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Original Research Article

INFLUENCE OF STOCKING DENSITY ON GROWTH PERFORMANCE TRAITS, BLOOD CHEMISTRY AND THE EXPRESSION OF *HSP*70 AND *IGF*-I GENES IN NEW ZEALAND WHITE RABBITS

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Abstract: This research was done to mainfest the effect of stocking density on growth performance traits, blood chemistry and the expression of heat shock protein (HSP) 70 and insulin-like growth factor I (*IGF*-I) genes in growing New Zealand White rabbits. A total of 75 rabbits at weaning (35 days of age) were randomly assigned into three stocking densities of 12, 20 and 28 rabbits/m^2 from weaning until 13 weeks of age. Rabbits housed at 28 rabbits/m^2 had the lowest feed intake, live body weight and body weight gain, but they had the highest feed to gain ratio when compared with the lower densities. Moreover, rabbits housed at 28 rabbits/m^2 showed the highest serum creatinine, glucose and cortisol levels compared with rabbits housed at 20 and 12 rabbits/m^2. High stocking density up-regulated the expression of *HSP*70 gene when compared with the lower densities. Meanwhile, *IGF*-I mRNA expression was significantly reduced in the rabbits housed at 28 rabbits/m^2. In conclusion, high stocking densities (28 rabbit/m^2) had negative impacts on growth performance traits and the stress-related parameters (serum glucose, cortisol and creatinine). Moreover, the expression of *HSP*70 gene was increased, with a remarkable reduction in *IGF*-I gene expression in the high stocking group.

Key words: rabbits; growth performance; *HSP*70; *IGF*-I; biochemical parameters

Introduction

Domestic rabbits (oryctolaguscuniculus) are considered a non-conventional livestock species that appear to be a cheap and a common source of high quality animal protein in developing countries. This may be attributed to the established prolificacy, rapid growth rate, high feed conversion ratio, early maturity, small housing area and high genetic selection potential of rabbits (1). The rabbit's meat is of high quality protein with lower calories, fat and cholesterol levels when compared with other sources of meat (2).

One of the most important ways to increase kit survivability and growth performance in the rabbit farms is to create a conductive environmental condition, among which is the appropriate stocking rate (3). The optimal cage space should allow each growing rabbit to stretch along one side of the cage and sit up straight at all age intervals (4). Stocking density in rabbits affects growth and litter size at weaning. Feed intake and daily gain of growing rabbits were higher in less crowded housing (5).

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The European Food and Safety Authority (6) recommended a minimum space of 625 cm^2/rabbit; not more than 40 kg/m^2 at the end of fattening in order to avoid disturbance of rabbit behavior. Growth performance of growing rabbits housed at density lower than 16 rabbits/m^2 was not affected (7,8).

Heat shock proteins (HSP) are considered as molecular chaperone proteins, which have the ability to interact with other proteins reversibly and to promote the folding, the formation and the transmembrane transport of these proteins (9). When an animal is exposed to harmful environmental condition (stress), the metabolism tends to synthesize more HSP, which help the cell to recognize the damaged protein for consequent repair. In addition, HSP has an essential role in the conservation of cellular life as they inhibit apoptosis (10).

Insulin-like growth factor-I is manufactured by almost all tissues and has a vital role in cell growth, differentiation and transformation (11). I*GF*-I gene expression in liver is affected by physiological (12) and nutritional status (13). To our knowledge, no previous scientific researchs that investigated the influence of stocking density on *HSP*70 and *IGF*-I genes` expressions in rabbits. Thus, the present work was aimed to manifest the effect of stocking density on growth performance, some biochemical parameters and expression of *HSP*70 and *IGF*-I genes of New Zealand White (NZW) rabbits.

Material and methods

The present study was carried out at a rabbit farm of Animal Wealth Development Department, Zagazig University, Sharkia-Egypt. This study was carried out during the period from the first of April to the end of May 2017.

