Adrenocortical responses in Japanese quail classified by their permanence in proximity to either low or high density of conspecifics

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ABSTRACT The density-related permanence test (DRP) is used to classify young birds (while in groups) according to their individual permanence in proximity to either a high or low density of conspecifics (HD or LD, respectively). The birds' performance in DRP was associated with underlying differences in the social responses of their individuals. Quails in homogeneous groups of LD residents responded with less compact groups and higher levels of agonistic interactions to the presence of an intruder, and showed higher levels of agonistic interactions among cagemates than the homogeneous groups of HD birds. This study deepen the characterization of DRP evaluating whether contrasting behavior during test is associated with birds' stress responses to challenging situations during rearing. Plasma corticosterone responses were individually assessed after submitting DRP categorized birds to a brief (10 min) partial restraint (mechanical stressor; experiment 1), or after introducing them during 5 min as intruders in homogeneous groups of 6 unknown same category (HD or LD) conspecifics (social stressor; experiment 2). Experiment 1 showed that basal corticosterone levels were similar in all groups and the partial mechanical restraint induced an increase in plasma corticosterone concentration also in all groups. However, the increase induced by the stressor was higher in the LD birds than in their HD counterparts. In experiment 2, compared to controls, social stressor showed no plasma corticosterone changes in the intruders that were introduced in an unfamiliar group of HD conspecifics. However, intruders (both HD and LD) that were visiting the LD residents showed an increased corticosterone response compared to their control counterparts. Results suggest that categorization of birds in the DRP test could have relevance for selection programs oriented to obtain birds better suited to intensive rearing conditions that includes high density of animals and exposure to unavoidable stressors.

Key words: social behavior, density-related test, stress response, corticosterone

2018 Poultry Science 0:1–6
http://dx.doi.org/10.3382/ps/pey269

INTRODUCTION

Challenging situations capable of altering the homeostasis of an individual have been defined as stressors. Independently from the nature of the stressor (physical, chemical, social, etc.) all of them induce a highly conserved physiological reaction defined as stress response (de Kloet, 2003; Koolhaas, 2008). This reaction is triggered by the perception of the stressor at the central nervous system (hypothalamus) and culminates in the release of stress response mediators (mainly corticosterone) from the adrenal glands to the blood stream. These mediators are aimed at promoting the physiological and behavioral adjustments required to overcome the homeostasis alteration induced by the threatening situation (Kuenzel and Jurkevich, 2010; Scanes, 2015).

The current poultry husbandry routines often involve situations reported to be stressful. Abnormal social groups conformed by individuals of the same sex and age, dysfunctional social structures, and high stocking density could lead, for example, to deleterious interactions (with conspecifics or with the environment) as registered in different species (Jones, 1996; Guibert, et al., 2010; Nazar, et al., 2017). In this context, suboptimal housing or rearing conditions can facilitate feather pecking or cannibalism (de Haas, et al., 2013). Selection based on a particular trait of interest has been shown to be an effective way to overcome some of the previously stated problems. However, selection for a

Received April 10, 2018.
Accepted May 28, 2018.

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particular attribute has also led to modifications or effects on other variables which are directly or indirectly related to the attribute on focus. For example, selection on low mortality showed promising results toward reducing maladaptive (feather pecking) behavior in laying hens (Bolhuis, et al., 2009; Rodenburg, et al., 2009a,b), while also altered corticosterone and serotonin levels after a restraint test (Rodenburg, et al., 2009a). Lines selected for high or low tonic immobility response also differed in other fear related traits as well as hormonal responses (i.e., corticosterone) (Hazard, et al., 2008).

