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INFLUENCE OF AGE, SEX AND REARING SYSTEMS ON SERUM CORTICOSTERONE AND HETEROPHIL TO LYMPHOCYTE RATIO AS A STRESS PROFILE IN PEARL GUINEA FOWL

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Abstract– The present study was designed to evaluate the effect of age, sex and rearing system on serum corticosterone and hetyerophil to lymphocyte ratio in pearl guinea fowl. Twenty four Pearl Guinea fowls each were placed in cage and on floor and the birds were maintained under standard managemental conditions. Once every four weeks from the fourth week onwards blood was collected from these birds up to 16 weeks of age. Two milliliters of blood was collected from all 48 birds using a 3ml syringe. Drop of blood was used for smear preparation and staining with Modified Leishman- Giemsa stain for the differential count to obtain heterophil to lymphocyte ratio and the remaining blood was kept undisturbed for two hours for separation of serum for performing ELISA test for serum corticosterone estimation. The heterophil to lymphocyte ratio was significantly (P<0.01) affected by age and rearing system of guinea fowl. Older birds of 12 and 16 weeks of age had a higher heterophil lymphocyte ratio of 1.4 ± 0.16 and 1.38 ± 0.19 respectively. Caged reared birds had a significantly (P<0.01) higher heterophil to lymphocyte ratio. All the interactions except rearing system × sex × age were highly significant (P<0.01). Other than rearing system, neither age nor sex had influenced the serum corticosterone level in pearl guinea fowl. Birds reared on floor had higher corticosterone levels of 1.12 ± 0.03 ng/ml than those raised in cages (1.00 ± 0.15) ng/ml and this was highly significant (P<0.01). The result of the present study revealed that a higher heterophil to lymphocyte ratio identified in older birds (12-16 week) to be more stressed than the younger (4-8week) guinea fowls. Guinea fowls reared on floor had higher serum corticosterone levels than those reared on cages while heterophil to lymphocyte ratio was higher in cage reared birds.

INTRODUCTION

Guinea fowl derived its name from place of origin the 'Guinea' on the western coast of Africa. The name 'Guinea' came into use in the 1400s. Possibly it has been derived from the 'Berber' word meaning land of blacks. According to Zeuner (1963) guinea fowls were present in Greek and Roman barnyards as early as about 500 BC and were called Numidian birds. Hence scientific name of the family. France, Italy, U.K. and Russia are the pioneers in raising guinea fowl as a commercial venture. India has a large guinea fowl population. Although organized commercial venture is non existent in India, yet flocks of 150-200 on droving is a common sight in guinea fowl producing areas like Punjab, Haryana, Andhra Pradesh and few places in Tamilnadu. Guinea fowl is nicknamed as 'day wathchman' as it makes noise on seeing intruders in the premise and they predate on a few rodents. Guinea fowl rearing is an excellent source of agricultural prosperity, hitherto overlooked by the poultry breeders in developing countries. Earlier, throughout the world it was considered as an enterprise profitable only for small farmers. Recent developments have established that they are reputable meat producing birds of definite commercial value. They have a flocking instinct and are of gregarious in nature when raised on grazing and can serve as low input sustainable agriculture for landless labourers, small and marginal farmers and so raising guinea fowl will be advantageous under range condition for the

rural people. Intensive guinea fowl production has provided poultry farmers an opportunity to diversify their business and to consumers an extended variety of poultry meat.

In India, guinea fowl raising was only a sporadic rural occupation of weaker sections in many states (Harpreet Singh and Panda, 1984). It has made spectacular progress in the last two decades as guinea fowls are considerably less susceptible to heat stress and highly resistant to dietary aflatoxins. These two characteristics alone make them immensely suitable to the rural indigenous conditions prevailing in India. Despite their immense suitability for commercial farming not much effort have been taken to study guinea fowl production on scientific lines.