Animals, housing and management

Seventy five male NZW rabbits weaned at 35 days of age with an average weight of 545±3.4 g were randomly assigned to three stocking densities (12, 20 and 28 rabbits/m^2). The allowed cage space was adjusted to provide the required stocking density with equal group size (five rabbits per cage) and each stocking density was replicated 5 times. Rabbits were ear-tagged, and housed in wire cages equipped with feeder and automatic nipple drinker. About 14 hours photoperiod was maintained, the housing temperature was 26±2°C and the relative humidity was 68±4 % throughout the experimental period. All rabbits were fed ad libitum a commercial pelleted diet, which consisted of 18% crude protein, 2.5% crude fat, 2651 kcal/kg digestible energy and 12.65% crude fiber from weaning until 13 weeks of age. The experimental rabbits were exposed to the same environmental condition and managed according to the rabbit's management standards.

Growth performance

Biweekly, the live body weight (LBW) was recorded individually for each rabbit at 5, 7, 9, 11 and 13 weeks of age, as well as the feed consumption. The feed conversion ratio and the body weight gain (BWG) were calculated at different week intervals from 5 to 13 weeks of age. The experimental unit was individual cage to calculate the average feed consumption and feed conversion ratio.

Blood sampling

At the end of the experimental period (13 weeks of age), five rabbits from each group were randomly taken (one rabbit from each replicate) and blood samples were collected in plain tubes to obtain serum. Separation of serum was done by centrifugation at 3000 rpm for 15 min, then kept at -20 °C. The total serum protein, albumin, triglycerides, cholesterol, glucose and creatinine were estimated using a commercial diagnostic kit (Elitech Clinical System SAS- Zone Industrielle- 61500 SEES, France); globulin level was estimated by subtracting the obtained albumin level from the obtained total protein level (14). The concentration of serum cortisol was detected (Cortisol ELISA Kit, Cayman Chemical).

Gene expression

RNeasy Mini kit (Qiagen, Germany) was used for RNA extraction following the manufacturer's protocol, 25 mg of thigh muscle were taken from the slaughtered rabbits and homogenized. Quantitect Reverse Transcript-

ion kit (Qiagen, Heidel-berg, Germany) was used for reverse transcription of the first-strand cDNA from total RNA following the manufacturer's protocol. The Real-Time PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using Quantitect SYBR Green PCR kits (Qiagen, Heidel-berg, Germany). The forward and reverse primers (15,16) for each gene are summarized in Table (1). The Q-PCR mixture contained of 12.5 ml 2x SYBR Green PCR Master Mix, 1µl of each primer (10 Pmol/ml), 2 µl cDNA and 8.5 µl RNase-free water in a total volume of 25 µl. The cycle counts and amplification conditions were as follows: initial activation at 95 C˚ for 15 min and then 40 cycles of denaturation at 94 C˚ for 15 sec, annealing at 60 C˚ for 15 sec and elongation at 72 C˚ for 15 sec.

Table 1: Oligonucleotide primer sequences used for semi-quantitative reverse transcription polymerase chain reaction

Gene	Primer	Sequence (5′-3′)	Reference
***IGF*-I**	forward	AGGAGGCTGGAGATGTACTG	(15)
	Reverse	AAATGTACTTCCTTCTGAGTCT	
***HSP*70**	forward	GAGGTCACCTTCGACATCGA	(16)
	Reverse	CTTGCCCGTGCTCTTGTC	
GAPDH	forward	ATTGCCCTCAATGACCACTTTG	(15)
	Reverse	TCTTACTCCTTGGAGGCCATGT	

IGF-I = Insulin-like growth factor I; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; HSP = Heat shock protein

Fold changes in the target genes` expressions were calculated by comparing $2^{-\Delta\Delta CT}$ (CT: cycle threshold) method (17) with the GAPDH housekeeping gene. The difference between mean ΔCT of the treatment group and the control group is ΔΔCT, where the difference between the mean ct gene of the interest and the internal control gene in each sample is ΔCT then statistical analysis was applied.