A challenge that still remains open to welfare ethologists is to help birds to form functionally successful groups over time under the current intensive rearing conditions. Searching for phenotypic variability, we developed a density-related permanence (DRP) test that enables us to classify young birds (while in groups) according to their individual permanence in proximity to either a high or a low density of conspecifics (HD or LD, respectively) (Guzmán et al., 2013). The birds' performance in DRP was associated with underlying differences in the social responses of their individuals. A previous report showed that homogeneous groups of LD-resident birds responded to the presence of an intruder spreading their groups (less compact spatial distribution) and showing higher levels of agonistic interactions among cagemates than the homogeneous groups of HD birds (Guzmán et al., 2013). It was then proposed that the early individual permanence near to a high or a low density of conspecifics in the DRP test could be considered a consequence of their differential capacity to interact with conspecifics. This study deepen the characterization of DRP test evaluating whether contrasting DRP behavior is also associated with birds' stress responses to challenging situations during rearing. Plasma corticosterone responses were assessed after submitting birds individually to a brief (10 min) partial restraint (mechanical stressor; experiment 1), or after introducing during 5 min HD and LD individuals as intruders in homogeneous groups of 6 unknown same category (HD or LD) conspecifics (social stressor; experiment 2).

MATERIALS AND METHODS

Animals and Rearing Conditions

This study was conducted with 1,479 Japanese quail (*Coturnix coturnix japonica*) taken from a larger population of animals obtained from 7 incubation series (batches). Housing conditions were similar to those used elsewere (Pellegrini, et al., 2015; Caliva, et al., 2017). Immediately after hatching, chicks were leg-banded for individual identification and housed in mixed-sex groups of 70 to 75 birds, in white wooden home boxes (90 × 90 × 60 cm, length × width × high, respectively). A wire-mesh floor (1 cm grid) was raised 5 cm from the base to allow the passage of excreta. Each box had a feeder covering the entire front of the box, 16 automatic nipple drinkers, and a lid to prevent birds from escaping and heat loss. Each box was also provided with a heating system that allowed maintaining brooding temperature at 37.5°C during the first week of life, with a weekly decline of 3.0°C. Quail were subjected to a daily cycle of 14 h light (300 to 320 lx):10 h darkness during the study. Lights were turned on at 08:00 h and turned off at 22:00 h. Leg bands were replaced with permanent wing bands at 21 d of age. A quail starter diet (28% CP; 2,800 Kcal ME/kg) and water were provided ad libitum throughout the study.

Experimental Details and Procedures

Density Related Permanence Test (DPR) Classification of quail in the density-related permanence (DRP) test was conducted when they were 11 d of age. A full description of the test system including a figure representation of the apparatus is presented elsewhere (Guzmán et al., 2013). Briefly, the classification apparatus consisted of 2 boxes interconnected by a central region delimited by 2 sliding doors. Each box contained at its distal end either 12 or 3 conspecifics confined behind a glass. At 11 d of age and every 1 h, the sliding doors were closed and experimental birds (36) were identified and released back in the central region of the device. According to where they were found (box containing high density, low density, or in the central region) each bird received a 1, –1, or a 0 score, respectively. The procedure was repeated 9 times and the scores summed. Birds with final values of ≥3 or ≤–3 were respectively categorized as HD or LD. Birds with intermediate scores were categorized as showing no preference (**NP**). After the classification process, all birds (except those used as stimulus birds) were housed back in their home boxes. Stimulus birds were not further used in the study.

Blood Collection Blood (approximately 1 mL) was withdrawn via jugular vein puncture. All samples were taken within 2 min of handling, a time recommended by Romero and Reed (2005) to assess reliable baseline corticosterone concentrations. After refrigerate centrifugation (1,000 *g* for 10 min at 4°C), plasma was removed and 0.2 mL aliquots were frozen at −20°C until assayed.

