) in plasma concentrations of CORT between turkeys that had been shackled for 30 and 240 s (Table 1, Figure 1).

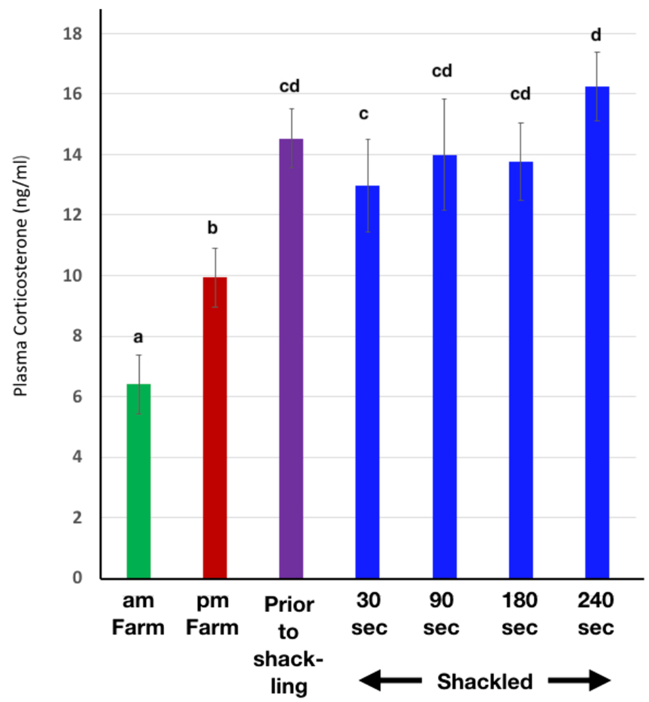

Figure 1. Effects of processing procedures (including shackling and time of shackling) on plasma concentrations of corticosterone (studies 1 and 2 combined) [vertical lines indicate SEM (standard error of the mean); a, b, c, d Different superscript letter indicates difference $P < 0.05$]

Effect of Catching/transportation/lairage and Shackling on H : L Ratios

Figure 2A shows a frequency distribution for H : L ratios for blood samples taken at farms from studies 1 and 2 (Figure 2A). Medial H : L ratios were between 0.75 and 1.00. Data from studies 1 and 2 were not combined as there were differences in the H : L at several times points. For instance, there were differences in the H : L ratio both from the evening on farm time point ($P = 0.0027$) and immediately prior to shackling ($P < 0.0001$). In study 1, there were no differences ($P > 0.05$) in H : L ratios between on farm, following transportation and lairage and with shackling, irrespective of its duration (Table 2). In study 1, the H : L

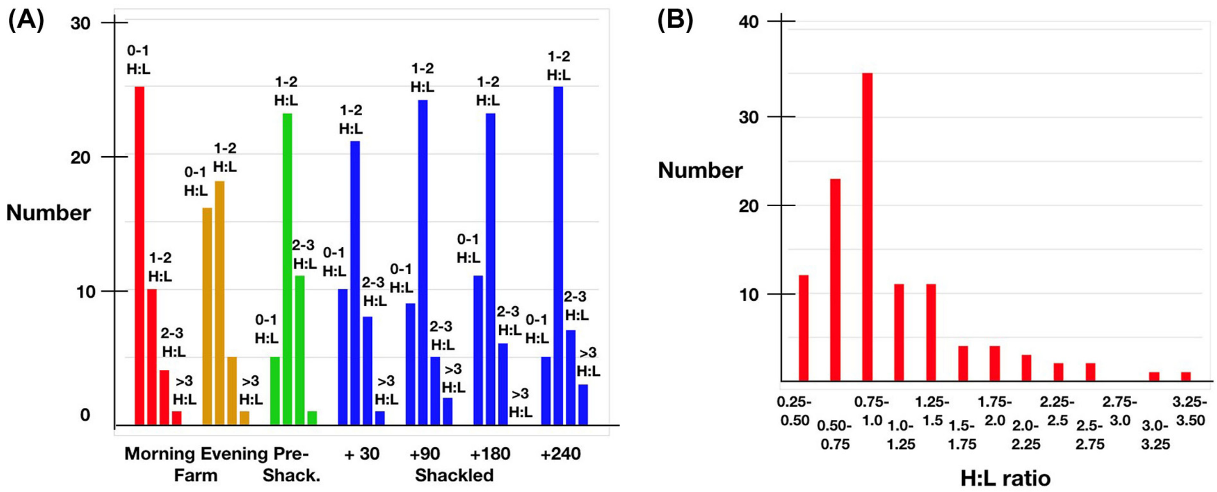

Figure 2. Frequency distribution of H : L ratios in turkeys A. Frequency distribution of H : L ratios in birds on farms in studies 1 and 2 B. Frequency distribution of H : L ratios on farms, following transportation/lairage and shackling in study 2

Table 2. Effects of processing procedures including transportation/lairage, shackling and time of shackling on blood H : L ratios.

