Level of stress in relation to emotional reactivity of hens

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ABSTRACT

The aim of the study was to associate behavioural reactions of laying hens with the level of stress and to answer the question whether it is possible to select reactions that will facilitate phenotypic assessment of the level of stress in these birds. The investigations were carried out in 50 Green-legged Partridge, 50 Polbar and 50 Leghorn birds. A modified open-field test (MOFT) and a tonic immobility test were applied. Next, the quality of feathers was evaluated and the level of corticosterone in the feathers was determined. The corticosterone level was assessed in relation to the genetic group and feather quality and in relation to the probability of the presence/absence of specific physiological–behavioural reactions. The analysis of the results revealed no correlations between the corticosterone level in the feathers and the quality of the feathers. In turn, there was a correlation between the hen breed and the corticosterone level in the feathers: a significant difference was found between Zk and Pb hens. It was demonstrated that the corticosterone level was significantly higher in birds that exhibited such behaviours as preening in the open-field test. To sum up the results, it can be concluded that such behaviours as feather preening and latency until tonic immobility occurs can be indicators of stress in laying hens.

HIGHLIGHTS

- There was a correlation between the breed and the corticosterone level in the feathers: there was a significant difference between the Green-legged Partridge (3.97 ± 0.17) and Polbar (4.50 ± 0.19) hens.
- No significant correlations were found between the corticosterone level and the behavioural reactivity in the analysed hens.
- It was demonstrated that the corticosterone level was significantly higher in birds that exhibited self-grooming behaviours in the open-field test (4.61) than in hens that did not show such behaviours (3.91).

Introduction

Farm breeding has so far been targeted at improvement of performance traits in laying hens with no consideration of their well-being and stress level. In recent years, the increasing consumers’ awareness has contributed to appearance of a positive trend in animal production towards reduction of stress levels (Bergmann et al. 2017).

Stress plays a very important role in animal biology. It determines animal health and fitness and facilitates adaptation to changing environmental conditions. The hypothalamic–pituitary–adrenal axis (HPA) of vertebrates responds to environmental disturbances by releasing glucocorticoids to the circulation, which trigger a number of physiological and behavioural changes, i.e. stress responses (Koch et al. 2002; Blas et al. 2007).

Physiological and behavioural changes allow the organism to cope with environmental challenges. Yet, despite the adaptive value of short-term increases in the glucocorticoid content, a constantly elevated level of stress hormones exerts a negative impact on cognitive abilities, growth, fitness and general health status in animals (Sapolsky et al. 2000; Kitaysky et al. 2003). Researchers have proposed various definitions of stress in hens; however, it is known that any changes in the animal environment evoke emotions but concurrently not all changes must exert negative effects disturbing the homeostasis of the organism (de Haas et al. 2013). Many changes, e.g. those associated with...
enrichment of the environment, may bring a very positive effect (Bergmann et al. 2017). Various investigations indicate considerable individual variability associated with the effect of stress on the organism and the strategy of coping with an adverse impact of environmental factors (Blas et al. 2007; Romero 2004; Campbell et al. 2016). An important element in animal husbandry is the ability to recognise individuals experiencing stress and having difficulty in maintenance of the homeostasis of the organism, as they can be specific bioindicators of a disturbed environment and welfare. Many indicators have been developed to recognise elevated levels of stress in companion animals. In contrast, there are not many standards for assessment of the stress level in a rapid and easy way in livestock animals and in poultry in particular. An objective and reliable indicator of animal stress is the level of corticosterone in blood, saliva, faeces, hair or feathers (Creel 2001; Blas et al. 2007; Cabezas et al. 2007; Weimer et al. 2018), but the measurement of this parameter is not a simple and fast assessment tool. Another indicator is the general fitness or quality of feathers (Dixon et al. 2008). However, these indicators are assessed subjectively, whereas unambiguous standards should be based on indicators that can be assessed in an objective, quick and simple way. Such indicators should also be simple to measure to be used for selection of birds targeted at increasing the threshold of susceptibility to stress in hens.