Corticosterone Quantification Corticosterone concentration (ng/mL) in plasma samples was quantified using the commercially available ^{125}I corticosterone-radioimmunoassay kit (**RIA** kit—MP Biomedicals, Costa Mesa, CA) developed for mice but successfully used to quantified corticosterone concentrations in birds species, included Japanese quail (Washburn, et al., 2002; Jones, et al., 2005; Lèche, et al., 2013). RIA was used following the procedure described in Lèche et al. (2009). Standard validation procedures and quality controls were used including tests of parallelism, accuracy (recovery of added corticosterone), and precision (O'Fegan, 2000). Parallel displacement curves were obtained by comparing serial dilutions (1:2 up to 1:200) of quail plasma pool with kit standard calibrators ($r^2 = 0.96$). Plasma samples were run at a dilution of 1:2 or 1:4 in the assay buffer provided with the kit. Recovery of known amounts of corticosterone added to a diluted pool of plasma was 92.3% ± 5.9% ($y = 12.79 + 0.84x$, $r^2 = 0.98$). The manufacturer's reported cross reactivity for corticosterone antisera was 100% and <1% for other steroid (desoxycorticosterone: 0.34%, testosterone: 0.10%, cortisol: 0.05%, aldosterone: 0.03%, progesterone: 0.02%, and less than 0.01% for all other steroids tested). Intra- and interassay coefficients of variation were 2.7% (n = 7) and 6.8% (n = 5), respectively.

Experiment 1: Adrenocortical Stress Response to a Mechanical Restraint in HD, LD, and NP Japanese Quails A total of 180 birds were categorized in the DPR test (31 HD, 44 LD, and 105 NP) as described above. At 31 d of age, 165 birds were identified and rehoused in 18 mixed category and mixed-sex groups of 9 to 10 birds (losses were due to natural mortality or deterioration of leg/wing bands). Quails were randomly selected but ensuring that each box contained representatives of all categories (HD, LD, and NP birds) and sexes. Boxes measured 115 × 43 × 44 cm (length × width × height, respectively), and contained a bell drinker, a feeding trough, and 2 8 W fluorescent lamps (placed at 40 cm high in each side of the box). Lamps provided between 300 and 320 lx illumination at ground level.

After a week of habituation, plasma corticosterone responses to a partial mechanical restraint (which also included a social isolation as stressor) were evaluated in all animals. Half of the birds per box were captured, taken to an adjacent experimental room and immediately blood sampled (control group) as previously described. The other half was also captured, taken to an adjacent experimental room and individually partially restrained by placing them in a black cloth bag (18 × 16 cm; long and width, respectively) for 10 min (stressed group). The bag prevents most gross movements, whereas the head, leg, and wing can still move. After this time, blood samples were collected. Within the same day, the procedure was repeated until birds from all boxes were sampled. Experimenters were careful to avoid disturbing animals while manipulating them. Plasma corticosterone concentrations (ng/mL) were determined as mentioned above.

Experiment 2: Adrenocortical Stress Response of HD and LD Birds After Including Them as Intruders in Groups of Unknown Same Category (HD or LD) Conspecifics This experiment was part of a larger study evaluating social behaviors in groups of birds of the same category (HD or LD). It was initiated with 1,224 birds taken from a larger population of animals obtained from 6 incubation series (batches). A total of 208 birds were categorized as HD, 220 birds as LD, and the rest were considered NP. As mentioned, after finishing the categorization process all birds were housed back in their home boxes. At 31 d of age, birds were rehoused in 8 white wooden home boxes (115 × 43 × 44 cm, length × width × height, respectively). Birds to be tested as "residents" were housed in same category (HD or LD) groups of 6 animals (3 females and 3 males, each). Birds to be tested as "intruders" were housed in identical boxes than their resident counterparts to avoid the novelty effect when tested the week after (see below). Because HD and LD quails differed in their social cohesion and/or agonistic behaviors (Guzman, et al., 2013), intruders were maintained in mixed-category-sex groups in a higher density (140 cm^2/bird) to minimize aggressions within boxes (Estevez et al., 2003) and therefore to avoid conditioning them to live in a particular social situation (either with less or high level of aggressions) that could further affect their behavioral responses when tested in the resident-intruder test. Quails from each batch were housed in one of 8 identical home boxes. One box was used to keep the group of intruders and the remaining 7 boxes were used to keep the groups of residents. To balance the treatments, 4 groups of HD residents and 3 groups of LD residents or 3 groups of HD residents and 4 groups of LD residents were housed alternately between each of the 6 batches.