Statistical analysis

One-way analysis of variance (ANOVA) procedure of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc, Chicago, IL, USA) was used for analysis of the obtained data. The multiple comparisons of means were done by using the Duncan's Multiple Range Tests (DMRT). Results were documented as means ± standard errors (SE), the value of $P < 0.05$ was used to indicate statistical significance. The statistical model that used as the following:

$$Yij = \mu + Si + eij$$

Where: Yij, The animal observation; µ, Overall population mean; S_i, Fixed effect of i^{th} stocking density and e_{ij}, Residual error.

Results

Growth performance traits

Rabbits housed at 28 rabbits/m^2 had the lowest LBW at 9^{th}, 11^{th} and 13^{th} week of age and the lowest BWG at 7-9, 11-13 and 5-13 week intervals compared with those housed at either 12 or 20 rabbits/m^2 (Table 2). The feed consumption of rabbits housed at 28 rabbits/m^2 was significantly lower at all age intervals when compared with the rabbits housed at 12 rabbits/m^2 (Table 2). In general, rabbits housed at 28 rabbits/m^2 had the highest feed conversion ratios at all age intervals studied compared with rabbits housed at 12 rabbits/m^2 and 20 rabbits/m^2 except for 5-7 weeks interval of age, but only significant results was obtained at 7-9 and 11-13 weeks interval of age.

Table 2: Growth performance traits of New Zealand White rabbits as affected by cage stocking density

Variable	Stocking density (rabbits/m^2)			*P*-value
	12	20	28	
Live body weight (g)				
5th week	714.00±23.75	651.00±22.54	656.37±12.33	0.082
7th week	1062.40±29.54	1036.20±29.83	1009.57±24.39	0.455
9th week	1475.07±29.41^a	1463.40±30.70^a	1365.37±29.17^b	0.022
11th week	1832.47±29.01^a	1753.40±31.20^{ab}	1658.46±31.93^b	0.001
13th week	2210.87±32.51^a	2014.68±36.29^b	1866.46±30.83^c	0.003
Body weight gain (g/d)				
5-7 weeks	24.24±0.86	27.51±2.02	25.21±1.33	0.399
7-9 weeks	28.81±1.38^a	30.51±1.09^a	25.43±0.93^b	0.002
9-11 weeks	24.84±1.71	20.71±0.95	20.93±1.54	0.182
11-13 weeks	27.03 ±1.24^a	18.52±1.08^b	14.86±0.72^c	0.001
5-13 weeks	26.73±0.69^a	25.14±0.70^a	21.61±0.44^b	0.001
Feed consumption (g/d)				
5-7 weeks	64.73 ±0.65^a	63.84 ±0.63^{ab}	62.43 ±0.31^b	0.036
7-9 weeks	86.26 ±0.915^a	73.72 ±0.91^b	72.79 ±0.51^b	0.001
9-11 weeks	90.09 ±1.23^a	75.15 ±0.98^b	73.83 ±1.06^b	0.001
11-13 weeks	89.33 ±0.91^a	89.43 ±0.59^a	81.67 ±0.37^b	0.001
5-13 weeks	82.60 ±0.44^a	75.54 ±0.54^b	72.68 ±0.38^c	0.001
Feed conversion ratio				
5-7 weeks	2.69±0.12	2.70±0.24	2.81±0.21	0.904
7-9 weeks	3.03±0.17^a	2.49±0.09^b	3.00±0.12^a	0.004
9-11 weeks	3.74±0.24	3.90±0.26	3.99±0.20	0.787
11-13 weeks	3.40±0.15^b	5.27±0.34^a	5.67±0.16^a	0.001
5-13 weeks	3.12±0.08	3.18±0.11	3.44±0.09	0.053

Means within the same row having different superscript letters are significantly different at (P<0.05)

Blood biochemical parameters

Rabbits housed at 12 rabbits/m^2 recorded the highest significant total serum protein, globulin and cholesterol concentrations compared with those housed at 20 and 28 rabbits/m^2 (Table 3). However, rabbits housed at 28 rabbits/m^2 recorded the highest serum creatinine, glucose and cortisol levels compared with rabbits housed at 20 and 12 rabbits/m^2. The serum albumin level was increased in rabbits housed at 28 rabbits/m^2 compared with their counterparts housed at 20 rabbits/m^2. The effect of stocking density on serum triglyceride level was non-significant.