Therefore, a question arises whether the stress level can be predicted based on behavioural responses exhibited by hens in specific situations. Such an indicator might facilitate the control of bird welfare and selection targeted at reduction of susceptibility to stress in farmed hens. Hence, the aim of the study was to associate behavioural reactions of laying hens with the level of stress and to answer the question whether it is possible to identify reactions that will facilitate phenotypic assessment of the level of stress in these birds.

**Materials and method**

All procedures used during the research were approved by the II Local Ethics Committee for Animal Testing at the University of Life Sciences in Lublin, Poland (Approval No. 69/2017 of 28 September 2017).

The research was carried out in a group of 150 laying hens, i.e. 50 Green-legged Partridge (Zk), 50 Polbar (Pb) and 50 Leghorn (Lg) birds. All birds were 30 weeks old at the time of the experiment. The hens were chosen randomly from the flock and kept in boxes for 2 months before the start of the experiments. The birds were kept in one pen divided into 6 compartments, with 25 hens of one breed in each at a density of 0.3 m²/individual. All compartments were equipped with nipple drinkers, feeders, nests, straw mulch and 16-hour illumination. The birds were fed and maintained in conditions compliant with applicable veterinary and husbandry standards (Act of 21 August 1997 on animal protection (Journal of Laws 1997 no. 111 item 724). Two behavioural tests were employed: a modified open-field test (OFT) and a tonic immobility test (TI). The tests were carried out between 8:00 and 15:00 for 6 days. Each day, 25 birds, i.e. all from one compartment, were assessed. All birds were evaluated once. The experiments were carried out by the same person. The experimental room was located in the same farm building where the hens were kept, but was separated. The birds were transferred to the experimental room directly from their compartments.

The modified open-field test (OFT) was carried out as in Rodenburg et al. (2003) and Kozak et al. (2019). To this end, a special observation box with a 1.25 × 1.25 m floor divided into 25 squares with an area of 25 × 25 cm each was constructed. A camera viewing the entire area available to the birds was mounted above the box and each bird was recorded separately during a 600-s test. The test was modified by placing additional environment-enriching elements, i.e. a container with water (W), a container with commercial feed (P), a container with feed supplemented with cereal grains, finely cut straw, and insect larvae (EF), a sandpit (SA), a mirror (M) and a shelter resembling hen nests (S). The birds were carried separately and placed in the centre of the experimental box to keep the same distance from the enriching objects. The recording started 1 minute after placing the bird in the box to allow the experimenter to leave the place (Table 1).

Each of the activities denoted by W7 was recorded separately to analyse each reaction as a separate phenomenon in further analyses.

The tonic immobility (TI) test was carried out immediately after the MOFT test, following the procedure proposed by Jones (1986). A special cradle was used in this test as in the experiments conducted by Forkman et al. (2007). Immobilisation was achieved by placing the bird on the back and pressing the sternum gently for approximately 10 s so that the head was hanging backwards freely. The following parameters were measured:
1. Time between restraining the bird and tonic immobility (TI1) measured in seconds (s)

2. Duration of tonic immobility (TI2): time between the onset of tonic immobility and the first head movement and/or righting measured in seconds (s) (Table 2)

Thirty of the 150 birds did not undertake exploration of the environment, i.e. 9-Zk, 11-PB and 10-L. The minimal value 0 for indicators W3–W6 was noted in the case of these 30 birds only.

Immediately after the tonic immobility test, the birds were weighed and the quality of the birds’ feathers was assessed on a 5-point scale, i.e. 1 – feathers without damage; 2 – less than 10 damaged feathers; 3 – more than 10 damaged feathers; 4 – bald patches, massive feather loss; and 5 – most of the body surface devoid of feathers. The quality of feathers was regarded as an indicator of disturbed welfare (de Haas et al. 2013). The birds were examined for the presence of external parasites but these were not found. The body weight of the birds was Lg = 1609 (±143), Pb = 1573 (±178) and Zk = 1574 (±147). This trait was excluded from further analyses, as it had no statistically significant effect on the other parameters.