Native guinea fowl appear to be well adapted to the environmental stress conditions under semi intensive and extensive husbandry systems. More evidence on scientific line is needed to substantiate these facts (Harpreet Singh and Panda, 1984). In recent years this alternative poultry species witnessed increasing emphasis for low input grain saving aviculture (LISA) (Muthukumar and Dev Roy, 2004).

MATERIALS AND METHODS

Broad outline of work

Twenty seven Pearl Guinea fowls each were placed in cage and on floor and the birds were maintained under standard managemental conditions. They were provided feed ad libitum and plenty of clean, potable water. Once every four weeks from the fourth week onwards blood was collected from these birds up to 16 weeks of age. In cages birds were provided 0.25 ft^2/ keet floor space upto 4 weeks of age and 0.5 ft^2/ keet upto 8 weeks of age and the space was later increased to 0.9 ft^2/keet. On floor the keets were provided with a floor space of 0.5 ft^2/per bird upto 8 weeks of age and later increased to 1.5 ft^2/ birds upto 16 weeks of age. Brooding continued upto fourth week of age. All the birds were provided brooder mash of 20.5 per cent CP with 3000 Kcal of ME / Kg with the same nutrient composition and at 9^{th} week of age grower mash of 17.5 per cent CP with 3000 Kcal of ME/Kg was provided. Every once in four weeks the body weights of the birds in cages and on floor was recorded. Blood collection was done during the morning hours to avoid diurnal variation and to lower stress. Effect of age, sex and rearing system on the haematological and biochemical parameters were studied.

Collection of blood

Two milliliters of blood was collected from all 48 birds using a 3ml syringe and was kept undisturbed for two hours for separation of serum. The extremity of the wing was pricked using a lancet for blood smear preparation. A drop of blood placed on a clean grease free glass slide was gently slid over using another glass slide to prepare the smears and were allowed to air and shade dry and fixed in methanol for two minutes to be used later for staining.

Differential count (DC)

The granulocytes in birds include heterophils, basophils and eosinophils while the agranulocytes are the lymphocytes and monocytes.

Preparation of 100 ml Modified Leishman-Giemsa stain and staining technique

Leishman's powder of 150 mg and 30 mg of Giemsa powder were dissolved in 100 ml of acetone free methanol and solution was stirred and mixed thoroughly for about one hour using a mortar and pestle. The solution was later filtered thrice in Whatman filter paper No.1.

A thin blood smear was made from whole blood. The smear was dried and later stained with Modified Leishman-Giemsa ((Bancroft and Marilyn, 2008) and held for two minutes. Distilled water was later added slowly over the stain until the water did not overflow and allowed to stand for 50 minutes. As per the method the time mentioned was only 30 minutes, but it was observed that the guinea fowl blood smear showed good staining result when it was stained and distilled water was allowed to stand for 50 minutes. The smear on the glass slide was washed, air dried and examined under oil immersion lens of the microscope. Moving the slide vertically and horizontally, a total of 100 leucocytes were counted using blood cell counter.

Heterophil to lymphocyte ratio

The value of heterophil lymphocyte ratio was obtained by dividing the heterophil value by lymphocyte value.

Corticosterone estimation by ELISA Test

Effect of rearing system, age and sex on the serum corticosterone was done by performing ELISA test

using commercial available corticosterone ELISA kit manufactured by Neogen R Corporation.

Extraction of Corticosterone

100 µl of serum was taken in a glass tube with 1 ml of ethyl ether. The tube was vortexed for 30 seconds to allow the phases to separate. The organic phase was transferred into a clean glass tube and the solvent was evaporated with a stream of nitrogen. The residue was dissolved in 100 µl of of diluted extraction buffer. The extract was diluted 100 fold by adding 10 µl of the above extract into 990 µl of diluted extraction buffer. The tube was vortexed and 50 µl in duplicates was assayed. The value obtained was multiplied by 100 to give final ng/ml concentration.