	H : L ratio (mean ± (n =) SEM)	
Time of sampling	Study 1	Study 2
On Farm—morning	-	1.094 ± (40) 0.101[a]
On Farm—evening	0.916 ± (30) 0.0585	1.342 ± (40) 0.110[a,b]
Immediately prior to shackling[#]—time 0	1.040 ± (30) 0.0764	1.862 ± (40) 0.149[d]
Shackled for 30 s	1.080 ± (20) 0.0993	1.505 ± (40) 0.142[b-d]
Shackled for 90 s	0.999 ± (30) 0.0962	1.573 ± (40) 0.151[b-d]
Shackled for 180 s	0.864 ± (29) 0.0498	1.437 ± (40) 0.091[b,c]
Shackled for 240 s	0.886 ± (29) 0.0680	1.772 ± (40) 0.147[c,d]

[#]After catching/placing in transportation crates/transportation/lairage with birds still in transport cages

[a–c]Different superscript letter in a column indicates difference, $P < 0.05$

ratio declined with duration of shackling (adjusted R^2 0.0267; $P = 0.0318$) as did the $\log_{10}$ H : L ratio (adjusted R^2 0.0212; $P = 0.0498$). In study 2, the H : L ratio was increased ($P < 0.05$) by 38.7% from that observed on the farm to that immediately prior to shackling, i.e., after catching, transportation, and lairage (Table 2). The H : L ratio declined 22.8% ($P < 0.05$) from prior to shackling to 180 s of shackling (Table 2). In study 2, there was no relationship between H : L and time of shackling (adjusted $R^2 = -0.0045$, $P = 0.740$). No wing flapping was observed in shackled (∼200) turkeys.

Figure 2B shows the frequency distribution of H : L ratios in male turkeys on farms, following catching/placing in transportation cages/transportation/lairage and during shackling in study 2. There were more ($P < 0.05$) H : L ratios between 0 and 1 in the morning on farm samples compared to either following transportation/lairage or after shackling. In addition, there tended to be more H : L ratios between 0 and 1 in the evening on farm samples than either following transportation/lairage or after shackling.

Relationship Between Plasma Concentrations of CORT and H : L Ratios

In study 1, there was no relationship between plasma concentrations of CORT and H : L ratios (adjusted R^2 -0.00421; $P = 0.580$). In study 2, there was a small positive relationship between plasma concentrations of CORT vs. H : L (adjusted $R^2 = 0.0266$, $P = 0.0036$).

Differences Between Houses and Farms

The plasma concentrations of CORT across farms/houses were 5.60 ± (4) 1.54 for the morning sampling and increased ($P < 0.05$) to 9.89 ± (7) 1.22 ng mL^{-1} for the evening sampling. There were some differences in the plasma concentrations of CORT and H : L ratios between farm/houses (Table 3). In study 1, there were higher ($P < 0.05$) plasma concentrations of CORT in 1 house than the others but no differences in H : L ratios. In study 2, plasma concentrations of CORT were greater ($P < 0.05$) on 1 farm than the others in the evening sampling. The turkeys in

Table 3. Effects of farm/house on plasma concentrations of CORT and H : L ratios [mean ± (n = 10) SEM].

	Plasma corticosterone (ng/mL)	H : L ratio
Morning sampling		
Study 2		
Farm 1 house 1	9.54 ± 1.99	0.846 ± 0.079[a]
Farm 1 house 2	5.60 ± 1.54	0.811 ± 0.074[a]
Farm 2 house 1	5.51 ± 1.79	0.782 ± 0.100[a]
Farm 3 house 1	5.07 ± 1.38	1.786 ± 0.255[b]
Evening sampling		
Study 1		
Farm 4 house 1	14.3 ± 2.60[b]	0.902 ± 0.042
Farm 4 house 2	10.7 ± 2.15[b]	0.777 ± 0.123
Farm 5 house 1	4.45 ± 0.49[a]	1.069 ± 0.107
Study 2		
Farm 1 house 1	7.59 ± 2.08[a,b]	1.247 ± 0.211[a,b]
Farm 1 house 2	15.3 ± 3.07[b]	0.961 ± 0.098[a]
Farm 2 house 1	7.59 ± 2.19[a,b]	1.369 ± 0.241[a,b]
Farm 3 house 1	5.07 ± 1.38[a]	1.726 ± 0.247[b]

[a–c]Different superscript letter indicates difference.

