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An integrated analysis of social stress in laying hens: The interaction between physiology, behaviour, and hierarchy

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ABSTRACT

Livestock is the category of animals that suffers the most severe welfare problems. Among these, physical, physiological, and behavioural distress caused by artificial grouping are some of the challenges faced by these animals. Groups whose members are frequently changed have been reported as socially unstable, which could jeopardise the welfare of animals. Here, we assessed the effect of social instability on aggression, stress, and productivity in groups of laying hens (*Gallus gallus domesticus*). We studied 36 females, distributed into three stable groups (without group membership change) and three unstable groups (where the dominant member was rotated every week) over the course of 10 weeks. We evaluated the frequency of agonistic interactions, glucocorticoid metabolites (GCM) concentrations, and egg production. In both treatments, dominant hens produced more eggs compared to intermediate and subordinates, and intermediate hens had the highest GCM concentrations. Socially unstable groups had lower productivity and higher frequencies of agonistic interactions than stable groups. Social instability also affected GCM of the animals: in stable groups, subordinate hens had higher concentrations than dominants; in unstable groups, this pattern was reversed. Our results point to a social destabilisation in groups whose members were alternated, and suggest the welfare of individuals in unstable groups was compromised. Our results pointed to a complex relationship between hierarchy, productivity, physiological stress and aggression in laying hens, and have implications for their husbandry and management and, consequently, for their welfare levels.

1. Introduction

Livestock is the most numerous category of animals in direct contact with humans, and these are the animals that present the most severe welfare problems (Broom and Molento, 2004). They are often kept in artificial groups that can either foster positive social behaviour or lead to aggression, injury and chronic fear (Fraser et al., 2013). Behavioural distress caused by artificial confinement has been frequently reported in laying hens (*Gallus gallus domesticus;* Broom and Molento, 2004). Although not common worldwide, some poultry producers regroup or divide groups of hens at different stages of the laying cycle in order to keep the same number of individuals in the cages (Hester and Wilson, 1986). Laying hens kept in small groups (6–10 individuals) exhibit social behaviour similar to that of their wild ancestor, the red jungle fowl (*Gallus gallus*; Collias and Collias, 1996). Social organisation in chickens is typically stable and hierarchical (McBride et al., 1969). In stable hierarchies, social dominance tends to settle to avoid the costs and risks of increased and continual fighting (Creel, 2001; Enquist and Leimar, 1990). Therefore, allowing animals to form and maintain stable associations can create a positive social environment and improve their ability to cope with new stressors (Fraser et al., 2013). However, when the stability of the social group is disturbed, higher levels of aggression and, consequently, greater stress loads are expected (Rose and Croft, 2015).

Aggression in groups of domestic hens is targeted more to newcomers than towards older members (Cloutier and Newberry, 2002). Aggressive behaviour directed to unfamiliar birds was observed for up to eight weeks after the introduction of new members (Guhl and Allee, 1944). Therefore, regrouping, dividing or introducing new members may induce social stress due to the establishment of a new pecking order every time a new hen is introduced into the group, likely affecting the welfare of the birds (Cheng and Fahey, 2009). Previous studies

Abbreviations: AgI, agonistic interactions; AIC_c, Akaike's information criterion corrected for small samples; DVR, digital video recorder; GC, glucocorticoids; GCM, glucocorticoid metabolites; GLM, generalised linear model

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considered either egg production (Hill, 1983), aggression (Cloutier and Newberry, 2002; Cordiner and Savory, 2001) or body condition and productivity (Guhl and Allee, 1944) as indicators of laying hens' health level. However, the measurement of only one or two stress parameters is often insufficient to evaluate the welfare of the animals because the interaction between these parameters may be complex (Scheiber et al., 2015), and lead to inconclusive results. In addition, differences in techniques and conditions used to measure stress parameters may prevent comparisons between studies. For instance, Guhl and Allee (1944) reported that unstable groups had higher levels of aggression and lower productivity than stable groups, and that hens that constantly switched groups had lower body weight and lower food consumption than individuals in stable groups. In contrast to these results, other studies comparing stability and egg productivity in laying hens found no correlation between these parameters (Feldkamp and Adams, 1973; Adams, 1974; Hester and Wilson, 1986). Studies evaluating the effects of stability in laying hens using different physiological parameters have also found different outcomes. For example, in two studies which were conducted by the same researchers, one study found no difference in stress levels between stable and unstable groups (Cheng and Fahey, 2009, based on the weight of adrenal glands and plasma concentrations of serotonin and tryptophan) while another study found lower concentrations of glucocorticoids (GC – used as a measure of stress levels) in the plasma of animals in unstable groups (Fahey and Cheng, 2008). Littin and Cockrem (2001) found no effect of mixing groups on plasma GC, and no correlation of GC responses to stressors with the birds' hierarchical positions.

For social species, the involvement in recurrent and/or prolonged agonistic interactions can lead to chronic stress (Sapolsky, 1992). In response to stressors, within seconds, adrenaline is released into the blood stream, promoting increases in blood pressure, heart and respiratory rate and, consequently, energy is mobilised and made available for immediate use. Within a few minutes, the adrenal cortex secrets GC that alter the metabolic pathway for the production of adenosine triphosphate, redirecting for immediate survival the energy that would otherwise be used for other physiological processes (such as digestion, growth and reproduction; Moberg and Mench, 2000). This response is beneficial to the animal in the short term, but it can become harmful if prolonged or repeated too often in conditions that preclude the animal from recovering from each stressful event. Animals that are constantly stressed exhibit higher basal GC concentrations, leading to inhibition of anabolic processes, which can result in reproductive problems, low disease resistance, neuronal loss and decreased life expectancy (Siegek, 1980; Sapolsky, 1992). Therefore, the evaluation of the stress-response system functioning may bring relevant information, and should be considered when evaluating welfare (Möstl and Palme, 2002).