ELISA test procedure

1. Preparation of standards as follows:

Standard	Preparation
A	1 µg/ml of stock solution
B	20 µl of standard A is taken and added to 980 µl of EIA buffer and mixed (final concentration = 20ng /ml)
C	200 µl of standard B is taken and added to 1.8 ml of EIA buffer (final concentration = 2 ng /ml)
D	200 µl of standard C is taken and added to 1.8 ml of EIA buffer (final concentration = 0.2 ng /ml)

Preparation of standards was continued following scheme 1

Scheme 1

The number of wells to be used was determined. The corticosterone enzyme conjugate was diluted by adding 1µl of enzyme conjugate into 50 µl total volume of EIA buffer for each well to be assayed. For the whole plate, 110 µl of the enzyme conjugate was added into 5.5 ml total volume of EIA buffer. The solution was mixed thoroughly. 50 µl of standards (S) or unknown (U) was added into the appropriate wells in duplicate. 50 µl of the diluted enzyme conjugate was added to each well. The plate was mixed by shaking gently. The plate was covered with plastic film and was incubated at room temperature for one hour. The concentrated wash buffer was diluted with deionized water by adding 20 ml of wash buffer to 180 ml of deionized water and it was mixed thoroughly. After incubation the contents of the plate was dumped out and tapped thoroughly on to a clean lint free towel. Each well was washed with 300 µl of diluted wash buffer. Washing was repeated for a total of three washings. 150 µl of substrate was added to each well and plate was mixed by shaking gently. The plate was incubated at room temperature for 30 minutes. The plate was shaken gently before taking a reading to ensure uniform colour throughout each well. Plate was read in a microplate reader at 650 nm. For accounting of substrate background, 2 to 8 wells were used as blanks using only substrate in the wells (150 µl/well). The average of these absorbance values were subtracted from the absorbance values of the wells being assayed. 50-100 µl of Neogen's Red Stop Solution was added to each well to stop enzyme reaction. The plate was read at 650 nm. The standard curve was plotted and the concentration of the samples were estimated from the curve.

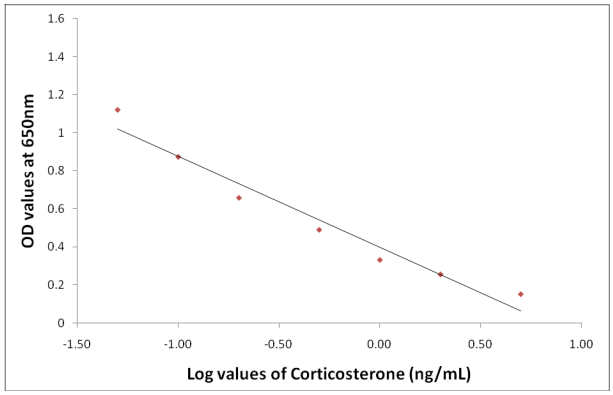


Fig. Standard curve for serum corticosterone in pearl guinea fowl

Standards	ng/ml	EIA buffer µl	B standards µl	C standards µl	D standard µl
S0	0	1000	-	-	-
S1	0.05	750	-	-	250
S2	0.1	500	-	-	500
S3	0.2	-	-	-	1000
S4	0.5	750	-	250	-
S5	1	500	-	500	-
S6	2	-	-	1000	-
S7	5	750	250	-	-

Statiscal Analysis

The experimental data was analysed statistically using factorial design (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Effect of age, sex and rearing system on heterophil to lymphocyte ratio in pearl guinea fowl

The effect of age, sex and rearing system on heterophil to lymphocyte ratio is presented in Table 1. Graphical representation on the effect of rearing system × age and rearing system × sex are presented in Figure 1 and 2. The heterophil to lymphocyte ratio was significantly (P<0.01) affected by age and rearing system of guinea fowl. Older birds of 12 and 16 weeks of age had a higher heterophil lymphocyte ratio of 1.4 ± 0.16 and 1.38 ± 0.19 respectively. Caged reared birds had a significantly (P<0.01) higher heterophil to lymphocyte ratio. All the interactions except rearing system × sex × age were highly significant (P<0.01).

