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Effect of Dietary Supplementation of an Antioxidant Blend on Performance, Egg Quality and Behavior in Bovans Brown Hen

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Abstract: In egg industry, transportations and mixing are common stressors for hens. The objective of this study was to determine if the dietary antioxidant supplement improves hen performance, egg quality and behavior following transportation and housing environment changes. At 28 week of age, floor pen housed Bovans Brown hens (n = 48) were transferred to 2-hen wire cages and randomly assigned to one of the four dietary treatments: standard layer ration (control) and antioxidant supplemented diets at 200, 400 and 600 ppm of Agrado for 2 weeks. Egg production was recorded daily. Egg quality and feed consumption were measured weekly on two consecutive days. Individual body weight and weight of internal organs were measured at the end of the study. Behaviors were recorded twice per week using scan sampling. All three treated groups had higher egg production and fewer broken eggs compared to the control group. Body weight loss was observed in all hens regardless of treatments. However, hens fed 400 ppm antioxidant tended to lose the least body weight. In addition, hens fed 400 ppm antioxidant displayed the lowest incidence of stereotypic pecking behaviors. There were no treatment effects on feed intake, liver and spleen weight, Haugh units and yolk color, heterophil to lymphocyte ratio and corticosterone concentrations. Adrenal weight tended to be lower in all treated hens compared to the controls but without dose effects. In conclusion, dietary supplementation of the antioxidant could be used as a practice strategy to improve hen productivity performance under management stress.

Key words: Antioxidant, egg production, egg quality, physiology, behavior, hens

INTRODUCTION

Stress is a response to various adverse simulations and can be caused by routine management practices in the farm animal production industry, such as transportation (Cheng and Jefferson, 2007; Marchewka *et al*., 2013; Fazio *et al*., 2015), changes of housing environments and regrouping (Edens, 1987; Fahey and Cheng, 2008). In the modern egg industry, pullets are transported from grower facilities to layer facilities with regrouping prior to egg laying. During transportation, chickens are exposed to a wide range of physical and mental stressors, such as capturing, handling, loading and unloading, overcrowding, feed restriction, dehydration, noise, changing of temperature (cold or hot ambient condition), vibration, novel environments and social disruption (fighting for a dominant position following mixing and relocation) (Mitchell and Kettlewell, 2004; Cheng and Jefferson, 2007; Ajakaive *et al*., 2010; Vosmerova *et al*., 2010). These stressful events could disturb chickens' behavioral and physiological equilibrium or homeostasis, leading to negative impacts on their welfare and health (Mstl and Palme, 2002; Adenkola and Ayo, 2010; Yue *et al*., 2010). In addition, regrouping by mixing unfamiliar chickens from different sources and relocating them to a novel environment has been proved to cause substantial growth depression and to change physiological functions and immunity (Stooky and Gonyou, 1994; von Borell, 1995; de Groot *et al*., 2001; Cheng and Fahey, 2009; Rydhmer *et al*., 2013; Wilcox *et al*., 2013). These stressors could possibly lead to oxidative stress (Piccione *et al*., 2013; Fazio *et al*., 2015), resulting from an imbalance between free radical production and antioxidant defenses (Tokarzewski *et al*., 2004; Zhang *et al*., 2010; Beaulieu *et al*., 2014). Oxidative stress causes a wide range damage of molecular species including lipids, proteins and nucleic acids (McCord, 2000; Markesbery and Lovell, 2007; Murray *et al*., 2008). Oxidative stress is a major cause of pathophysiological damage in animals including chickens (Mezes *et al*., 1997; Estevez *et al*., 2006).

Displays of abnormal behaviors are associated with elevated stress in animal populations (Strekalova *et al*., 2005; Wang *et al*., 2014). Stereotypic behaviors are often associated with impaired welfare status (Mason, 1991;

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Diez-Leon *et al*., 2013) and often observed in sub-optimal environments or in response to management stressors (Mason, 1991). Laying hens, for example, develop stereotypic pacing behaviors without accessing to a suitable nest site (Duncan, 1970; Wood-Gush, 1972; Vesterggard *et al*., 1997) and lambs increase stereotypic oral licking behaviors following transportation and rehousing in a novel environment with unfamiliar animals (Miranda-de la Lama *et al*., 2010).