1 farm/house had the highest H : L ratios consistently in the morning and evening sampling periods.

DISCUSSION

There is limited information available on the effects of production and other stressors in turkeys (Erasmus, 2017). The present studies examined the effects of shackling, duration of shackling (in a commercial processing facility), and the combined effects of catching, loading in crates, transportation, and lairage (in a holding pen) on plasma concentrations of CORT and H : L ratios in turkeys.

Plasma concentrations of CORT were elevated in the evening compared to morning (Table 1, Figure 1). This increase may reflect either feed withdrawal or diurnal changes. In growing poultry, there is evidence for diurnal changes in plasma concentrations of CORT with plasma concentrations of CORT reported to increase at the beginning or during the period of night (chickens: Skwarło-Sońta et al., 1983; Wilson et al., 1984; turkeys: Martin et al., 1982). In contrast in other studies of chickens, plasma concentrations of CORT were higher during the day than at night (Majsa et al., 1976; Ozkan et al., 2012).

Plasma concentrations of CORT were increased between levels in the production facilities and immediately prior to shackling (i.e., after catching/placing in transportation cages, transportation and lairage) (Table 1, Figure 1). This is similar to reports on broiler chickens. For instance, plasma concentrations of CORT were elevated after catching broiler chickens and placing them in containers for transportation (Nijdam et al., 2005; Bedanova et al., 2007). Similarly, plasma concentrations of CORT were elevated in broiler chickens by rough handling and duration of handling (Chloupek et al., 2011). Moreover, there was a progressive large response of increased plasma concentrations of CORT with longer times in the crates (Bedanova et al., 2007; Chloupek et al., 2008). Plasma concentrations of CORT in broiler chickens rise progressively reaching a maximum after 7 min after being placed in crates (Voslarova et al., 2011). Subsequently, plasma concentrations of CORT decline to a new nadir after 45 or 60 min within the crates but remain higher than before crating (broiler chickens: Voslarova et al., 2011; Bedanova et al., 2014). In addition, large increases in plasma concentrations of CORT have been reported between catching and after transportation, shackling, and stunning (broiler chickens: Nijdam et al., 2005). Plasma concentrations of CORT were reported to be elevated in transported broiler chickens in one study (Cheng and Jefferson, 2008) but not another (Yue et al., 2010). Moreover, plasma concentrations of CORT were markedly greater in broiler chickens after catching, crating, and loading than after the birds were transported for 10, 70, or 130 km (Vosmerova et al., 2010). Similarly, in livestock, the effects of transportation on plasma concentrations of the endogenous glucocorticoid, cortisol, vary being decreased in cattle (Mitchell et al., 1988) but increased in sheep (Leme et al., 2012). There are reports of transportation influencing novel indices of stress in turkeys. There is increased hepatic expression of the acute phase proteins, namely α_1-acid glycoprotein and C-reactive protein (Marques et al., 2016). Plasma concentrations of 3 microRNA (**miRNA**), namely miRNA, miR-22, miR-155, and miR-365 were elevated after road transportation in turkeys (Lecchi et al., 2016). In another studies, peripheral blood cell expression of lysozyme, IL-1β, IL-6, and HSP-70 were elevated after transportation on turkeys (Wein et al., 2017). These are potentially exciting developments but require further validation. Unfortunately, neither plasma concentrations of CORT nor H : L ratio were reported in these studies. This would have allowed comparison with plasma concentrations of CORT and H : L ratio; together these being the best available methods to assess stress in poultry at this time.

The increases in both plasma concentrations of CORT (studies 1, 2, and combined) and H : L ratios (in study 2) after catching/placing in transportation cages/transportation/lairage is consistent with an acute stress response. In turkeys, H : L ratios have been reported to be markedly increased after a stress, i.e., following transportation and *E. coli* challenge or dexamethasone treatment together with *E. coli* challenge (Huff et al., 2005) or cold stress (Huff et al., 2007). Similarly, plasma concentrations of CORT are increased following exposure of turkeys to various stressors (for details see introduction). There is other evidence that turkeys respond to catching, crating, and loading as a stress. There is an acute increase in heart rate in turkeys after loading (Prescott et al., 2000); the increase in heart rate presumably reflecting release of epinephrine/norepinephrine.