Studies evaluating stress levels have used numerous methods, such as measuring glucocorticoid concentrations in blood, evaluating immune system functioning, etc. Non-invasive sampling methods are the best suited, since the negative effects of invasive collections – e.g. blood samples – impose limitations to this kind of analysis. Beuving and Vonder (1978) reported an increase in serum corticosterone only 45 s after restraining hens, which could influence the results and mask the effects of other variables on stress levels. The measurement of faecal glucocorticoid metabolites (GCM) is one of the non-invasive methods used more often (Möstl and Palme, 2002; Palme, 2012; Sheriff et al., 2011). Faeces are the matrix that offers the greatest advantages due to their ease of collection, and its collection being feedback free (Touma and Palme, 2005). Non-invasive techniques for GCM monitoring have been extensively used by researchers and conservationists with numerous species (Touma and Palme, 2005; Sheriff et al., 2011; Kersey and Dehnhard, 2014), and have been already validated for chickens to assist in assessing welfare levels (Rettenbacher et al., 2004). However, as stress mechanisms may interact with other physiological systems – introducing confounding variables to the results (Scheiber et al., 2015), the measurement of other variables in tandem with physiological parameters will bring accuracy to the evaluation of welfare. Stress measurements analysed together with hierarchy, productivity, and behaviour could provide a comprehensive evaluation of long-term stress levels in laying hens which are submitted to changes in their social environment.

The relationship between GC levels, aggression and social status has been studied since the 1950s (Creel et al., 2013). In the early years of study, it was believed that subordination represented a more significant stressor than dominance. However, it is not always so. In some societies, the actions required to maintain dominance can be, per se, stressors (Creel et al., 1992; Sands and Creel, 2004). The endocrine profiles of baboons (*Papio anubis*) in different social positions may change, depending on the type of hierarchy. During periods of unstable hierarchy, dominant baboons show higher GC levels than subordinates. However, in stable periods, they present lower GC concentrations than subordinates (Sapolsky, 1983). In bearded capuchins (*Sapajus libidinosus*), in both types of hierarchies – stable and unstable – the dominant male presented the highest stress loads (Mendonça-Furtado et al., 2014).

This study aimed at investigating the effects of social instability on the welfare of laying hens at a group level, and at distinct hierarchical levels, through the evaluation of: (1) egg productivity; (2) the frequency of agonistic interactions, and (3) stress levels, to perform a comprehensive analysis of the social stress experienced by these birds. Considering the well-established relationship between social stressors, physiological reactions and welfare levels (Hill, 1983; Creel, 2001; Broom and Molento, 2004), and based on studies carried out evaluating social stressors in livestock (Guhl, 1968; Cheng and Fahey, 2009; Matur et al., 2015), our hypotheses for this study were that a social instability would be connected to (a) higher GCM concentrations and (b) higher frequencies of conflicts, both leading to (c) a reduction in productivity. We also predicted (d) greater productivity for the most dominant hens compared to subordinates, since subordinates have been reported as being targets of aggression and having poor access to resources in poultry (Hill, 1983; Cunningham and von Tienhoven, 1984).

2. Material and methods

Procedures described herein were approved by the Committee of Ethics for Animal Research from the Pontifical Catholic University of Minas Gerais, Brazil (approval number 001.2015) and are in accordance with the ethical guidelines published by the International Society for Applied Ethology (ISAE, 2002).

2.1. Subjects

We studied 36 female hens (ISA Brown hybrids), acquired from a commercial breeder (Agropecuária Vila Verde, Betim-MG) at the age of 19 weeks. From acquisition until the start of the experiments (when the birds were 34 weeks old), the animals underwent 15 weeks of habituation (we waited until all hens were steadily laying) kept together in a 90 m^2 fenced space, containing eight feeders and eight water drinkers. During habituation, as well as the experimental period, every bird had water *ad libitum,* and received 300 g of commercial laying feed and around 70 g of corn grains, divided into two meals (at 08:00 h and at 16:00 h). Before the start of the experiment, all hens were clinically checked, and marked in different colours using non-toxic cloth dye (Acrilex®) for visual identification. Hens are less likely to identify a target when all of them are marked similarly, which is particularly important if they are in small groups (Marin et al., 2014), which is the case of the subjects of this study.

2.2. Facilities

The study was conducted in a property located in the municipality of Sete Lagoas, in the central region of Minas Gerais state, Brazil. After the habituation period described above, the birds were randomly allocated into six groups, containing six members each. To define the number of individuals in each group, we followed the study of Guhl and Allee (1944) and the average number of individuals in feral fowl social groups (McBride et al., 1969). In order to allow accurate observations of social interactions, and to maintain the animals with acceptable levels of welfare, the birds were housed in enriched enclosures, instead of battery cages, a usual housing system for laying hens. Each group of animals was allocated in a 3.50 × 4.00 × 3.50 m triangular enclosure (1.04 birds/m^2), surrounded by chicken wire fences, partly covered by PVC tile, containing two perches (1 m from the floor), one nest, one feeder, grass (*Brachiaria* sp.) and a water drinker. The dirt floor enabled the birds to dustbathe. The enclosures were cleaned thoroughly once a week and were arranged so that different groups of birds had no physical or visual contact with each other. The hens were kept under natural regimen of light and temperature (mean sunrise 6:40 and sunset time 17:45 [11L:13D]; mean temperature ± SE 19.5 °C ± 0.25). All birds were daily visually inspected for behavioural/clinical signs of health problems (e.g., diarrhoea, coryza etc.).