Dietary supplementation of antioxidants has become one of the most preferred and practicable management strategies in the farm animal industry, playing a critical role in maintaining animals' health, production and/or reproduction (Surai, 2006; Spears and Weiss, 2008), especially under various husbandry-associated stressors (Tavarez *et al*., 2011; Felver-Gant *et al*., 2014). Various antioxidants have shown positive effects on chicken growth and production performance (Wang *et al*., 1997; Lu *et al*., 2014a, b).

Agrado™ (Novus International Inc., St Charles, MO), an antioxidant, is a blend of ethoxyquin, propylene glycol and propyl gallate, with efficiency against lipid oxidation and harmful free radical formation (Duffy and Murphy, 1998; Kraft *et al*., 2004). Ethoxyquin (1, 2-dihydro-6-thoxy-2, 2, 4-tri-methylquinoline) is commonly used for protecting feedstuffs against oxidation, oil rancidity and degradation of fat soluble vitamins and pigments. In broiler chickens, ethoxyquin supplement benefits growth performance (Dibner *et al*., 1996; Wang *et al*., 1997; Lu *et al*., 2014a) and stabilizes the lipid in meat (Webb *et al*., 1978; Lu *et al*., 2014b). Propyl gallate, a phenolic antioxidant, improves stability of vegetable oils, fats and food against oxidative deterioration (Hawrysh *et al*., 1992). Ethoxyquin and propyl gallate combination has shown positive effects on broiler performance and meat shelf life (Tavarez *et al*., 2011). Propylene glycol, a glycogenic precursor, has been used as a dietary energy source to improve performance in dairy (Moallem *et al*., 2007) and poultry (Persons *et al*., 1967; Waldroup and Bowen, 1968). In poultry, Agrado supplementations at 135 mg/kg (or 135 ppm) reduced negative effects of oxidized oil on broiler performance and improved meat shelf life (Tavarez *et al*., 2011). Lu *et al*. (2014a, b) also observed dietary addition of Agrado blend in broilers was effective in improving growth, liver function and reducing inflammation in fat. Felver-Gant *et al*. (2014) reported that Agrado at 160 ppm attenuated the oxidative stress response in laying hens following heat stress. Therefore, we hypothesized that dietary supplementation of Agrado improves laying hens' ability to adapt management-associated stressors, such as transportation from grower facilities to layer facilities and regrouped. However, limited information is available on effects of Agrado on laying hens under routine management practices and its effective dose. The objective of this study was to determine the effects of Agrado as a dietary supplement on laying hen performance, egg quality and behavior following transportation and rehousing processes.

MATERIALS AND METHODS

Birds and treatments: Forty-eight 28-wk old Bovans Brown laying hens were transferred from furnished floor pens with perches and nest boxes (10 hens/pen, 3,716 cm^2/hen) to conventional cages (2 hens/cage). Hens were manually caught and kept in crates and then transported by a pick-up truck for 20 min to reach another housing facility. Hens were unloaded upon arrival and pair-housed in conventional cages. The cages were randomly assigned into 1 of the 4 treatments for 2 weeks: control (standard layer ration) and Agrado at 3 different concentrations: 200 ppm, 400 ppm and 600 ppm with 6 replications per treatment. Each cage had dimensions of 38.1 x 50.8 cm, a floor space of 968 cm^2 per hen. The room temperature was set at an average of 21°C and relative humidity at 63%. Lighting was set at a 16 h light: 8 h dark cycle. Water and feed were provided *ad libitum* throughout the experiment. The experimental protocol was approved by the Purdue Animal Use and Care Committee (PACUC Number: 1111000262).

Physical and physiological sampling: Body weight was measured individually at the beginning and the end of the experiment; body weight gain (BWG) was calculated afterwards.

Eggs were collected and recorded daily. Egg weight, albumen height and yolk color were measured two days per week using digital balance, albumen height gauge and yolk colorimeter, respectively (Technical Services and Supplies, England). The haugh unit was calculated using egg weight and albumen height following the equation below (Monira *et al*., 2003).

$$HU = 100 \times \log(H - 1.7W^{0.37} + 7.6)$$

where: HU = Haugh unit, H = observed height of the albumen in millimeter and W = weight of egg in grams. Feed intake (FI) was monitored weekly following the protocol published previously (Felver-Gant *et al*., 2014). Briefly, trough feeders were emptied before the test and a weighed portion of feed was added daily to the troughs. A liner was placed in a tray under each cage to collect feed waste during the test. At the end of the test, feed remaining in the feeders and waste feed inside the tray was weighed after being separated from manure.