Studies have noted that heterophil to lymphocyte ratio is effected by stressors and it could be used as an indicators of stress in birds. Age had a significant effect on heterophil to lymphocyte ratio on guinea fowl as age advanced there was an increased in heterophil to lymphocyte ratio. No literature could be traced on this parameters in guinea fowl. In chicken Singh *et al.* (2009) partially agreed with the above finding. Patterson and Siegel (1998) and Moneva *et al.* (2009) working on chicken and Schmidt *et al.* (2009) working on turkeys disagreed with above finding present. In chicken it has been observed that birds after 20 weeks of age are less stressed in cages due to adaptation to the cage environment (Singh *et al.*, 2009). This study was only upto 16 weeks of age and may be the birds were yet to adapt to a stressed life in cages. This is observed in Figure 1.

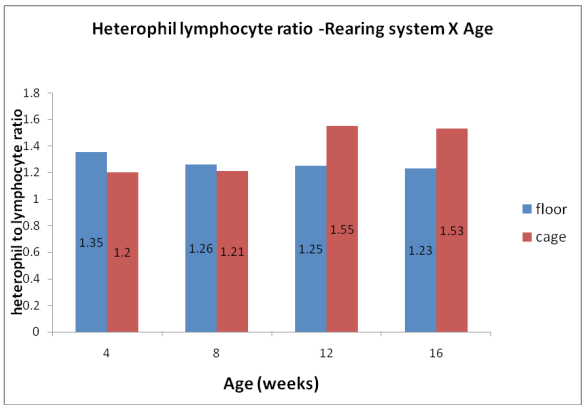


Fig. 1. Interaction of rearing system and age in heterophil to lyphocyte ratio in pearl guinea fowl.

Sex had no significant influenced on heterophil to lymphocyte ratio in guinea fowl. This agreed with the finding of Simaraks *et al.* (2004) who had studied the effect of sex on heterophil to lymphocyte ratio on chicken and Schmidt *et al.* (2009) working on turkeys. Females seemed to be more stressed in cages when compared to males. Most probably they are less adaptive to long term stress. This could be attributed to the hormone prolactin. This is observed in Fig. 2.

Guinea fowl reared in cages had significantly higher (P<0.01) heterophil to lymphocyte ratio of (1.37 ± 0.11) as compared to those on floor (1.27 ± 0.09). Work done on chicken by Campo *et al.* (2008), Shini (2003) and Onbasilar and Aksoy (2005) observed significant changes in the heterophil to lymphocyte ratio as influenced by rearing system. Moe *et al.* (2010), Fouad *et al.* (2008), Tactacan *et al.*

Table 1. Mean (± SE) Effect of age, sex and rearing system on heterophil to lymphocyte ratio in pearl guinea fowl

Age(weeks)	Rearing system				Mean for Age**
	Floor		Cage		
	Male	Female	Male	Female	
4	1.62 ± 0.09	1.06 ± 0.04	1.10 ± 0.03	1.28 ± 0.05	1.27^{de} ± 0.12
8	1.60 ± 0.06	0.91 ± 0.04	1.13 ± 0.03	1.29 ± 0.05	1.23^{e} ± 0.14
12	1.36 ± 0.08	1.13 ± 0.04	1.23 ± 0.02	1.85 ± 0.05	1.39^{c} ± 0.16
16	1.46 ± 0.07	0.99 ± 0.05	1.18 ± 0.02	1.87 ± 0.05	1.38^{c} ± 0.19
Mean for Rearing system**	1.27^{b} ± 0.09	1.37^{a} ± 0.11			
Mean for Sex^{NS}					
Male	1.34 ± 0.07				
Female	1.30 ± 0.13				

**- Highly Significant (P<0.01), NS- Not significant (P>0.05). Mean values sharing any one common superscript in a row or column for age, rearing system and sex do not differ significantly

