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D-Aspartate amends reproductive performance of aged roosters by changing gene expression and testicular histology

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Abstract. Male broiler breeders ($n = 32$) of 55 weeks of age were administered four different doses of capsulated D-aspartate (DA; 0, 100, 200 or 300 mg kg^{-1} day^{-1}, p.o. (DA0, DA100, DA200 and DA300 respectively)) for 12 successive weeks to assess reproductive performance, blood testosterone, testicular histology and transcript levels of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), androgen receptor (AR), LH receptor (LHR), 3β-hydroxysteroid dehydrogenase (3BHSD), proliferating cell nuclear antigen (*PCNA*), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1) and glutamate ionotropic receptor NMDA type subunit 2B (GRIN2B). Blood samples and ejaculates were collected, and bodyweight was recorded weekly for 10 weeks. AI was performed weekly for the last 2 weeks to determine the number of sperm penetration holes in the perivitelline layer, fertility and hatchability. Testes histology and transcript levels were evaluated in the 12th week. Bodyweight, numbers of Leydig cells and blood vessels, testis index and levels of sperm abnormalities were not affected ($P > 0.05$) by the treatment. However, sperm total and forward motility, plasma membrane integrity and functionality of sperm, ejaculate volume, testosterone concentration and fertility were higher ($P < 0.05$) in both the DA200 and DA300 groups compared with the other groups. In the DA100 and DA200 groups, sperm concentration, number of spermatogonia, thickness of the seminiferous epithelium and the diameter of tubules were significantly higher ($P < 0.05$) than the other DA-treated groups. The number of penetration holes, hatchability and malondialdehyde concentration were higher in the DA200, all DA-treated and DA300 groups respectively compared with the control and other treatment groups. Except for P450scc, AR, LHR and PCNA transcript levels in the DA300 groups, the relative expression of the genes evaluated improved significantly in the other DA-treated groups. Based on these experimental findings, it is concluded that DA improves reproductive performance of aged roosters.

Additional keywords: fertility, hatchability, spermatogenesis, steroidogenesis.

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Introduction

Aging is defined as a natural phenomenon among creatures observed during life from the scale of cell to organism. Reproductive aging is referred to as a postpubertal decline in the reproductive performance of both sexes. In males, it is manifested by decline in sexual behaviour, serum androgens, semen quality and quantity and fertility (Gunes *et al.* 2016). Despite comprising only 10% of commercial broiler breeder flocks, roosters contribute equally in the production of fertile eggs and hatched chicks (Silveira *et al.* 2014). In broiler breeder roosters, fertility begins to decline at around 40 weeks of age. Male replacement (or spiking) is used to sustain fertility performance in older

flocks and has become a standard practice in broiler breeder operations despite the fact that this approach is costly, threatens biosecurity and disrupts social flock behaviour (Brillard 2004). The proposed reasons for this age-associated decline in fertility include: (1) animals are overweight (Silveira *et al.* 2014); (2) sperm retention by Sertoli cells in seminiferous tubules (Rosenstrauch *et al.* 1994); (3) a decrease in testosterone and an increase in oestradiol concentrations in the plasma (Rosenstrauch *et al.* 1998; Weil *et al.* 1999; Sarabia Fragoso *et al.* 2013); and (4) elevated serotonergic axis (serotonin–vasoactive intestinal peptide (VIP)–prolactin (PRL)) activity (Avital-Cohen *et al.* 2015). Chen *et al.* (1994) suggested the age-associated decline in blood testosterone concentrations in rats is related to impairment of the steroidogenic pathway rather than Leydig cell numbers. Age-related decreases in steroidogenic machinery enzyme levels, such as steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc; Luo *et al.* 2005), 3β- and 17β-hydroxysteroid dehydrogenase (HSD; Luo *et al.* 1996), as well as in the number of LH receptors (LHR; Chen *et al.* 2002), have been reported in Leydig cells. Histological analysis of testes belonging to roosters of different ages showed that, compared with testes from younger roosters, testes from older roosters (55 weeks) had a moderate reduction in seminiferous tubule diameter and a decrease in germinal epithelium, which were concurrent with impaired sperm production and maturation (Sarabia Fragoso *et al.* 2013).

Reproductive performance of senescent roosters has been improved using a male-specific diet (Silveira *et al.* 2014) and by incorporating by-products with known antioxidant properties (Akhlaghi *et al.* 2014). Recently, parachlorophenylalanine (PCPA) and active immunisation against VIP (avian prolactin-releasing factor) successfully improved both semen quality and the plasma hormone profile of aged broiler breeder roosters (Avital-Cohen *et al.* 2015). One of the biggest challenges faced by poultry production today is to extend the fertility of modern broiler breeders during the breeding period. Finding a practical strategy that targets the endocrine and spermatogenetic pathways related to the age-associated decline in rooster fertility would have a considerable economic effect on worldwide poultry production.