Afterwards, the corticosterone level was determined in the feathers according to the method modified by Bortolotti et al. (2008). Briefly, the second quill of the first row from the left wing of each bird was taken for the study. The feathers were washed in isopropyl alcohol, dried for 24 h in free air, and weighed. Vanes were cut into pieces, placed in 2 mL Eppendorf tubes, and 1.5 mL of methanol was added to each tube. The samples were ground with steel balls in a homogenizer (Tissuelyser II, Quagen). The supernatant was filtered on a syringe filter (φ0.2 μm Millipore millex–GN) and the level of corticosterone was determined with the immunoenzymatic method using the Chicken CORT (Corticosterone) ELISA Kit (Fine Test) according to the manufacturer’s procedure.

### Statistical analyses

The level of corticosterone was determined in relation to the genetic group of the birds and the quality of feathers. The level of corticosterone in the groups was presented as the mean least squares (lsq) together with standard errors (se). The significance of differences between the groups was verified using the GLM multivariate analysis of variance. The model included the fixed effect of the genetic group of the hens and the quality of the feathers.

The corticosterone level in relation to the probability of the presence/absence of the specific physiological–behavioural reactions was determined. It was presented as the least square mean (lsq) with standard errors (se). The significance of differences between

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### Table 1. Indicators assessed during recording analysis.

| Indicator | Measurement | Units |
|-----------|-------------|-------|
| W1 | Time between placing the bird on the floor in the experimental box and the first movement, e.g. head movements, looking around, but no locomotor activity | Measured in seconds (s), 600 points were assigned when the bird did not undertake any activity throughout the test (600 s) |
| W2 | Time between placing the bird on the floor in the experimental box and the beginning of locomotor activity | Measured in seconds (s), 600 points were assigned when the bird did not undertake exploration throughout the test (600 s) |
| W3 | Interest in the enrichment elements: water container, commercial feed container, enriched feed container, shelter, mirror; bird’s interest was manifested by approaching the object and exploration thereof with the beak, looking at the object standing next to it, or entering the object | Minimum value – 0 if no exploration of the environment was undertaken; the maximum value depended on the number of squares crossed by the hen |
| W4 | Number of squares crossed by the bird | Measured in seconds (s) |
| W5 | Time spent by the bird on exploration and showing interest in the enrichment elements | Measured in seconds (s) |
| W6 | Time spent by the bird on locomotion throughout the test, excluding the time of exploration of the objects | Point scale 1–0, where 1 denoted approach and interest in the enrichment elements, and 0 – no interest; max 6 points |
| W7 | Physiological–behavioural activities, i.e. defaecation, shaking, preening, vocalization | |

### Table 2. Results of behavioural tests.

| Indicator | N | Mean | SE | Median | Minimum | Maximum |
|-----------|---|------|----|--------|---------|---------|
| W1        | 150 | 73.6 | 161.5 | 9.0 | 1 | 600 |
| W2        | 150 | 281.6 | 236.7 | 169.0 | 1 | 600 |
| W3        | 150 | 1.9 | 1.6 | 2.0 | 0 | 6 |
| W4        | 150 | 12 | 20 | 5.0 | 0 | 112 |
| W5        | 150 | 161.2 | 160.9 | 124.0 | 0 | 561 |
| W6        | 150 | 217.0 | 145.6 | 212.0 | 0 | 544 |
| W7        | 150 | 1.5 | 0.8 | 1.8 | 0 | 4 |
| TI1       | 150 | 44.9 | 49.4 | 34.0 | 0 | 322 |
| TI2       | 150 | 25.4 | 46.2 | 3.5 | 0 | 278 |

The indicators are defined in Table 1.
the groups (presence/absence of reaction) was verified using the GLM multivariate analysis of variance. The model included the fixed effect of the genetic group and the fixed effect of the reaction types (defaecation, vocalisation, shaking and preening).