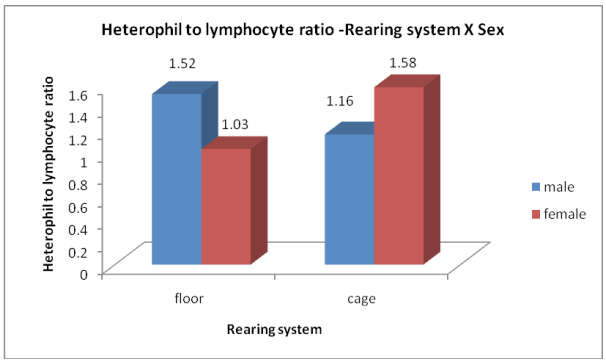


Fig. 2. Interaction of rearing system and sex in heterophil to lymphocyte ratio in pearl guinea fowl.

(2009) and Salamano *et al.* (2010) in chicken and Erisir *et al.* (2009) in ducks disagreed with above finding while Singh *et al*.(2009) observed high heterophil to lymphocyte ratio in cages upto 22 weeks, later birds on floor had higher heterophil to lymphocyte ratio . Guinea fowls are known to enjoy space. Very often it is felt that guinea fowls have still not changed their feral behaviour. Therefore having excess to more space on floor provided the birds with more comfort as compared to the birds in cages who may have found the cage environment more stressful. A lack of stress also improved immunity as a result of early adaptation in floor reared guinea fowl. It has been observed that depriving hen of litter leads to stress and increased heterophil to lymphocyte ratio (El Lethy *et al.* 2003). Heterophil to lymphocyte ratio are commonly used to indicate long term stress (Maxwell and Robertson, 1998). As studied by Onbasilar and Aksoy (2005) space may have been the threshold for a response in the heterophil to lymphocyte ratio in cages versus floor.

Effect of age, sex and rearing system on serum corticosterone in pearl guinea fowl

The effect of age, sex and rearing system on serum corticosterone levels in pearl guinea fowl is presented in Table 2. Graphical representation on the effect of rearing system × sex is presented in Fig. 3. Other than rearing system, neither age nor sex had influenced the serum corticosterone level in pearl guinea fowl. Birds reared on floor had higher corticosterone levels of 1.12 ± 0.03 ng/ml than those raised in cages (1.00 ± 0.15) ng/ml and this was highly significant (P<0.01). Highly significant (P<0.01) result was observed in rearing system × sex interaction where female reared on floor had a very high corticosterone level of 1.16 ± 0.06ng/ml while female reared in cages had the lowest corticosterone level of 0.93 ± 0.07 ng/ml.

No significant changes in the serum corticosterone level in guinea fowl was found at various ages. This agreed with Turkyilmaz (2008) and Tactacan *et al.* (2009) who found no significant difference due to age on serum corticosterone level in chicken. However, Pohle and Cheng (2009) working on chicken observed significantly higher levels of corticosterone in older birds. The study was taken up between a wider age group between 30 to 60 weeks of age where birds could have experienced stress for a longer period.

No literature on the effect of age, sex and rearing system on guinea fowl could be traced. Sex did not have any significant effect on serum corticosterone level in guinea fowl. Huff *et al.* (2007) studying the effect of transportation and stress on turkey observed high corticosterone level in male. Males in cages and floor (Fig. 3) related to interaction of rearing system and sex seemed to be equally affected by the stress of handling with lower floor space. While females showed higher serum corticosterone levels on floor than in cages. The immediate stress of catching and handling affected female guinea fowls more than space restriction.

Table 2. Mean (± SE) Effect of age, sex and rearing system on corticosterone (ng/ml) in pearl guinea fowl

Age(weeks)	Rearing system				Mean for Age^{NS}
	Floor		Cage		
	Male	Female	Male	Female	
12	1.08 ± 0.04	1.22± 0.01	1.08 ± 0.03	1.00 ± 0.03	1.09 ± 0.05
16	1.09 ± 0.03	1.11± 0.03	1.09 ± 0.03	0.86 ± 0.12	1.04 ± 0.06
Mean for Rearing system**	1.12^{a} ± 0.03		1.01^{b} ± 0.05		
Mean for Sex^{NS}					
Male	1.09 ± 0.03				
Female	1.06 ± 0.08				