Gene expression

The expression of *HSP*70 mRNA was significantly higher in rabbits housed at 28 rabbits/m^2 compared with those kept at 20 and 12 rabbits/m^2 (Figure 1). The *IGF*-I gene showed a reduction of the transcript level for rabbits housed at 28 rabbits/m^2 than those kept at lower densities (Figure 2).

Table 3: Effect of stocking density on blood biochemical parameters of New Zealand white rabbits at 13 weeks of age

	Stocking density (rabbits/m^2)			
Variable	**12**	**20**	**28**	***P*-value**
Total protein (g/dl)	5.94±0.03^a	4.82±0.01^c	5.60±0.05^b	0.001
Albumin (g/dl)	3.70±0.05^a	3.07±0.07^b	3.79±0.06^a	0.001
Globulin (g/dl)	2.24±0.04^a	1.74±0.07^b	1.81±0.08^b	0.003
Creatinine (mg/dl)	0.41±0.01^b	0.40±0.012^b	0.45±0.01^a	0.013
Cholesterol (mg/dl)	126.67±0.67^a	100.33±0.39^b	76.71±0.89^c	0.001
Triglycerides (mg/dl)	147.02±25.99	149.22±1.35	173.63±0.37	0.433
Glucose (mg/dl)	76.63±0.28^c	79.45±0.34^b	81.83 ±0.81^a	0.001
Cortisol (µg/dl)	5.59±0.54^b	6.01±0.13^b	8.35±0.24^a	0.001

Means within the same row having different superscript letters are significantly different at (P <0.05)

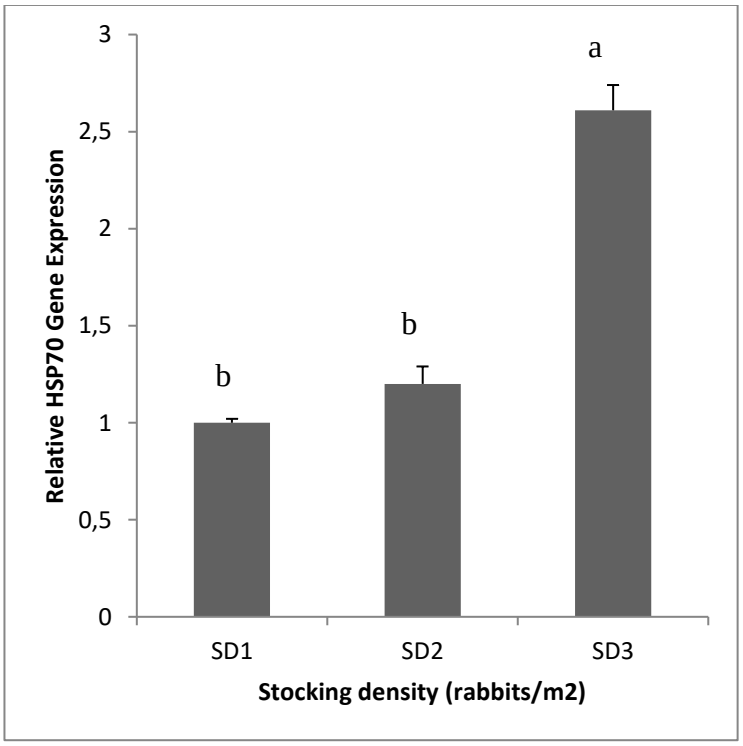

Figure 1: Effect of stocking density on heat shock protein (HSP) 70 mRNA expression in New Zealand White rabbits. SD1, 12 rabbits/m^2; SD2, 20 rabbits/m^2; SD3, 28 rabbits/m^2.Values were means±SEM. The bars represented the treatment with different superscripts (a, b) were significantly different at p <0.05