2.3. Treatments

Three of the six groups were randomly defined as control groups (Stable groups), where there would be no introductions or removals of individuals until the end of the experiment. The remaining three groups were designated experimental groups (Unstable groups), from which the dominant bird was removed and moved to another experimental group every week, for ten weeks. One-week intervals were defined in order to promote constant instability and to measure the effect of these regroupings in the birds' stress levels, since these are shorter than the time necessary to promote a decrease in aggressiveness against newcomers in hens (eight weeks, Guhl and Allee, 1944). The rotated hen would return to a group it had been previously part of after three weeks. This would not interfere with the experiment since hens were shown not to distinguish known from unknown conspecifics after two weeks of separation (Chase, 1982). During the whole experimental period, we: a) recorded the agonistic behaviours performed by the animals, in order to evaluate the level of aggressiveness in each group and to assess the incipient hierarchies, so than the dominant hen was always the rotated one in the unstable group; b) collected all eggs laid by every hen in all groups; c) collected the birds' droppings for GCM evaluation.

2.4. Behavioural observations

Behaviours were recorded five days per week, in two daily sessions: between 08:00–10:00 h and between 16:00–18:00 h. Each group was observed for 10 min per session, through Continuous Recording of all agonistic behaviours (Behaviour Sampling, Martin and Bateson, 2010), including fights, pecks directed towards the head of another bird, threats and chases – behaviours usually observed in "establishment fights" in laying hens (Biswas and Craig, 1971; Cloutier and Newberry, 2000). The actors and targets of each interaction were defined according to Table 1. The mean frequencies of agonistic interactions recorded in each group during the whole experiment were used for statistical analysis. Because aggression naturally occurs when unfamiliar hens are grouped, we monitored the birds during the whole experiment – especially when there were introductions – and were ready to intervene if any of them got seriously injured, which did not happen.

2.5. Dominance hierarchy assessment

The assessment of the hierarchies was performed by weekly calculating the ranking of every bird in each group, based on the spontaneous agonistic interactions recorded during the sessions of behavioural observations. The calculations were made through the Elo-rating method (Albers and de Vries, 2001; Neumann et al., 2011). Elo-rating values were calculated for all group members, and consider the probability that bird A beats bird B in a given contest. After each contest, the hierarchical position of the winner increases (and the loser's decreases), based on the expected probability of this individual come out as the winner in a given interaction. For example, if a hen with a higher dominance index, i.e., with a higher Elo-rating, wins an interaction against a lower indexed one, its Elo-rating will increase slightly (and the losers' will decrease in the same proportion). But, on the other hand, if the bird with the lowest index beats the dominant, the Elo-rating of both will have a more drastic change (Neumann et al., 2011). All hens started the experiment with 1000 points, and the dominant ones that moved to other groups started in the new group with 1000 points. Elo-Rating values were calculated in the end of each week.

2.6. Egg collection

The eggs laid by each individual were collected daily. To enable individual identification of the hens who laid the eggs, the birds were monitored by video-cameras (Bullet Infra AHD CHD cameras 4013 1,3 MP, Pyxel Electronics), positioned in front of the nests, and connected to a DVR (Digital Video Recorder Stand Alone 8 channels AHD HDVR 4008, Pyxel Electronics).

2.7. Collection and analysis of droppings

Once a week, droppings from all members in each group were collected individually, homogenised and frozen for later analysis. Before collection, the enclosures were thoroughly cleaned right after the hens perched for the night (18:00 h), and all droppings found beneath the roosts until 20:00 h were collected to assess the stress levels of each group. Individual analyses were also made from hens in the positions 1, 3 and 6 in the dominance hierarchy, to evaluate a possible relationship between social rank position and the animals' stress levels. For this sampling, the faeces were collected also once a week, in the mornings, but upon observation of defecation, in order to identify each animal's samples. Even though studies show that there is no daily pattern in GCM concentrations in hens (Rettenbacher et al., 2004), no comparisons were made between group (night samples) and individual (morning samples) GCM.

Dropping samples were individually stored in labelled tubes and immediately frozen for later extraction, according to a methanol-based

Table 1
Ethogram of agonistic behaviours of *Gallus gallus domesticus* used in this study to evaluate dominance hierarchies.[a]

Behaviour	Description
Fight	Two hens perform a series of aggressive acts towards each other in rapid succession, including leaps and pecks. We considered as loser the animal that retreated.
Peck	A hard fast stab with the beak at another hen, usually at the back of the head or comb. We considered as winner the animal who pecked and as loser, the one who was pecked.
Threat	A hen raises her head and looks at another hen, or makes a slight intention movement towards the other hen, and the other bird submits to the former by lowering her head, crouching, looking away or moving away. There is no physical contact. The hen that threatened was considered the winner if the other retreated.
Chase	One hen runs towards her target. The hen that ran towards the other was considered the winner.

[a] (adapted from Cloutier and Newberry, 2000).

protocol (Palme et al., 2013). Briefly, a portion of 0.5 g of each well homogenised sample was extracted with 5 ml of 60% (v/v) methanol by shaking it for 1.5 min. After centrifugation for 15 min, aliquots (0.5 ml in duplicates) were dried and sent to the University of Veterinary Medicine, Vienna, Austria for measuring GCM with a cortisone enzyme immunoassay previously developed and validated for use in chicken (for details see Rettenbacher et al., 2004). Glucocorticoid metabolites measures are given in nanograms per gram of droppings (ng/g).