At the end of the experiment, blood and organ samples were collected from each hen. Hens were sedated using an intravenous injection of sodium pentobarbital (30 mg/kg of body weight), then a 10 mL blood sample was collected by cardiac puncture followed immediately by cervical dislocation. The liver, spleen and right adrenal

gland were collected and weighed after dissection. The relative weight of each organ was calculated using the formula: Relative organ weight = Absolute organ weight (g or mg)/Body weight of the hen (kg).

Behavior measures: Live behavioral observations (eating, drinking and pecking) were conducted by scan sampling on Tuesday and Wednesday weekly for 2 h starting at noon each day (Mack *et al*., 2013). All measured behaviors were observed and recorded by individuals trained in observing and analyzing poultry behavior. Descriptions of the recorded behaviors are described in Table 1.

Radioimmunoassay (RIA): Total plasma corticosterone (CORT) was measured in duplicate using a commercial ^{125}I CORT radioimmunoassay kit (MP Biomedicals, LLC, Costa Mesa, CA 92626) with a procedure modified for chickens previously (Cheng *et al*., 2001). Briefly, in order to validate for parallelism and recovery in chickens, plasma samples were diluted 5 times (i.e., 20 ul of sample to 80 ul of steroid diluents). The concentration of CORT was calculated from a reference curve that ranged from 0.1 ng/ml (95.4% binding) to 4.0 ng/ml (14.9% binding) and the correlation coefficient was 0.9995. A well recovery of exogenous CORT was determined by adding known amounts of unlabeled CORT to aliquots of steroid diluent to produce theoretical concentration of 0.5, 1.0 and 2.0 ng/ml which yielded recovered concentrations of 0.48, 1.08 and 1.97 ng/ml, respectively. The sensitivity of the assay was 0.02 ng/ml.

Heterophil to lymphocyte ratio: The heterophil to lymphocyte (H: L) ratio was determined from 2 blood smears per hen (Harmon, 1998). Blood smear slides were air dried and then stained with Wright's staining solution (Fahey and Cheng, 2008). One-hundred leucocytes, including granular (heterophil, eosinophil, basophils) and nongranular (lymphocytes, monocytes), were counted from each slide (i.e., 200 white cells per bird) using microscopy with 4,000 X magnification. The H: L ratio was calculated by the formula dividing the total number of heterophils by lymphocytes per hen. H: L ratio = Total Number of Heterophils/Total Number of Lymphocytes.

Statistical analysis: The cage was used as an experimental unit. Diet treatment was considered as a fixed effect, hens within the cage were considered random effects. Analysis was done using PROC MIXED model with SAS 9.3 software (SAS Institute Inc. Cary, N. C). If data lacked homogenous variances, transformations of arcsine square root were used and the data reanalyzed. Statistical trends were similar for both transformed and untransformed data, therefore untransformed results will be presented. Means reported were the least square means (LSM) with standard error of the mean (SEM). Significant statistical differences were reported when $p<0.05$ and statistical trends were reported when $0.05<p<0.1$.

RESULTS AND DISCUSSION

The results of present study provide evidence that dietary supplementation of Agrado reduces the negative effects of transportation and regrouping associated physical and social stresses on hen performance, egg quality and behavioral exhibition, especially, at the 400 ppm level, which proves our hypothesis that Agrado, an antioxidant, improves laying hen ability to adapt to certain management-associated stressors.

Stressors applied on hens: The hens used in this study were housed at 10-hen floor pens, at 3,716 cm^2 per hen, from 16 to 27 week-old before the current experiment. The hens were then subjected to the stressful stimuli of transportation, regrouping and caging. Transportation subjects the chickens to stress, resulting from an accumulation of stressors during the process, including, capturing, loading and unloading, overcrowding, feed restriction, dehydration, change in temperature (cold or heat), vibration and noise (Schaefer *et al*., 2001; Bejaei and Cheng, 2014). Hens were rehoused in two-bird cages with an unfamiliar bird, resulting in the need to re-establish social stability within the cage (Stooky and Gonyou, 1994; Ison *et al*., 2014). In the current study, transportation and rehousing stress included moving the hens from floor pens to conventional cages as that density became much higher (floor pen size vs. cage size per hen) within a barren area (eliminated to use perch and nestbox provided within the floor pens). These stressors lead to a deviation from physiological homeostasis, in turn, impairing hen well-being as evidenced by the display of abnormal behaviors and reduced performance (Craig and Adams, 1984; Lewis and Hurnik, 1990; Estevez *et al*., 2006).