**- Highly Significant (P<0.01), NS- Not significant (P>0.05). Mean values sharing any one common superscript in a row for age, rearing system and sex do not differ significantly

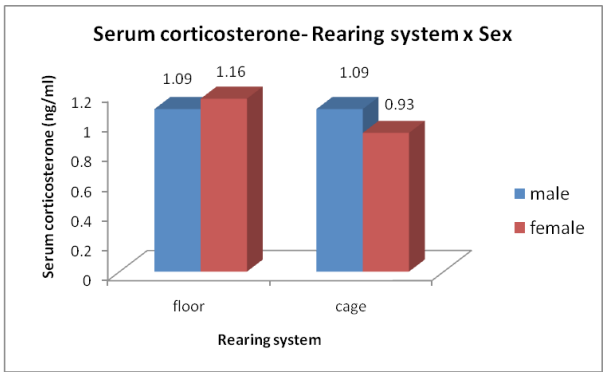
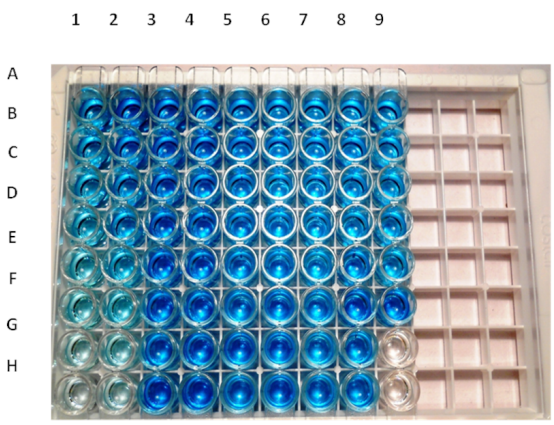


Fig. 3. Interaction of rearing system and sex in serum corticosterone level in pearl guinea fowl.

Plate – ELISA for estimation of Serum Corticosterone in Pearl Guinea fowl



A1, A2 to H1, H2 - Standards in duplicates with concentrations in ascending order
A3 to F9 - Test sera samples
G9, H9 - Blank

Rearing system had a significant (P<0.01) effect on serum corticosterone level on guinea fowl. No literature on this criterion with regard to guinea fowl could be obtained. However in chicken, Koelkebeck and Cain (1984) and Mench *et al*. (1986) agreed with the above finding. Contrary to the above finding Tactacan *et al*. (2009) and Erisir *et al*. (2009) observed non significant difference in the serum corticosterone level of chicken and ducks reared under different rearing system. In the present study birds reared on floor had higher level of corticosterone (1.12 ± 0.03 ng/ml) when compared to those on cages (1.01 ± 0.05 ng/ml). This could have resulted from catching itself or handling birds which could have stressed the birds on floor. Birds deal with stress at physiological level by adrenocortical activation (Acute stress response) followed by period of adaptation. Corticisterone also known as stress hormone is the major glucocorticoid in birds. Handling of birds can increase the glucocorticoid production (Chloupek *et al*., 2009) which is mediated by release of adrenocorticotropic hormone from the pituitary gland. Corticosterone estimation is more on short term stress and disturbing the birds on floor, catching them and collection of blood sample would have increased glucocorticoid production thus increasing serum corticosterone level in floor reared guinea fowl. Females on floor seemed to be more stressed and this could be related to the hormone prolactin that augments corticosterone level and this is further enhanced by extracellular calcium which was higher in floor reared guinea fowls. It appears that significant quantities of ACTH are released from activated lymphocytes which were high in female guinea fowl.

A higher heterophil to lymphocyte ratio identified older birds (12-16 week) to be more stressed than the younger (4-8 week) guinea fowls.

Guinea fowls reared on floor had higher serum corticosterone levels than those reared on cages while heterophil to lymphocyte ratio was higher in cage reared birds

Conflicts of interest: There is no conflict of interest between the authors which is related to the works submitted for publication.

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