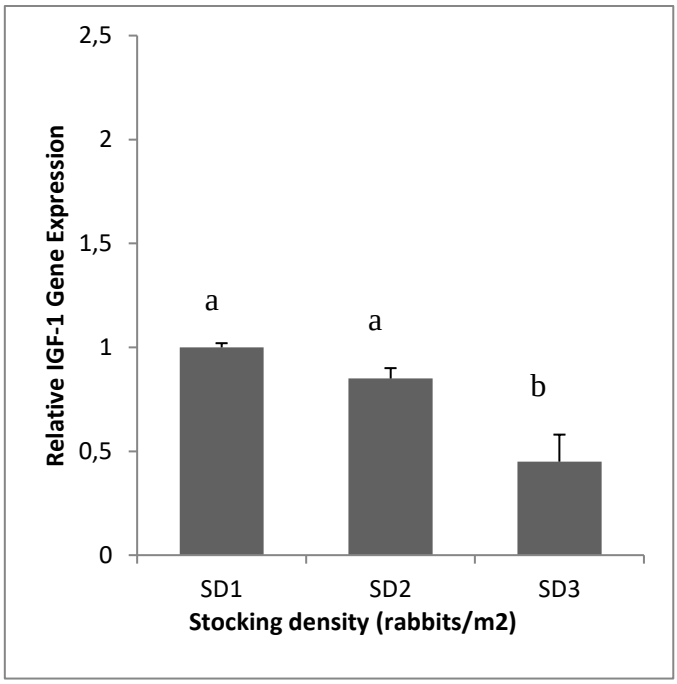

Figure 2: Effect of stocking density on IGF-I mRNA expression in New Zealand White rabbits. SD1, 12 rabbits/m^2; SD2, 20 rabbits/m^2; SD3, 28 rabbits/m^2. Values were means ± SEM. The bars represented the treatment with different superscripts (a, b) were significantly different at p <0.05

Discussion

The present results revealed that higher stocking density depicted a negative effect on growth performance traits of growing rabbits which were harmonious with those reported in previous studies (7,18-21). In another study by Mbanya et al. (22), lower results of final body weights and weight gains have been recorded in rabbits housed at 10 rabbits/m^2 than those housed at 5 rabbits/m^2. Among four stocking densities (6, 12, 18 and 28 rabbits/m^2), the last one had the greatest impairment effect on daily gain and feed intake (FI) than the others (23). But controversy results were repoted in various studies (8,24,25) in which the stocking density had no effect on growth performance of rabbits. Increasing the number of rabbits from 1 to 3 or 5 rabbits/cage (70x40x60 cm) led to a reduction in daily weight gain (4), also, groups having 5 rabbits/cage showed higher feed conversion ratio . The higher feed intake for pellet feed recorded in rabbits housed at lower density (4 rabbits/m^2) could be attributable to greater physical activity which increase the energy requirement (26). The lighter body weights that were found in the rabbits housed at higher density might be attributable to the lower feed intake, the lower feed utilization and the stresses that the rabbit exposed which are reflected in the higher glucose and cortisol levels in the serum of these rabbits.

In partial agreement with the present results, Ebru Onbasilar and Ílyas Onbasilar (4) detected that serum cholesterol and triglyceride levels in growing rabbits were not influenced by cage density. Contradicted with the present results, Kalaba (21) found that the levels of albumin in blood plasma of rabbits were not changed by stocking density (4, 8, 12 and 16 rabbits/m^2); but in parallel with the current study, he observed that plasma total protein, globulin and cholesterol levels were significantly reduced in rabbits kept at the highest density (16 rabbits/m^2) than those housed at a lower cage density, whereas plasma glucose and cortisol levels were apparently higher in rabbits housed at the same cage density. The total protein, globulin and creatinine concentrations were higher (p≤0.05) in rabbit housed at 1 and 2 rabbits/cage (50x60x30 cm) than those housed in 3 and 4 rabbits/cage, while cholesterol was decreased by increasing the cage density (3).