D-Aspartic acid (DA) is an endogenous amino acid present in endocrine tissues including the pituitary, adrenal, pineal, ovary and testis (Di Fiore *et al.* 2014). Studies on mammals and seasonal-breeding animals have defined different functions for this unusual amino acid in male reproduction (Raucci *et al.* 2004, 2014; Macchia *et al.* 2010; D'Aniello *et al.* 2012). Both oral and intraperitoneal administration of DA resulted in its accumulation in the rat testis, as well as increased serum LH concentrations and increased testis and serum testosterone levels (Di Fiore *et al.* 2014). It has been shown that this effect of DA on androgen production is mediated via modulation of the gene expression of some key molecules in the steroidogenic pathway. For example, DA-treated frogs show increased StAR mRNA expression during both reproductive and post-reproductive periods (Burrone *et al.* 2012). Both *in vivo* and *in vitro* administration of DA enhanced StAR, P450scc and 3BHSD mRNA levels in rat testis (Raucci *et al.* 2014). Improvement in sperm production parallels the accumulation of DA in Leydig and Sertoli cells, rete testis fluid, spermatogonia, elongated spermatids and spermatozoa (Sakai *et al.* 1998; D'Aniello *et al.* 2005; Raucci and Di Fiore 2009). Culturing spermatogonial GC-1 cells in DA-containing medium induced both cellular proliferation and survival pathways (Santillo *et al.* 2016). *In vivo* studies also confirmed the stimulatory effect of DA on sperm concentration, total and forward motility and plasma testosterone concentrations in human (D'Aniello *et al.* 2012) and buck rabbit (Macchia *et al.* 2010).

Although a growing body of evidence points to an effect of DA on male reproduction in several species (Di Fiore *et al.* 1998, 2008; Raucci *et al.* 2005; Boni *et al.* 2006; Raucci and Di Fiore 2009; Topo *et al.* 2009; D'Aniello *et al.* 2012), the effectiveness of this amino acid on the reproductive fitness in the roosters has not been investigated. Thus, the aim of the present study was to evaluate the effects of orally administered DA on the reproductive performance of aged male broiler breeders.

Materials and methods

Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich and Merck.

Bird husbandry and treatments

All birds (32 roosters, 120 hens) were bought from the Babolkenar Arian Line Breeding Center (Babolkenar, Iran). Male broiler breeders (55 weeks old) were randomly assigned to one of four treatment groups ($n = 8$ per group) that were provided either 0, 100, 200 or 300 mg kg^{-1} day^{-1}, p.o., capsulated DA (Trec Nutrition; DA0, DA100, DA200 and DA300 respectively) for 12 successive weeks. Roosters were maintained in individual pens (1.2×1.2 m^2) on a 14-h light : 10-h dark cycle and fed standard diet (Table 1). Female broiler breeders were also caged individually ($30 \times 40 \times 50$ cm^3) on a 14-h light : 10-h dark cycle and fed standard diet (15% crude protein, 2800 kcal kg^{-1} diet metabolisable energy, 3% calcium and 0.35% available phosphorus). This trial was performed following approval given by the Department of Animal Science, University of Tehran, Iran.

Experimental design

Semen and blood sampling, along with bodyweight, were recorded weekly during the first 10 weeks of the experiment. AI was performed in Week 11 (twice) and Week 12 (once), and eggs from inseminated hens were collected for 12 days. The first two eggs recovered 48 h after insemination were used to assess sperm penetrability of the inner perivitelline layer. At the end of study (Week 12), roosters were killed and two samples were taken from same testicle and processed for either mRNA expression analysis or histological assay (see below).

Semen traits

Gross evaluation

Semen samples were collected form conditioned birds once a week using the abdominal massage technique (Burrows and Quinn 1937). Quality parameters, namely ejaculate volume and

Table 1. Ingredients and chemical composition of the standard diet fed to broiler breeder roosters
ME, metabolisable energy; CP, crude protein

	Content (%)
Ingredients	
Corn	69.5
Soybean meal	9
Wheat bran	19.5
Dicalcium phosphate	0.18
Calcium carbonate	0.85
Sodium chloride	0.35
DL-Met	0.12
Vitamin premix[A]	0.25
Trace mineral premix[B]	0.25
Digestible amino acids	
Lysine	0.46
Methionine	0.39
Methionine and cystine	0.49
Tryptophan	0.12
Arginine	0.67
Valine	0.5
Leucine	0.53
Isoleucine	0.4
Threonine	0.37
Chemical composition	
ME (kcal kg^{-1})	2754.5
CP (%)	12
Ca (%)	0.7
Available P (%)	0.35
Na (%)	0.15
Cl (%)	0.15
K (%)	0.6