2.8. Data analysis

We used generalised linear models (GLMs) to test which variables explained productivity, GCM concentrations, and the frequency of agonistic interactions (AgI). We fit two separate sets of GLMs: for GLMs 1, we used data of all individuals in the groups, and the fixed effects considered were STABILITY (Stable or Unstable Group) and AgI on productivity; and WEEK, STABILITY and AgI on GCM, and the interactions between these factors; for GLMs 2, we considered only data from the birds occupying the positions (1) Dominant, (3) Intermediate and (6) Subordinate at the moment of each Elo-Rating calculation, whoever they were. Even if the individuals occupying these positions changed during the experiment, data was always collected from the birds in those positions. Fixed effects considered were GCM and RANKING on productivity; AgI, STABILITY and RANKING on GCM; STABILITY and RANKING on AgI, and the interactions between these factors.

Minimal adequate models were obtained by comparing models with dropped terms using Akaike's information criterion corrected for small samples (AIC_c), with the lowest AIC_c value indicating the best model fit. Standard errors were corrected using a quasipoisson-GLM model, where variance is given by $ø*\mu$, where μ is the mean and the ø is the dispersion parameter (Zuur et al., 2009). We used "lme4" (Bates et al., 2015), "MASS" (Venables and Ripley, 2002), "car" (Fox and Weisberg, 2011), and "AICcmodavg" (Mazerolle, 2015) packages to predict distribution of data and fit GLM models in the R statistical software, version 3.1.1 (R Core Team, 2014).

We used a T- (normal data) or Mann-Whitney test (non-normal data) to check for differences in baseline parameters between treatments, differences between pecks and threats in both treatments, and differences in AgI between treatments (since our best GLM model for these parameters was not good enough). We also used a Wilcoxon test to evaluate differences in the number of dominants' aggressive acts towards intermediates and subordinates in both treatments. All results were analysed based on statistical significance ($\alpha \leq 0.05$) and, regarding GLMs, also on the magnitude of the effects (*estimates* greater than 0.01).

3. Results

Baseline records did not show differences in the evaluated parameters between groups selected for different treatments during the first week (Agonistic interactions: $t = -0.56$, $p = .57$, stable groups [mean ± SE] 30.72 ± 5.54, unstable groups 35.22 ± 5.70; Productivity: $U = 157$, $p = .87$, stable groups [median = 2, quartiles $Q_1 = 1$ and $Q_3 = 3$], unstable groups [median = 2, $Q_1 = 0$, $Q_3 = 3$]; MGC concentrations: $U = 151.50$, $p = .73$, stable groups [median = 79.16, $Q_1 = 41.59$, $Q_3 = 93.81$], unstable groups [median = 77.68, $Q_1 = 43.67$, $Q_3 = 99.84$]).

3.1. Productivity

We collected a total of 1006 eggs during the 10 weeks of the study. The best model for individual egg productivity is shown in Table 2. STABILITY affected the number of eggs laid by the hens. In stable groups, the hens laid more eggs during the study (mean ± SE: 31.26 ± 2.06) than in unstable groups (24.35 ± 2.47, Fig. 1). There was also an effect of AgI on productivity; however, the effect was negligible (*estimate* 0.0009).

Table 2
Results from the final models for GLMs 1 to evaluate factors affecting individual parameters in female ISA Brown hens.[1]

Parameters	Estimate ± SD	t value	P
Productivity[a]			
(intercept)	3.2393 ± 0.1238	26.150	< .0001
stability[b]	−0.3740 ± 0.1296	−2.886	.007**
AgI	0.0009 ± 0.0004	2.053	.05*
GCM[c]			
(intercept)	4.8900 ± 0.1575	31.031	< .0001
week	−0.0142 ± 0.0187	−0.757	.45
stability[b]	0.1394 ± 0.1244	1.121	.26
AgI	−0.0019 ± 0.0014	−1.365	.17

[1] Parameters and interactions not shown were removed during the model selection process. Best models for: Productivity AICc = 88.28, GCM AICc = 669.73.
[a] Explanatory variables included in the full model: STABILITY, AgI, STABILITY*AgI.
[b] Stable or unstable: unstable is the reference group.
[c] Explanatory variables included in the full model: STABILITY, WEEK, AgI, STABILITY*WEEK, STABILITY*AgI, WEEK*AgI.
* P ≤ .05.
** P ≤ .01.

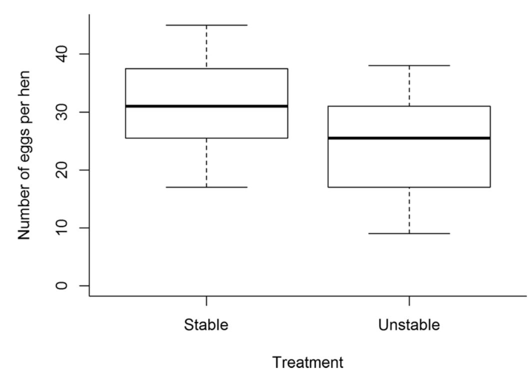

Fig. 1. Total number of eggs laid per hen in stable and unstable groups during the study. Boxes represent 1st and 3rd quartile, the thick lines are the medians and the whiskers extend to most extreme data points.

3.2. Productivity according to social ranking

As STABILITY had no measurable effect on the productivity of specific hierarchical positions, egg production of both groups were pooled. RANKING had an effect on egg productivity (Table 3). Top ranking hens laid more eggs (34.0 ± 3.49) than intermediate (27.66 ± 2.74) and low ranking birds (23.33 ± 4.47, Fig. 2). There was also an effect of GCM concentrations on productivity per ranking; however, the effect was negligible (*estimate* −0.0041).