There were no effects of either shacking or duration of shackling up to 4 min on plasma concentrations of CORT in turkeys in a commercial processing plant

(Table 1; Figure 1). The absence of increases in plasma concentrations of CORT (studies 1, 2, and combined) and H : L ratios (studies 1 and 2) following shackling was somewhat surprising particularly with the increases in plasma concentrations of CORT reported in broiler chickens following experimental shackling (Kannan and Mench, 1996; Kannan et al., 1997; Bedanova et al., 2007). In one study with broiler chickens, there were no effects of duration of mock shackling on plasma concentrations of CORT: 0 min—15.4 ng mL^{-1}; 1 min—16.8 ng mL^{-1}; 3 min—17.6 ng mL^{-1}; 4 min—17.6 ng mL^{-1} (Kannan et al., 1997). However, in another study by the same authors, plasma concentrations of CORT were greater after 4 compared to 2 min of shackling (Kannan et al., 1997). It is also noted that there are also increases in the plasma concentrations of CORT in broiler chickens that are treated in a manner mimicking being shackled; these being handled and inverted or handled and inverted multiple times (Kannan and Mench, 1996; Kannan et al., 1997). Plasma concentrations of CORT were elevated by shackling in a static experimental shackling system with the magnitude of the response increasing with duration of shackling (Bedanova et al., 2007). The H : L ratio was not increased in broiler chickens after 30 or 60 s of shackling but was markedly increased with 120 s of shackling (Bedanova et al., 2007). Another index of stress is tonic immobility. Shackling for either 60 or 120 s increased the duration of tonic immobility (Bedanova et al., 2007). Parenthetically, it noted that H : L ratios are frequently considered as delayed responses to stress. However, H : L ratios were increased within minutes in broiler chickens by handling/venipuncture (Wein et al., 2017) and shackling (Bedanova et al., 2007).

There is one ready explanation for the lack of an increase in plasma concentrations of CORT in shackled turkeys compared to the increase reported in chickens (Kannan and Mench, 1996; Kannan et al., 1997). This is that there are simply species differences in the response to shackling. This is supported by the lack of wing flapping observed in shackled turkeys compared to that in broiler chickens. Moreover, in a controlled situation in a research farm, mock shackling did not markedly influence plasma concentrations of CORT (C.G. Scanes and colleagues, unpublished observations). Another explanation is that turkeys perceive shackling as being equally or less stressful compared to being in a transportation cage following catching with concomitant social disruption together with transportation and lairage without either feed or water. The possibility that shackling is less stressful than transportation/lairage in transportation cages is supported by the linear decline in H : L ratios with duration of shackling (study 1) and lower H : L ratio with 180 s of shackling compared to immediately prior to shackling (study 2) in turkeys. In contrast, in broiler chickens, H : L ratios were increased with 120 s of shackling (Bedanova et al., 2007).

Alternatively, it might be argued that plasma concentrations of CORT were at or close to a *maxima* at about 15 ng mL^{-1} immediately prior to shackling and, hence, that shackling cannot increase plasma concentrations of CORT further (Table 1). The elevated plasma concentrations of CORT immediately prior to shackling are presumed to reflect the stresses of catching, loading, transportation, and lairage in a holding shed. There is, however, evidence against plasma concentrations of CORT being at physiological maximum immediately prior to shackling. Firstly, plasma concentrations of CORT in excess of 15 ng mL^{-1} were observed with levels of 21 ng mL^{-1} observed in turkeys after the production stress of herding and 29 ng mL^{-1} after an acute challenge with turkey ACTH (C.G. Scanes and colleagues, unpublished observations). It is also unclear whether combination of stresses exerts additive, synergistic, or subtractive effects on plasma concentrations of CORT. In turkeys, similar increases in plasma concentrations of CORT when birds were subjected to feed and water withdrawal for 12 h or fasting for 18 h combined with cold stress or fasting for 18 h combined with heat stress and crowding (Bartz et al., 2018). Another explanation for the lack of an effect of shackling on plasma concentrations of CORT is that the hypothalamic-pituitary-adrenocortical axis is already down-regulated by the stresses of catching, loading, transportation, and lairage. In chickens, there is evidence that the plasma concentrations of CORT response to immobilization stress are attenuated by repeated stressing (Kang et al., 2017). Down-regulation does not appear to be the case. There was a very similar effect of shackling on plasma concentrations of CORT in turkeys in a controlled situation where the birds were not subjected to being placed in crates or transportation or lairage (C.G. Scanes and colleagues, unpublished observations) to those in the present study. However, the effect of shackling turkeys in a controlled study contrasts with the reports of shackling on plasma concentrations of CORT in broiler chickens (Kannan and Mench, 1996; Kannan et al., 1997; Bedanova et al., 2007). It is suggested that this may be due to there being sufficient cervical flexion to enable the head to move from an inverted to vertical or upright position.