At 38 d of age (after a week of habituation), 2 HD and 2 LD birds from each batch were randomly captured from the intruder box, taken to an adjacent experimental room, and immediately blood sampled (control group) for basal corticosterone determinations. Another 3 to 4 HD and LD birds from the intruder box were tested as intruders by placing them individually, and only once, during 5 min in either HD or LD resident home boxes. Then they were immediately blood sampled (stressed group). In only one opportunity, we could not sample an HD bird within the 2-min timeframe. Thus, corticosterone was determined in a total of 24 (12 HD and 12 LD) control birds and 41 (20 HD and 21 LD) intruder birds.

Statistical Analysis

In experiment 1, corticosterone concentration values were analyzed with ANOVAs that evaluated the effects of the category of the birds (HD, LD, and NP), stress treatment (control and stressed), and sex (male and female) as well as their interactions. Because no sex effect was found, data were reanalyzed without considering that factor. In experiment 2, ANOVA also evaluated the effects of the category of the intruder (HD and LD) into resident home box, the effects of the category of the residents in the home box (homogenous groups of HD or LD birds), and the sex effect (male and female) as well as their interactions. Because no sex effect was found, data were reanalyzed without considering that factor. In all cases test assumptions were verified. Whenever specific effects reached singificance ($P < 0.05$), Tukey tests were performed for post hoc analysis. Values were expressed as the mean ± standard error.

Table 1. Effects of partial mechanical immobilization on plasma corticosterone (ng/mL) concentration in Japanese quail categorized by their permanences in proximity to either a high or a low density of confined conspecifics (HD or LD, respectively) and in birds that showed no preferences (NP).

	Stress treatment		*P*-value		
Bird category	Control	Stress	Category $F_{2,159} = 3.16$	Stress $F_{1,159} = 46.71$	Interaction $F_{2,159} = 3.09$
HD[1]	$2.75^{a} \pm 1.12$	$6.76^{b} \pm 1.12$			
NP	$3.61^{a} \pm 0.58$	$7.25^{b,c} \pm 0.56$	0.04	< 0.01	0.05
LD	$3.37^{a} \pm 0.90$	$10.77^{c} \pm 0.93$			

[1]Values are expressed as means ± standard error.
[a–c]Different letters represents differences between groups ($P < 0.05$; Tukey test).

RESULTS

Experiment 1: Adrenocortical Stress Response to a Partial Mechanical Restraint in HD, LD, and NP Japanese Quails

Analysis showed a main interaction effect between stress treatment and bird category on plasma corticosterone concentrations (Table 1). Post hoc analysis showed that basal corticosterone levels were similar in all groups and partial mechanical restraint induced an increase in plasma corticosterone concentration also in all groups. However, the increase induced by the stressor was higher in the LD birds than in their HD counterparts with the NP-stressed group showing intermediate values.

Experiment 2: Adrenocortical Stress Response of HD and LD Birds After Including Them as Intruders In Groups of Same Category (HD or LD) Unfamiliar Conspecifics

Analysis showed a main effect ($F_{2,54} = 4.93$; $P = 0.01$) of the category of residents in the home box on the plasma corticosterone response of the intruders that were visiting them (Figure 1). No direct effects of the category of the intruders ($F_{1,54} = 0.03$; $P = 0.86$) and no interaction ($F_{2,54} = 0.33$; $P = 0.72$) between the category of the intruder and the residents were found. Specifically, compared to controls, no plasma corticosterone changes were found in the intruders that were introduced in an unfamiliar group of HD conspecifics. However, intruders (both HD and LD) that were visiting the LD residents showed an increased ($P < 0.05$) corticosterone response compared to their control counterparts.