3.3. Agonistic interactions

We recorded 5370 agonistic interactions in all six groups during the 10 weeks of study. The proportion of pecks was higher than threats in both treatments (0.65 and 0.61 pecks versus 0.30 and 0.34 threats in stable and unstable groups respectively; $F = 138.80$, $p < .05$ and $F = 116.14$, $p < .05$). STABILITY affected the number of agonistic interactions (Fig. 3). In stable groups, hens participated in fewer AgI (212.40 ± 20.22) than in the unstable groups (334.85 ± 43.29, $t = -2.56$, $p = .01$, Fig. 4).

3.4. Agonistic interactions according to social ranking

No variables analysed had an effect on AgI when we analysed data

Table 3
Results from the final reduced models for GLMs 2 to evaluate factors affecting parameters per ranking in female ISA Brown hens.[1]

Parameters	Estimate ± SD	t value	P
Productivity[a]			
(intercept)	4.0201 ± 0.2336	17.203	< .0001
ranking	−0.0717 ± 0.0330	−2.168	.04[*]
GCM	−0.0041 ± 0.0019	−2.134	.04[*]
GCM[b]			
(intercept)	4.4896 ± 0.5431	8.267	< .0001
ranking[d]	−0.0792 ± 0.1058	−0.748	.47
stability[e]	2.0947 ± 0.6097	3.435	.005[**]
AgI	−0.0002 ± 0.0020	−0.142	.88
ranking*stability	−0.2796 ± 0.1078	−2.594	.02[*]
ranking*AgI	0.0007 ± 0.0004	1.752	.10
stability*AgI	−0.0041 ± 0.0014	−2.772	.01[*]
AgI[c]			
(intercept)	5.6136 ± 0.2618	21.441	< .0001
ranking	−0.0653 ± 0.0714	−0.915	.37
stability[e]	0.3800 ± 0.3338	1.156	.26
ranking*stability	0.0448 ± 0.0895	0.501	.62

Best models for: Productivity AICc = 75.60, GCM AICc = 142.67, AgI AICc = 383.46.
[1] Parameters and interactions not shown were removed during the model selection process.
[a] Explanatory variables included in the full model: STABILITY, RANKING, GCM, STABILITY*RANKING, STABILITY*GCM, RANKING*GCM.
[b] Explanatory variables included in the full model: STABILITY, RANKING, AgI, STABILITY*RANKING, STABILITY*AgI, RANKING*AgI.
[c] Explanatory variables included in the full model: STABILITY, RANKING, STABILITY*RANKING.
[d] Ranking: 1(most dominant), 3(intermediate) and 6(most subordinate).
[e] Stable or unstable: unstable is the reference group.
* P ≤ .05.
** P ≤ .01.

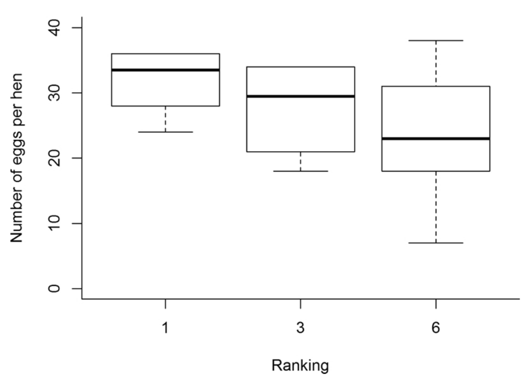

Fig. 2. Number of eggs per hen as a function of ranking. Total number of eggs laid per hen during the study in: (1) top ranking (3) intermediate ranking and (6) low ranking. Boxes represent 1st and 3rd quartile, the thick lines are the medians and the whiskers extend to most extreme data points.

from individuals in three specific hierarchical positions: dominants, intermediates and subordinates.

3.5. Dominants' aggression towards group mates

We found that dominant hens (N = 6) directed their aggressive acts more often to individuals right below them (N = 12) than to most subordinate ones (N = 12) in both treatments (Stable: Z = 1.68, p = .04, median 31 aggressive acts against intermediates, quartiles $Q_1 = 29$ and $Q_3 = 33.50$ versus 17 aggressive acts against subordinates, quartiles $Q_1 = 12.25$ and $Q_3 = 20.75$; Unstable: Z = 1.73, p = .04, median 59 aggressive acts against intermediates, quartiles $Q_1 = 50.5$; $Q_3 = 65.5$ versus 33 aggressive acts against subordinates, quartiles $Q_1 = 25.75$ and $Q_3 = 44.5$).

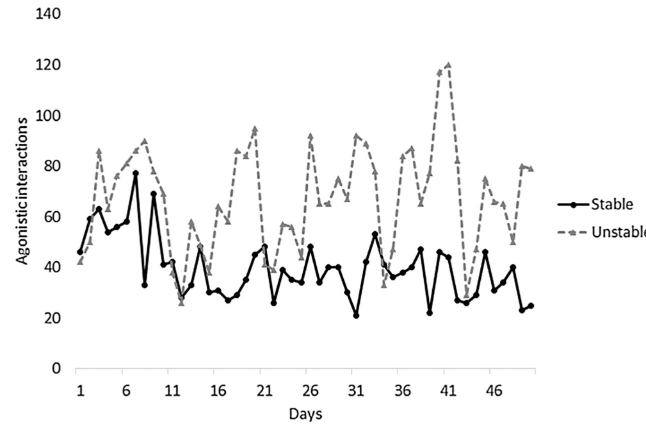

Fig. 3. Mean number of agonistic interactions in ISA Brown hens per day in stable (solid line) and unstable groups (dashed line) during the study.