Effects of the agrado supplementation on physical measurements in laying hens: Body weight was reduced in all hens following the transportation and rehousing regardless of treatments (Fig. 1); but 400 ppm fed hens tended to lose less weight in response to the rehoming stress than controls ($p = 0.06$), which was in the order of: 400<200<600 ppm <control hens. There was no difference in FI among treatments ($p>0.1$; Table 2). With similar FI, hens from the 400 ppm fed group may have better feed efficiency according to the body weight change following transporting and rehousing. In general, body weight loss is the result of a negative cascade following various stressful events in animals (Freeman, 1976; Hill, 1983; Bejaei and Cheng, 2014). The current results are in agreement with the findings from previous studies conducted in broilers (Lu *et al*., 2014a) and feedlot cattle (Krumsiek and Owens, 1998),

Table 1: Ethogram of Behavioral activities to be recorded

Behavior	Description
Feeding	The bird's head is extended from the cage and its beak has passed the lip of the feeder
Drinking	The bird's beak is in contact with the nipple drinker and then lifts its head
Stereotypic pecking	The bird pecking the cage or the cage floor repeatedly without an objective or goal (a meaningless movement)

Table 2: Effects of the antioxidant supplementation on relative organ weights and daily FI of the hen[1]

Treatment	LW[1] (g/kg)	SW[1] (g/kg)	AW[1] (mg/kg)	FI (g)
Control	2.81±0.12	0.10±0.01	4.11±0.15[†]	107.01±2.29
200 ppm	2.82±0.12	0.10±0.01	3.69±0.15	114.25±2.29
400 ppm	2.53±0.12	0.09±0.01	3.96±0.15	109.81±2.29
600 ppm	2.74±0.12	0.11±0.01	3.60±0.15	115.15±2.29
p-value	0.33	0.55	0.06	0.14

All means reported are LSM±SE created by mixed model analysis

[1]Relative organ weight = absolute organ weight (g or mg)/BW (kg)

AW = relative spleen weight; FI = Feed intake; LW = relative liver weight; SW = relative spleen weight[†], p<0.1

Table 3: Effect of AOX supplementation on H: L ratio, plasma CORT concentrations and egg quality of hens

Treatment	H: L ratio[1]	CORT[2] (ng/mL)	Albumen height (g)	Haugh units	Yolk color
Control	1.13±0.05	411.72±55.29	8.30±0.29	113.3±0.92	7.36±0.19
200 ppm	1.13±0.05	496.61±55.29	8.24±0.24	113.0±0.67	7.67±0.13
400 ppm	1.19±0.05	485.53±55.29	8.23±0.21	111.9±0.69	7.85±0.14
600 ppm	1.17±0.05	436.73±55.29	7.90±0.22	113.3±0.77	7.94±0.15
p-value	0.78	0.67	0.59	0.50	0.10

All means reported are LSM±standard error created by mixed model analysis

[1]H: L ratio = heterophil to lymphocyte ratio. [2]CORT = Corticosterone

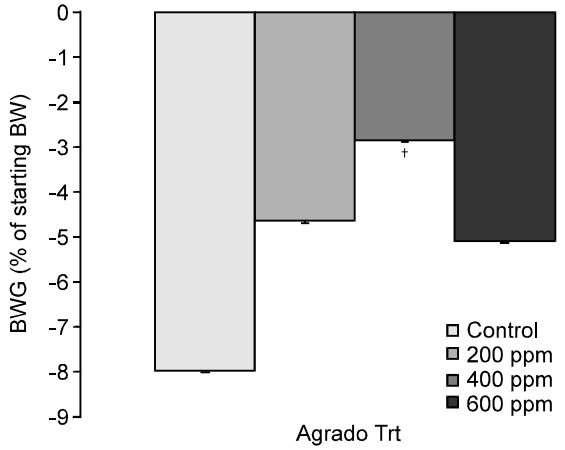

Fig. 1: Effect of the antioxidant supplementation on BW. Effects of Agrado on BW following combined transportation and rehousing associated stressors. Hens of 400 ppm treated group tended to maintain an optimal weight than control hens [adjusted (adj) p = 0.06]. However, no difference was observed in hens from both 200 and 600 ppm groups compared to controls. Values represent the least square means±SEM. [†]Denotes a trend difference between Agrado treatment hens and control hens (p<0.01)

in which animals fed with Agrado supplemented diets have better growth performance and feed efficiency. In addition, Radwan *et al.* (2008) reported that Agrado supplementation improved feed conversion in adult laying hens.