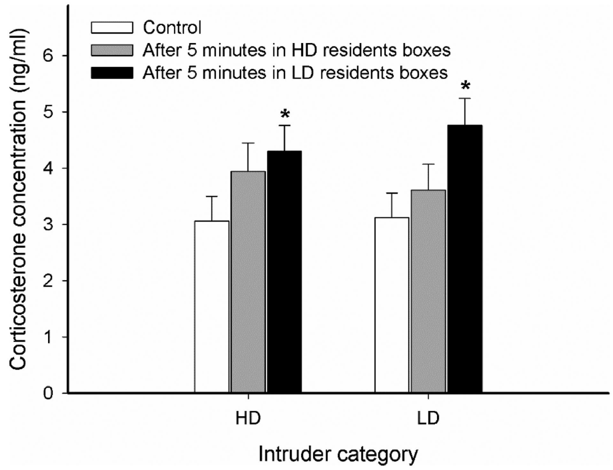

Figure 1. Plasma corticosterone concentrations (ng/mL) in Japanese quail categorized by their permanences in proximity to either a high or a low density of confined conspecifics (HD or LD, respectively) that were included into homogeneous groups of HD or LD unknown conspecifics. Values are expressed as means ± standard error. *$P < 0.05$ compared to control counterparts (Tukey test).

DISCUSSION

It is well recognized that the exposition to stressors (including brief mechanical restrain and social challenges) induces the elevation of corticosterone concentrations in poultry species, including *Coturnix coturnix japonica* (Faure, 1981; Dantzer and Mormede, 1983; Jones, et al., 1988, 1994; Beuving, et al., 1989; Jones, 1996; Jones and Satterlee, 1996; Hemsworth and Coleman, 1998). Therefore, in our study, the overall elevation of corticosterone concentrations showed in stressed groups was not unexpected. It is also recognized that the birds' performance in behavioral test can help to discriminate between animals showing different characteristics including divergent adrenocortical responses to stressors (Jones and Hocking, 1999; Marin and Jones, 1999; Hazard, et al., 2008). We found no differences in the plasma corticosterone levels of undisturbed (control) quails classified in the DPR test. However, the plasma corticosterone release induced by a restraint stressor triggered a much stronger (≥50%) response in the birds classified as LD than in their HD and NP counterparts. Therefore, the performance of young quail during the DPR test appears also related with later adrenocortical responses to an unknown stressor. We might tentatively suggest that the stronger responses shown in the LD quail either represent an exacerbated perception of the threatening properties of the restraint stressor or that they were not well suited to cope with such challenge. As mentioned, the releases of mediators (i.e., corticosterone) after a stressor exposure are aimed at promoting the physiological and behavioral adjustments required to overcome the threatening situation (Wingfield, et al., 1998; Kuenzel and Jurkevich, 2010; Scanes, 2016). Once the perturbation disappears, corticosterone levels return back to a baseline range. However, if the stressor does not disappear, the animal does not habituate to it, or it has an exacerbated response, the prolonged increased corticosterone levels could lead to a state of homeostatic overload, generating deleterious effects in the individual (Romero, et al., 2009). Although in our study we did not determine corticosterone responses after chronic stress exposure in a long-term study, it is conceivable that the exacerbated responses shown in LD quail would lead to difficulties in the homeostasis recovery after the stressor ended, at least in the short term.