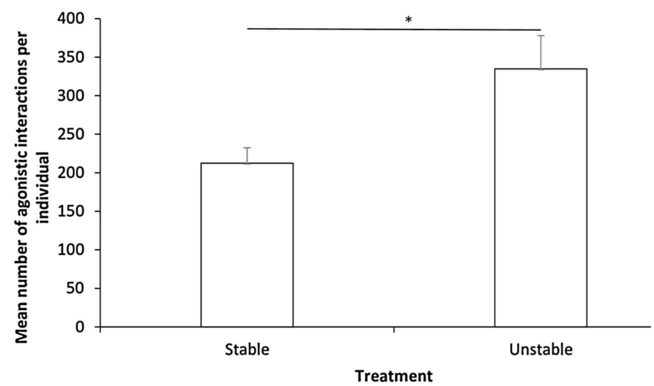

Fig. 4. Mean number of agonistic interactions per individual of ISA Brown hens in stable and unstable groups during the study. Asterisk indicates significant difference between treatments (p < .05).

3.6. GCM concentrations

No variables analysed had an effect on Group GCM.

3.7. GCM concentrations according to social ranking

In both treatments, intermediate hens (rank 3 in the hierarchy) had the highest GCM concentrations (136 ± 24 ng/g in stable groups and 123 ± 38 ng/g in unstable groups, respectively; Fig. 5). An interaction between RANKING and STABILITY showed that considering top and low ranking individuals, GCM concentrations had opposite patterns, depending on the treatment (Table 3). In stable groups, top ranking hens had lower GCM concentrations (116 ± 22 ng/g) than low ranking ones (84 ± 15). In unstable groups, dominant hens had higher

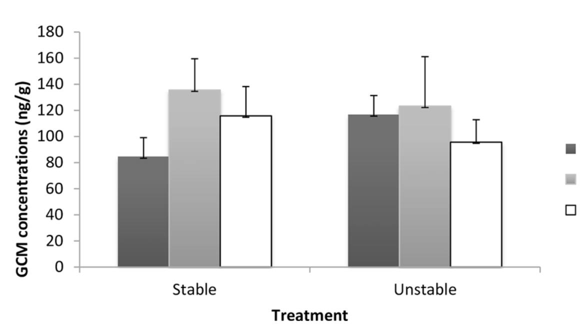

Fig. 5. Concentrations (ng/g dropping) of glucocorticoid metabolites (GCM) as a function of STABILITY (Stable vs. Unstable) and RANKING (1 – top ranking, 3 – intermediate ranking and 6 – low ranking animals) in ISA Brown hens.

concentrations than lower ranking ones (117 ± 15 vs 96 ± 17, Fig. 5). There was also an interaction between STABILITY and AgI; however, the effect was negligible (*estimate* −0.0041). No other measured variables affected GCM concentrations.

4. Discussion

In this study, we experimentally manipulated groups of hens to determine whether social instability negatively affected hen welfare. We investigated whether the substitution of the dominant member in groups of hens was associated with decreased productivity, increased aggression, and increased glucocorticoid levels. We also evaluated whether these effects may depend upon the hierarchical position of individuals. We found several differences between socially stable and unstable groups. Socially unstable groups had lower productivity and higher frequencies of agonistic interactions than stable groups. Social instability also affected GCM: in stable groups, subordinate hens had higher levels than dominant ones; in unstable groups, this pattern was reversed. In both treatments, intermediate hens had the highest GCM concentrations and dominant ones produced more eggs than hens in the other two positions studied.

4.1. Productivity

We recorded, in both groups, low egg production for 34-week-old ISA Brown hens (Hegelund et al., 2006). This might have occurred because our experiment took place in winter, and the hens were under natural regimen of light, which means they had only 11 h of light per day. Usually, laying hens are put under a light programme of about 14–16 h light per day and additional 2 h of artificial light given in the middle of night in hot climates (Leesson and Summers, 1980; Etches, 1994; Freitas et al., 2010; A Hendrix Genetics Company, 2017). Besides, but less probably, birds might have not totally recovered from the transportation stress, or fully habituated (despite the 15 weeks habituation) to the changes in housing system (from battery cages to open housing), drinking and feeding system (from nipples to usual drinkers) and environment (from artificial to natural light and temperature). However, these possible influences were not likely to affect our within- or between-group comparisons, since all birds were submitted to the same environmental conditions during the whole experiment.

Groups where the dominant member was rotated every week produced fewer eggs than the ones whose members were kept the same throughout the study. This result supported our third hypothesis (c). It is also in accordance with the study of Guhl and Allee (1944) in which, in addition to lower productivity, lower food consumption and more weight loss were recorded in unstable groups. Anthony et al. (1988) also found lower body weight after flock disruption. Once instability promotes weight loss through reduced food intake, it makes sense that unstable groups have lower productivity, because egg production is energetically costly (Morris and Taylor, 1967; Bornstein et al., 1984; Perrins, 1996). In order to avoid a possible stressful procedure for the animals, we did not weigh our birds. This could have brought additional data on the effects of instability; future studies might collect such data with the use of automated devices, non-stressful for the animals. Other studies carrying out similar experiments found no change in productivity due to social instability (Feldkamp and Adams, 1973; Adams, 1974; Hester and Wilson, 1986). Adams (1974) performed rotations at longer intervals than ours – rotations every 16 weeks. Studies have shown that after eight weeks, hen groups are already stabilised and have lower frequency and intensity of agonistic interactions (Guhl and Allee, 1944; Guhl, 1968; Cloutier and Newberry, 2002). The groups in the study of Adams (1974) may have not remained unstable throughout the experiment, which may have dissipated the effect of instability on productivity. Other authors (Feldkamp and Adams, 1973; Hester and Wilson, 1986) rotated randomly chosen hens. In hierarchies that are non-linear and, as a consequence, non-steep (i.e., where there is no group member which dominates all other members), even removing the dominant bird, no measurable effect is found in the birds' stress levels (Matos et al., 2016). Therefore, randomly choosing the hen to be rotated may lead to, sometimes, rotating subordinate hens, whose translocation may have had little to no measurable impact on either the group they left or the one they entered.