Overall, there was no difference in liver weight and spleen weight among the treatments. Compared to control, adrenal gland weight tended to be lower in Agrado groups without dose effects (p = 0.06; Table 2). Adrenal gland weight, as a stress indicator, has been used to assess the pathophysiological status of birds following various stressors (Siegel, 1980; Ulrich-Lai *et al.*, 2006; Spencer *et al.*, 2009). Adrenal gland weight was greater in chickens following thermal (heat or cold), physical (handling and caging) and social (grouping and competition) stimulations (Harvey *et al.*, 2005). In addition, previous studies suggest that antioxidants reduce negative physiological response to stress in broilers (Taniguchi *et al.*, 1992; Nain *et al.*, 2008). Similarly, in the current study, the antioxidative compounds in Agrado may reduce the effects of the physical and social stimulation associated with transportation and rehousing on the functions of the adrenal glands although there was no treatment effects on CORT concentrations (Table 3).

There was no treatment effects on albumen height, haugh unit and yolk color measured in hens (p = 0.1, respectively; Table 3). However, the egg production was significantly affected by treatments (Fig. 2). Compared to the control hens, daily egg production was higher (p<0.0001) and the incidence of broken eggs was lower (p = 0.004) in all Agrado treated hens, without dose effects (p>0.1). Similar to the current findings, higher egg production were reported previously in laying hens fed with dietary supplementation of antioxidants under both normal (Radwan *et al.*, 2008; Hayat *et al.*, 2009) and

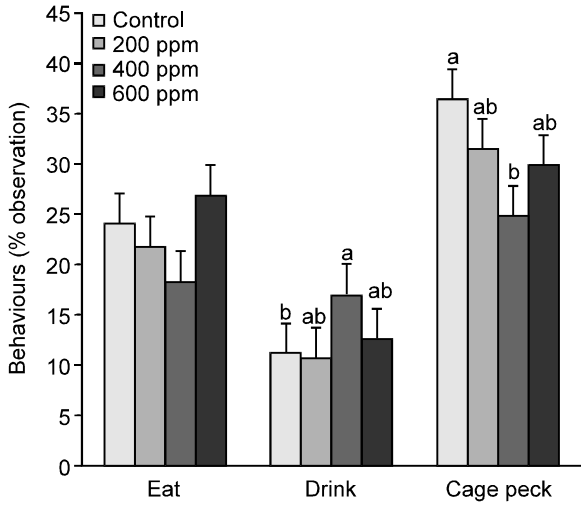

Fig. 2: Effect of the antioxidant supplementation on egg production and broken eggs. Difference effects of Agrado treatments on egg production. Compared with untreated controls, all Agrado treated hens had higher daily egg production [adjusted (adj) p<0.0001] and less broken eggs [adjusted (adj) p = 0.0004], but without dose effects. Values represent the least square means±SEM. [a,b]Different letters denote a significant difference between AOX treated hens and control hens (p<0.05)

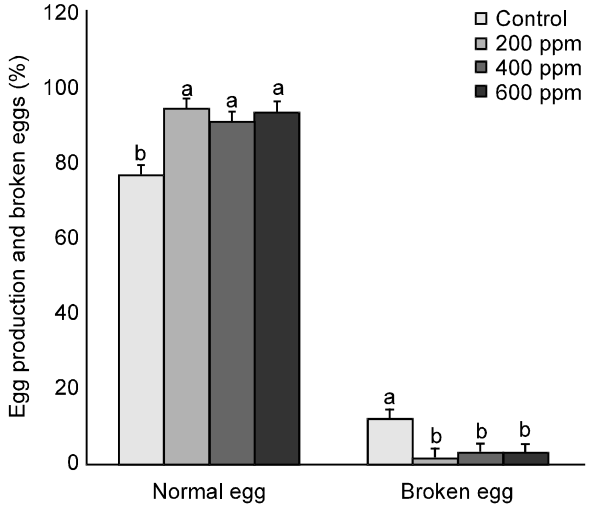

Fig. 3: Effect of the antioxidant supplementation on Behaviors. Eating, drinking and cage pecking behaviors in hens following Agrado treatment. Overall, hens of 400 ppm treated group tended to express more drinking behaviors and less cage pecking than hens from untreated control group [adjusted (adj) p = 0.0001]. No difference was observed in eating behaviors cross treatments. Values represent the least square means±SEM. [a,b]Different letters denote a significant difference among treatments (p<0.05)

stimulated conditions, such as heat stress (Ma *et al*., 2005). Both present and previous findings could be due to reduced oxidative stress and improved nutrient absorption by supplementing antioxidants.