Controversy results were reported by Abd El-Monem et al. (27) who noted that there was no difference among rabbits housed at four stocking densities (2, 3, 4 and 5 rabbits/cage) in plasma total protein, albumin, globulin and creatinine levels. The cage density of 8.6, 12 and 17.2 rabbits/m^2 had a non-significant effect on blood parameters (total protein, albumin, cholesterol and glucose) in 13-week-old rabbits (28). On the contrary, there were no significance differences between single and group housed female rabbits in corticosterone level (29).

The changes in *HSP* gene expression levels are considered an essential biomarker for detection of stress and ideal environmental condition (30). HSPs are essential for permitting cells to cope with acute stressor, especially those affecting protein synthesis (31). Moreover, previous studies have reported that synthesis of HSPs (Hsp27, Hsp70, and Hsp90) has increased under heat stress and these proteins play an important defensive role against stress-induced tissue harmful metabolites so maintaining integrity of organs in broilers (32,33). The *HSP*70 mRNA expression was significantly increased in rabbits kept at 20 and 28 rabbits/m^2 compared with those kept at 12 rabbits/m^2, which could be explained by the higher temperature at which the rabbits were kept in conjunction with high stocking density which increase this heat stress and these results were in agreement with Yu and Bao (34) and Gu and coauthors (35) who found that exposure of broilers to high temperature led to an increase in expression of *HSP* compared with those kept under normal temperature. *Hsp*27, *Hsp*70, and *Hsp*90 mRNA expressions in bursa and spleen of black-boned chickens kept at high temperature were significantly higher (P <0.01) than those kept under normal temperature (36). Similarly, Ramsay et al. (37) and Dhabhar (38) reported an increase in the expression of *HSP*70 after exposure to crowding in European Sea bass, Dicentrarchus Labrax and Atlantic cod and Gadusmorhua. expression of *HSP* gene in rainbow trout was significantly affected by stocking density (39, 40). *HSP*70 gene expression was increased by increasing stocking density from 0.0578 to 0.077 and 0.116 m^2/bird and from 0.100 to 0.063 m^2/bird (41,42). On the contrast, expression of *HSP*70 gene in the liver and head kidney of Senegalese sole was declined by increasing stocking density from 7 to 30 kg/m^2 (43).

Previous authors reported that *IGF*-I has an essential role in the growth of broiler as broilers exhibited slow growth had lower levels of *IGF*-I (44). High blood concentrations of *IGF*-I were associated with higher protein synthesis levels and lower protein breakdown levels and, thus larger skeletal muscle mass (45). In addition to this, higher expression of *IGF*-I gene in the liver and higher level of *IGF*-I in blood have been detected in broilers choosen for higher growth rate compared with those selected for slower growth rate (46).

The present results revealed a reduction in growth performance of rabbits that were kept at high stocking density and relatively high temperature (28-33°C) which could stimulate a physiological process related to exposure to stress that resulted in reduction of IGF-I mRNA expression and serum IGF-I concentration. IGF-I showed reduced transcript level for rabbits housed at higher stocking density and these present results were in agreement with Brockmark et al. (47) who detected that Atlantic salmon reared at low density had higher levels of IGF-I than those kept at higher density. Similarly, Salas-Leiton et al. (43) found that total IGF-I mRNA levels reduced significantly at higher density (30 kg/m^2) in liver but not in the kidney in Senegalese sole. Black-boned chickens reared at high temperature (37±2°C) had low serum concentration of IGF-I (P < 0.01) than those reared at normal temperature (24±2°C) (36).

Conclusion

In conclusion, high stocking densities (28 rabbit/m^2) had a negative impact on growth performance traits and the stress-related parameters (serum glucose, cortisol and creatinine). Moreover, the expression of *HSP*70 gene has increased, while the expression of *IGF*-I gene has decreased in rabbit housed at high stocking density.

Conflict of interest

The authors declare no conflict of interest

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