Finding a non-familiar individual has been shown as an aversive stimulus for hens (Freire et al., 1997). Hens surprised during pre-laying behaviour with non-familiar individuals next to the nest are slower to get into it and make more attempts to find an alternative route to enter the nest box than when they meet familiar individuals. Therefore, besides the decrease in food consumption, the aversive stimulus of daily encounters with strange hens can also reduce productivity over time, through a delay in egg laying every day, postponing the production of the next egg. The relationship between productivity and social stability has also been described in other livestock, such as dairy cows (Raussi et al., 2005; Von Keyserlingk et al., 2008), pigs (Büttner et al., 2015) and horses (taking into consideration body condition – Rubenstein, 1986).

4.2. Agonistic interactions

In line with our second hypothesis (b), socially unstable groups exhibited a higher frequency of agonistic interactions than stable ones. This relationship between instability and aggression has also been observed in other farm animals, such as in Japanese quail (*Coturnix japonica*, François et al., 2000) and pigs (Büttner et al., 2015). In pigs, agonistic interactions increased after mixing groups, but over time (even when holding the mixing of the animals constant) the frequency of aggression dropped, indicating a possible habituation of the animals through a coping strategy to limit energy costs and injuries (Büttner et al., 2015). That was not the case of our study, since aggression remained higher in the unstable groups than in the stable ones. Several studies in laying hens have found increased aggression after the introduction of individuals (Cloutier and Newberry, 2002) and mixing groups (Cordiner and Savory, 2001; Fahey and Cheng, 2008; Ringgenberg et al., 2015). The higher frequency of aggression in unstable groups of hens may be due to the "establishment fights", where individuals initially engage in costly fights to build or maintain their position in a hierarchy, with greater number of agonistic interactions (Pagel and Dawkins, 1997). After hierarchy establishment, individuals may only need to reaffirm their positions with less intense interactions, without the need to fight for resources. In our study, maintaining individuals in stable groups may have promoted better welfare conditions, since individuals could thus be recognised easily without going into "establishment fights" every time they meet each other.

In the study of Guhl and Allee (1944), few weeks after being together, hens were shown to reduce the frequency of aggression, even with high competition for food. In that study, in stable groups, the intensity of agonistic interactions was weaker than in unstable ones, and aggression was sometimes ritualistic – more a threat than actually fights. Although in our study, pecks, considered a more intense interaction (Guhl, 1968), were twice as frequent as threats in both groups, aggression also decreased with time in stable groups. In our unstable groups, aggression was high even when the rotated individual was known from previous weeks. Hens were shown not to distinguish known from unknown conspecifics after two weeks of separation (Chase, 1982); because our top-ranked hens were returned back to their original group after three weeks, their pen-mates might not have recognised or remembered them.

4.3. GCM concentrations

Our first hypothesis (a) was partially supported by our data. Despite our expectations, there was no effect of social instability on the GCM concentrations, when we considered overall group average stress levels.

However, we found a complex interaction between GCM concentrations, hierarchical position, and instability, which indicates a potential effect of instability on the social structure of the group. A detrimental effect of instability on the animals' stress levels has been reported in feral horses (*Equus caballus*, Nuñes et al., 2014) and bearded capuchins (Mendonça-Furtado et al., 2014). No previous study on hens has related hierarchy instability to GCM concentrations, considering hierarchical position. Fahey and Cheng (2008) rotated hens in battery cages every week, and observed higher mortality (due to aggression) and worse feather coverage in birds of unstable groups, although the weight of birds' adrenal glands in both treatments was similar. In their study, GC concentration decreased after social disruption, but droppings collection has not always been conducted using the same individuals. Other studies used different physiological parameters. One study measured heterophil/lymphocyte ratio, and found increased stress in hens submitted to instability (Anthony et al., 1988; Matur et al., 2015); another study measured plasma serotonin concentration, and found no difference between stable and unstable groups (Cheng and Fahey, 2009). We suggest that the reason for such a discrepancy and/or lack of effect of instability in previous studies is the absence of animal rank position as a variable in the analysis. Considering our findings regarding the effect of instability on rank position (see below, in 4.4), this is an important factor to be considered in studies on social animals.

Another factor that might have prevented an overt effect of instability on the group GCM in our study is the enrichment of the enclosures. Heterophil/lymphocyte ratio (Matur et al., 2015) and GC concentrations (Pohle and Cheng, 2009) were found lower in hens living in enriched cages than in conventional cages, with or without social stress. In our study, individuals of both treatments lived in enriched enclosures containing perches, sand, nest boxes and forage. It is possible that if their enclosures were more restrictive, i.e., if the birds were housed in battery cages, social stress could have affected individuals more profoundly. Even though instability did not affect overall GCM levels in experimental groups, data on productivity and agonistic interactions indicate a certain level of distress related to the social situation.

It is not clear at which level of social distress physiological responses are triggered. Vestergaard et al. (1997) studied hens with and without access to sand for dustbathing, a natural behaviour that is considered important for these birds. Although GC levels were the same in both groups, hens without access to sand had higher frequency of stereotypies. In addition, the frequency of stereotypies decreased after the hens received sand, showing that GC concentrations should be interpreted jointly with behavioural data. Several studies have emphasised that measuring only one or two stress parameters is often insufficient to evaluate the well-being of animals (Dawkins, 2003; Scheiber et al., 2015), indicating the need of an integrated analysis to expand our understanding of animal welfare.