The relationships between the level of corticosterone and the hens’ behavioural reactions in the MOFT and TI tests were assessed using Spearman’s rank correlations.

All analyses were carried out using statistical software SAS Institute (Cary, NC).

Results

The investigation results indicate that the corticosterone level in the feathers was not related to their quality.

There was a significant effect of the genetic group of the hens on the corticosterone level in the feathers (Table 3). The lowest level of the hormone was detected in the Green-legged Partridge hens (3.97 ± 0.17); it differed significantly from the corticosterone level in the Pb breed (4.50 ± 0.19).

No significant correlations were found between the corticosterone level and the assessed indicators, except for the latency until tonic immobility occurred (Table 4). There was a significant correlation between this indicator and the level of the stress hormone. The correlation was low (0.21), yet positive and statistically significant.

In the study, the level of corticosterone was analysed in relation to the presence or absence of selected physiological and behavioural responses (Table 5).

Table 5 shows the mean corticosterone level in two groups of birds: hens exhibiting the reactions assessed in the test and those that did not exhibit such reactions.

The level of the stress hormone was shown to be significantly higher in birds that exhibited grooming behaviours, e.g. preening, during the MOFT test. No differences were found between birds that exhibited and those that did not exhibit such behaviours as defaecation, shaking or vocalisation.

Discussion

It was found in the present study that the Zk hens were characterised by the lowest corticosterone level in the feathers, which was significantly different from the level detected in the Pb breed. It should be emphasised that both breeds (Zk and Pb) are reared in closed, unselected populations in accordance with the phenotypic and usability assumptions developed to protect the animal genetic resources. Differences in the corticosterone level are associated with other factors than the potential selection trend or maintenance system. They may be related to the fact that Green-legged Partridge hens are the most primitive breed of all the studied birds. The breed is often reared on organic farms due to its excellent adaptation to extensive farming conditions. Therefore, these birds can cope with various environmental challenges. As a rule, primitive animal breeds are clearly more resilient to stress and adverse effects of environmental factors (Blas et al. 2007; Cabezas et al. 2007), as confirmed by the present results.

Feather pecking is a frequent hens’ reaction to unfavourable changes in their environment (Dixon et al. 2008). Therefore, it might seem that farmed hens that are more frustrated and stressed by environmental constraints have poorer quality plumage. Yet, the analysis of the present study results showed no correlations between the plumage quality and the level of corticosterone. It should be underlined that the
experimental hens exhibited a number of grooming behaviours, e.g. preening, flapping the wings, or shaking. Grooming behaviours in animals help to maintain hygiene but may also represent displacement activity aimed at reducing agitation or providing relaxation (Spruitt et al. 1992). This was confirmed by the present results showing the highest corticosterone level in birds that exhibited preening behaviour in the open-field test (Table 5). In this case, such reactions may serve as a displacement behaviour reducing the level of stress. This is confirmed by the fact that the frequency of grooming behaviours was found to increase with the increasing frequency of conflict situations (Manning and Dawkins 1992). An analogous situation has been reported in mammals (Herskin et al. 2004).

It is also believed that grooming in various animal species is more common in a sterile environment (Berthelsen and Hansen 1999; Hansen and Berthelsen 2000). The increase in the corticosterone level in individuals exhibiting grooming behaviours such as preening is an important finding of this study. This type of behaviour is usually regarded to be a manifestation of hens’ welfare or, possibly, an element that enables the birds to regain homeostasis. The results of this present study confirm that this specific behaviour (preening) allows birds to relieve emotional tension, as it was observed in hens characterised by elevated corticosterone levels. Hence, this type of behaviour can be the first indicator of disturbed homeostasis, especially in comparison with the grooming behaviours observed in natural conditions, i.e. mainly dust bathing and shaking off the dust can be observed in free-range birds. Other types of grooming behaviour are observed less frequently. In the present experiments, the birds exhibited intensive preening. The behaviour referred to as shaking was not associated with the level of corticosterone in the feathers (Table 5). Frequently, intensive grooming behaviour can be evoked by the presence of external parasites, which disturb the homeostasis of the organism and increase susceptibility to stress. The behaviour observed in such a case can be associated with the corticosterone level (the presence of parasites induces severe stress) but does not represent displacement behaviour. However, the birds examined in the experiment did not have external parasites.