Adrenocortical response and fearfulness have also been previously positively correlated in a variety of species including Japanese quail (Faure, 1981; Dantzer and Mormede, 1983; Jones, et al., 1988, 1994; Beuving, et al., 1989; Jones, 1996; Jones and Satterlee, 1996; Hemsworth and Coleman, 1998). For example, quails with elevated stress responses to restrain consistently show elevated fear responses when compared with birds with lower responses to restrain (Jones, 1996; Jones and Satterlee, 1996). Birds with artificially elevated corticosterone also showed exacerbated fear responses when exposed to alarm stimuli (Gross, et al., 1980; Jones, et al., 1988). Interestingly, HD quail walked more, faster, and a greater distance in an open-field test, and required more inductions to develop shorter tonic immobility responses than their LD quail counterparts (Guzman and Marin, 2013). Because both stressful and frightening situations are many times unavoidable along productive processes (Jones, 1996; Jones, et al., 2005), the mentioned findings strengthens the idea that birds classified as HD could be better prepare to deal with these situations that their LD counterparts.

The placing of intruder birds into unfamiliar groups is considered a stressor because it combines the effects of the exposure to a novel environment and forced social interactions with unfamiliar conspecifics (Jones, 1996; Guzmán et al., 2013). In our study, both LD and HD intruders responded similarly to that situation increasing their plasma corticosterone concentration only when they were included into groups of unfamiliar LD conspecifics but not when included into groups of HD birds. A previous report (Guzman, et al., 2013) showed that the groups of same category LD birds performed more aggressive pecks (about an order of magnitude greater) than the groups of same category HD birds, suggesting that LD birds were more aggressives than HD birds (e.g., they require or perform more aggressive pecking during social interactions). It is known that both adult quail and chicken in small groups increased aggressive pecking interaction as a consequence of regrouping (possibly tending to establish hierarchy ranges) (Wood-Gush, 1971; Edens, et al., 1983; Zayan, 1987; Bradshaw, 1992; Odén, et al., 2000). Thus, the literature on this subject and the set of results mentioned above suggest that the differential aggressiveness (social stressor component) between HD and LD quail is underlying the induced corticosterone responses shown by the intruders.

During intensive rearing conditions, birds are housed in high stocking densities ($\geq$8 birds/m^2). There is a wide consensus that birds' inappropriate levels of sociality, when housed in groups, can exert undesirable effects on all aspects of social interactions, including affiliation, aggression, dispersal, as well as their ability to cope with social disruption, such as isolation, exposure to strangers, or crowding (Vallortigara, 1992; Jones, 1996; Jones, et al., 1996, 2002; Jones and Mills, 1999). Furthermore, a mismatch between a bird's underlying sociality and its social environment could elicit either a series of acute stress responses or chronic social distress with associated negative effects on performance (Mills, et al., 1993; Jones and Hocking, 1999; Jones and Mills, 1999). Thus, low-sociality birds might be poorly suited for housing in very large or in very confined groups. Considering that the birds that remained in close proximity to a high density of conspecifics (HD) compared to LD birds—a) performed behaviors that are considered adequate for group living (i.e., reduced level of aggressiveness while in groups; Guzman, et al., 2013), b) showed a reduced fear responses both in open-field and tonic immobility tests (Guzman and Marin, 2013), c) showed reduced corticosterone stress responses when restrained in isolation (current study), and d) induced lowered corticosterone responses in the intruders that were included in their groups (current study)—our finding suggests that categorization of birds in the DRP test could have relevance for selection programs oriented to obtain birds better suited to current intensive rearing conditions that includes high density of birds and exposure to stressors.

ACKNOWLEDGMENTS

The study complies with applicable Argentinian laws, with the local Argentinian Association for Science and Technology Laboratory Animals (AACyTAL Bulletins number 15 and 16, 2001), and meets the Institutional Animal Care and Use Committee guidelines. The authors wish to thank Biol. Julia Ortiz for the animal care assistance and Dr. Juan Busso for discussions and help during an early stage of this study. This work was supported by grants from Secretaría de Ciencia y Técnica of Universidad Nacional de Córdoba (SECyT-UNC), Agencia Nacional de Promoción Científica y Tecnológica (FONCYT-PICT-2016–1337), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. DAG, FNN, and RHM are Career Members from the later institution.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

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