4.4. Hierarchical position effects

In accordance with our fourth (d) hypothesis and supporting other studies, dominant birds had greater egg production than subordinate ones (Hill, 1983; Cunningham and von Tienhoven, 1984). Cunningham and von Tienhoven (1984) also observed that dominant hens laid heavier eggs, possibly due to their higher food intake and lower levels of "anxiety" compared to subordinates. Hens that are pecked more often when in the nest (mostly subordinates) spend less time inside it, which often delays oviposition (Lundberg and Keeling, 1999). Dominant hens also exhibit more nest-building movements and less frequent nest-searching activities, as they are interrupted less often than subordinates (Kite, 1985; Freire et al., 1998; Ringgenberg et al., 2015). The nest searching phase takes longer for subordinates, probably because dominants occupy the nests first, and due to a reported increased aggression concomitant with the daily production peak, suggesting competition for laying sites (Hunniford et al., 2014). Additionally, the hierarchical position of an individual, in general, is related to its fitness, as dominance gives priority access to mates and other resources (Höjesjö et al., 2002; Ratcliffe et al., 2007). When analysing our data according to social ranking, we observed that GCM had a small negative effect on egg productivity, meaning that hens with higher GCM concentrations tended to lay fewer eggs. Chronic stress, represented by higher GCM concentrations, can have an inhibitory effect on reproduction, delaying sexual maturity and undermining oogenesis (Blumstein et al., 2016), and thus lowering egg productivity in hens.

Intermediate hens were the most physiologically stressed birds in both treatments (highest GCM concentrations). Several studies have shown higher GC levels in either dominant (Creel et al., 1996; Sands and Creel, 2004; Blumstein et al., 2016) or subordinate individuals (Rohwer and Wingfield, 1981; Mendl et al., 1992; Goymann et al., 2001). However, studies reporting higher physiological stress in individuals that are neither at the top nor at the bottom of the hierarchy are less frequent. In our study, most aggression acts performed by dominants were directed to individuals at intermediate positions, and not to the most subordinate individuals, as one would expect. Forkman and Haskell (2004) found that 95% of aggressions between hens were directed to individuals immediately below them in the hierarchy, and they interpreted this fact based on the "Suppression Hypothesis" (Drummond and Osorno, 1992; Berdoy et al., 1995; Dugatkin, 1997). This hypothesis predicts that the alpha individual attacks the subdominant group member more frequently in order to condition it to lose. This would reduce the likelihood of a reversal in positions, but would force the dominant hen to continually impose herself over individuals who, because of similar conditions, would have more possibilities of outperforming her. Probably, intermediate individuals in our study had higher GCM levels because they lacked the dominance benefits (priority in access to resources), and yet, were continuously harassed by the top-ranking individuals.

Glucocorticoid-metabolite concentrations of dominant and subordinate hens were opposed, and depended on the treatment to which they were exposed. In stable groups, subordinates had higher physiological stress levels; in unstable groups, dominant individuals had higher GCM concentrations, a pattern similar to what was described in wild baboons (Sapolsky, 1992). The lack of control and predictability that dominants have in groups where they have to constantly fight for dominance may be the cause of this stress load. Unpredictability would remove the dominants' control over social stimuli (e.g., unknown group mates), because they would not be able to modulate their behaviour based on previous interactions, and would have to make greater adjustments to cope with the intense social events (Waitt and Buchanan-Smith, 2001). Reversals that occur in unstable groups have more intense consequences to dominants because they have a higher number of individuals below them and have more to lose than subordinates (Sapolsky, 1992). Moreover, dominants engage in more conflicts and, even if they win all, fighting is stressful. Furthermore, maintaining dominance requires constant 'policing' of others' behaviours (more intensively in the case of unstable groups) than the subordination requires (Creel et al., 2013). Several researchers found higher GC levels in alpha individuals, in different taxa, but they have not correlated it to the hierarchy type – stable or unstable (Florida scrub jays [*Aphelocoma coerulescens*] – Schoech et al., 1991; African wild dogs [*Lycaon pictus*] – Creel et al., 1996; yellow bellied marmots [*Marmota flaviventris*] – Blumstein et al., 2016). In stable groups, where reversals are rare, subordinates would have the greatest GC levels, since dominants would be less exposed to psychological stressors (Sapolsky, 1992), and subordinates would have the disadvantages of having limited access to resources, and being attacked more than any individual in another position (i.e., by dominants and intermediates). Additionally, in captivity, subordinates have still other setbacks, as they could not avoid dominants due to space restrictions (Creel, 2001). Thus, the "burden of the subordinate" is more commonly found in species living in stable groups (pigs [*Sus scrofa domesticus*] – Mendl et al., 1992; Harris' sparrow [*Zonotrichia querula*] – Rohwer and Wingfield, 1981; spotted hyenas [*Crocuta crocuta*] – Goymann et al., 2001).

5. Conclusions

In line with most of our predictions, our results indicate that a decrease in group stability in laying hens had a negative effect on the number of eggs laid, and promoted an increase in agonistic interactions. Although there had been no generalised differences in GCM concentrations when we compared group GCM averages between treatments, based on the effects observed in behaviour and productivity, we believe that the welfare of individuals in the unstable groups was compromised. Simple changes in group management can improve living conditions of laying hens, avoiding, for example, introduction of unfamiliar individuals in the enclosures. Our study, the first evaluate the effects of social stress by integrating productivity and behavioural, hierarchical, and physiological parameters using non-invasive techniques in domestic fowl, also highlights the importance of considering different parameters in order to generate a comprehensive evaluation of social processes. Our results may have implications also for the welfare of layers maintained in battery cages, since the environmental restrictions of birds in such conditions are even greater than in our study.

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