Effects of the agrado supplementation on physiological measurements in laying hens: Heterophil to lymphocyte ratio has been widely used to evaluate chickens' responsiveness to various stressors (Gross and Siegel, 1983; Al-Murrani *et al*., 1997; Puvadolpirod and Thaxton, 2000). In addition, H: L ratio has also been used as biometric for genetic selection of chickens with high resistance to stress (Al-Murrani *et al*., 1997). Elevated H: L ratios reflect an increased inflammatory immune response and have been seen in response to heat stress (Prieto and Campo, 2010). Antioxidant treatment has been shown to reduce H: L ratios in response to heat stress (Prieto and Campo, 2010). Indicating that heat stress-induced oxidative damage could be alleviated or remediated by antioxidant supplements (Altan *et al*., 2010; Lin *et al*., 2004). In the current study, H: L ratio did not show any differences among treatments (p = 0.78; Table 3). Potentially indicating that hens' immune response to transportation and rehousing is short-lived. The hypothesis is consistent with the previous findings that birds have the capability to adapt to their environments (Cheng, 2010) and their adaptability is based on multiple factors, such as the types of stressor as well as its frequency and duration.

Corticosterone concentrations have been used as an indicator for measuring adaptability of chickens to various stressors, such as emotional, physical and environmental stressors (Gross and Siegel, 1983; Yan *et al*., 2013). In the current study, there was no difference in the plasma CORT levels between hens fed Agrado supplemented and control diets (p = 0.67; Table 3). This result is possibly due to adaptation of the hens to the new environment after 2 wk post transportation and rehousing. As Siegel (1995) suggested that the changes of CORT concentrations may be a better indicator of acute or life-threatening stress.

Effects of the agrado supplementation on behavioral measurements in laying hens: Animals' behavioral patterns reflect their response to a particular moment and are related to both internal (physiological) and external (environmental) factors that affect their health. Animals change their behavioral patterns (behavioral adaptability) in response to their living conditions (Wolf and Linden, 2012). Alterations of behaviors in animals have been used as indicators for estimating their

welfare status (Broom, 1991; Phillips and Petherick, 2015). In the current study, hens fed 400 ppm of Agrado performed more drinking activities than hens from control group ($p = 0.0001$; Fig. 3) and both 200 and 600 ppm Agrado groups ($p<0.05$). There was no treatment effect on eating behaviors ($p>0.1$).

There were treatment effects on cage pecking behavior (Fig. 3). Cage pecking, as a type of stereotypic behavior in chicken, was reduced in hens fed 400 ppm Agrado but not in hens from both 200 and 600 ppm Agrado groups compare to control hens ($p<0.001$). Previous studies evidenced that developing stereotypic behaviors indicates that the environment may not meet the animals' physical, physiological and mental needs or represent the current suffering of the animals (Mason, 1991; Polverino *et al*., 2015). In rodents, for example, stress-induced oxidative damage alters patterns of stereotyped behaviors (Mendez-Cuesta *et al*., 2001) and these stereotypic behaviors are reduced by antioxidant supplemented diets (Mitra *et al*., 1996; Cantuti-Castelvetri *et al*., 2000). Agrado has been shown to be an external source of antioxidant in mammals (Vazques-Anon and Jenkins, 2007) and birds (Felver-Gant *et al*., 2014). In the current study, behavioral changes in 400 ppm fed hens may indicate that Agrado has potential to relieve some of the effects of oxidative stress-associated with transportation and rehousing.

Conclusion: In conclusion, our data suggest that dietary supplementation of Agrado, as an antioxidant supplementation, has positive effects on hen performance, egg production, egg quality and behavior following transportation and rehousing. In addition, the current data indicate that dietary supplementation of Agrado at 400 ppm could be an ideal dose for improving hen productivity and health. These results provide evidence for further investigation into the mechanisms of antioxidant supplementation to prevent stress-associated damage in laying hens.

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