It is argued that the procedures developed in the turkey industry are reducing distress during shackling of turkeys in an analogous manner to the use of technology to enhance poultry welfare (Sassi et al., 2016). This is supported by absence of wing flapping when the turkeys were placed on the shackle line or as during transit as the line progressed or in the simulation with on-farm shackling (C.G. Scanes and colleagues, unpublished observations). This lack of flapping is in contrast to the situation with broiler chickens. Immediately after shackling, 5.7% broiler chickens were reported to be flapping their wings vigorously in 30 processing plants (Gregory and Bell, 1987). If data from one plant was removed from the analysis, the percentage dropped to 1.06% (Gregory and Bell, 1987). Furthermore, the percentage declined to 0.11% 2 m into the shackling line and thereafter (Gregory and Bell, 1987). It was suggested that "rough handling" and "loud noises" contributed to increased incidence of wing flapping in broiler chicken processing (Gregory and Bell, 1987). In contrast, vigorous wing flapping was reduced by gentle massaging/stroking, i.e., "*the shackler ran his hands down the legs or legs and body*" (Gregory and Bell, 1987). The absence of increases in plasma concentrations of CORT with shackling may also reflect good processing practice perhaps combined with optimal on-farm husbandry. Grandin (2017) concluded that some on farm welfare issues can be assessed at the processing plant by evaluating body condition, lesions, and injuries.

There were no effects of time of day (in the rearing houses) on the H : L ratio (Table 2). In contrast, there are marked diurnal changes in circulating numbers of lymphocytes and granulocytes (predominantly heterophils) in chickens with these diurnal shifts being out of phase (Skwarło-Sońta et al., 1983). In study 2, H : L ratios were increased after catching/placing in transportation cages/transportation/lairage (Table 2). Similarly, H : L ratios are reported to be increased following transportation stress in turkey poults (Huff et al., 2010) but not chickens (Yue et al., 2010). This acute effect on H : L ratios is in accord with the reports of rapid effects of stressors and/or the hypothalamo-pituitary axis on H : L ratio in other poultry species. For instance, the H : L ratio was increased 20 h after handling treatment and the effect was blocked by treatment with ascorbic acid (Zulkifli et al., 2000). Moreover, there were increases in the H : L ratio with 7 D exposure to electric shocking or heat stress or to ammonia (McFarlane and Curtis, 1989). In addition, H : L rations were increased by shackling for 120 s (Bedanova et al., 2007).

There were marked differences in H : L ratios between different pens and with age in laying hens (Lentfer et al., 2015). This led Lentfer et al. (2015) to conclude that H : L ratios may not be a useful "*indicator of stress under commercial conditions.*" Similarly, in the present studies, there were differences in H : L ratios between houses/farm with male turkeys. Moreover, there were differences in plasma concentrations of CORT between houses/farm in the present study (Table 3). The individual houses/farms with elevated H : L ratios were not the same as those with highest plasma concentrations of CORT. It is argued that there is a similar case for using either or both plasma concentrations of CORT and H : L ratios as indicators of stress in turkeys and other poultry.

It is concluded that turkeys experience catching/caging/transportation/lairage as a physiological stress(es) with elevated plasma concentrations of CORT and H : L ratios. There were no further increase in plasma concentrations of CORT either after shackling or with increasing duration of shackling. In contrast, there were decrease in H : L ratios following shackling. The present data suggest that extended duration of shackling should not be viewed as unduly stressful consistent with "*evidence-based decision and policy-making*" (Farm Animal Welfare Committee, 2018). However, there is a case for re-examining procedures for moving turkeys from grower facilities to processing plants to alleviate stress.

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