An important element in the present investigations was the assessment of the correlations between the stress level and the indicators of hens’ behaviour in the MOFT test (W1–W7). The relationships between the corticosterone level in the feathers and the analysed indicators were expected. The level of stress experienced by the bird in a specific situation can potentially be evaluated based on behavioural observations, i.e. by measurement of the distance that can be covered while approaching a bird before it escapes, the intensity of locomotion in the experimental box, or the intensity and quantity of the sounds produced by the bird (Stankowich and Blumstein 2005; Forkman et al. 2007; Zimmerman et al. 2011; de Haas et al. 2012). However, no correlation between the behaviour of hens and the corticosterone level was found in the present investigations. The absence of these relationships can be explained. It has been shown that animal reactions are indirect indicators of emotions but do not indicate the level of stress experienced by animals (Cockrem 2007). The behavioural indicators assessed in the present study can only indirectly reveal emotions experienced by the hens. Upon negative emotional stimulation, animals would not be able to explore the objects systematically for a long time but would rather move fast and chaotically (Forkman et al. 2007). Birds experiencing negative emotions would not show interest in the surrounding objects (Hocking et al. 2001; Rodenburg et al. 2003; de Haas et al. 2013). The birds’ behaviour in MOFT can be linked to negative or positive emotions (Kozak et al. 2019), but the level of stress cannot be assessed based on the hens’ reactions observed in the test. The behavioural response to a specific stimulus may be analogous in a situation of stress and in a situation when the HPA axis is not activated (Cockrem and Silverin 2002). Behavioural reactions may indicate emotions experienced by the animal, but they are typically not a clear indicator of the level of stress experienced by the individual (Cockrem 2007).

The reactions of the birds in the MOFT test may be related to the level of corticosterone in birds’ blood or faeces, as the indicator in this case is associated with the rate of hormone output in the animal organism in response to a stress stimulus (Blas et al. 2007). Yet, the level of corticosterone assessed in this way will indicate hens’ emotional reactivity rather than the level of stress. Investigations of the relationships between animals’ behaviour and the stress level should be carried out on material in which the hormone level does not depend on factors that affect the organism temporarily (Bortolotti et al. 2008). Corticosterone determined in feathers is an indicator of life-long stress experienced by the bird, in contrast to determinations in other biological materials, e.g. blood, saliva and faeces, in which the hormone level
is associated with strong but temporary emotional stimulation (Weimer et al. 2018).

The present results emphasise the significant correlation between latency until tonic immobility occurred (TI1) and the corticosterone level. The correlation coefficient was only slightly higher than 0.2; yet, it indicates that birds with a higher level of stress may need a longer time before the occurrence of tonic immobility. Concurrently, previous studies showed that tonic immobility was more difficult to achieve by excitable birds with increased emotional reactivity (Kozak et al. 2019). This reaction may also be used as an indirect indicator the stress level in hens.

Conclusions

To sum up, it can be concluded that such behaviours as preening can be indicators of stress in hens. This type of behaviour can be regarded as displacement behaviours associated with mitigation of emotions experienced by birds and simultaneously indicate an elevated corticosterone level. Latency until tonic immobility occurrence can serve as another indicator of stress in hens. The higher value of this parameter was associated with elevated corticosterone levels.

Disclosure statement

No potential conflict of interest was reported by the authors.

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