[A]Supplied per kilogram of diet: vitamin A 15 000 IU; vitamin E 100 IU; vitamin K_3 4 mg; vitamin B_{12} 25 μg; vitamin D 3000 IU; riboflavin 7.5 mg; niacin 50 μg; pantothenic acid 18 mg; pyridoxine 5.5 mg; biotin 50 mg; folic acid 1.5 mg.
[B]Supplied per kilogram of diet: Fe 90 mg; Mn 120 mg; Zn 110 mg; I 2 mg; Se 0.3 mg.

sperm concentration, were determined using a graduated 1.5-mL microtube and Neubauer chamber respectively. To evaluate total and forward motility, a diluted sample (1 : 20 in 2.9% sodium citrate) was loaded onto a slide and examined under a Zeiss compound microscope at a magnification of ×400. Sperm morphology and plasma membrane integrity were determined by the eosin–nigrosin staining method (Akhlaghi *et al.* 2014). Briefly, an equal proportion of diluted sample and dye (10 μL) was mixed, placed on a slide and smeared. Unstained spermatozoa had an intact plasma membrane. Spermatozoa with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails and protoplasmic droplets were considered abnormal. Sperm plasma membrane functionality was assessed by the hypo-osmotic swelling test. A raw sample was mixed with a solution (osmolality 100 mOsmol kg^{-1}) containing sodium citrate (1 : 20) in a 0.5-mL microtube and incubated at 37°C for 20 min. Then, 10 μL sample from the prepared solution was loaded onto a slide and the percentage of spermatozoa with a swollen 'bubble' around the curled flagellum was calculated (Łukaszewicz *et al.* 2008; Akhlaghi *et al.* 2014).

Thiobarbituric acid-reactive substances assay

Malondialdehyde is the most abundant aldehyde produced during lipid peroxidation, and its determination by thiobarbituric acid (TBA) is a common assay in lipid peroxidation studies (Esterbauer and Cheeseman 1990). Briefly, 1 mL diluted semen (1 : 2 in 2.9% sodium citrate solution) was mixed with 2 mL trichloroacetic acid (TCA; 612 mM), 1 mL ethylenediaminetetraacetic acid (EDTA;12.66 mM) and 1 mL butylated hydroxytoluene (BHT; 90.76 mM in 100% ethyl alcohol) and centrifuged (900*g*, 18°C, 10 min). Following centrifugation, 1 mL supernatant was reacted with 1 mL TBA (3.6 mM in acetic acid solution, pH 4) in a boiling water bath for 10 min. After cooling, absorbance was read at 532 nm using a spectrophotometer (UV-2100; Shimadzu; Akhlaghi *et al.* 2014).

Artificial insemination

Twenty roosters (five birds in each treatment group) and 120 broiler breeder hens (30 birds per treatment group, six hens per rooster) were allotted to AI during the last 2 weeks of the experiment. Hens were 36 weeks old at the time of AI with 75% egg production. Semen from each rooster was diluted with modified Beltsville Poultry Semen Extender and six hens were inseminated with a dose of 200 μL per hen (100×10^6 spermatozoa per dose; Ali *et al.* 2017). As per Nabi *et al.* (2016), with minor modification, the diluting extender was composed of potassium phosphate dibasic trihydrate (33.25 mM), sodium L-glutamate (51.26 mM), D-fructose (27.75 mM), sodium acetate trihydrate (23.51 mM), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic (13.95 mM), potassium citrate tribasic monohydrate (1.85 mM), potassium phosphate monobasic (5.14 mM) and magnesium chloride anhydrous (3.15 mM) with an osmotic pressure (Osmomat 030-D; Gonotec) and pH (3520; Jenway) of 310 mOsmol kg^{-1} and 7.4 respectively.

Sperm penetration assay

The sperm penetration (SP) assay is commonly used as an indicator of fertility because it reflects the number of spermatozoa that successfully arrive at the site of fertilisation (Gumułka and Kapkowska 2005). Briefly, each egg was broken and the yolk was separated from the albumen. The yolk was then submerged in a 1% NaCl solution for 10 min. After incubation, a 1-cm^2 section of the perivitelline layer (PL) overlaying the germinal disc (GD) was removed and vigorously shaken in the 1% NaCl solution to remove adhering yolk material. Each section was carefully positioned on a slide to avoid wrinkles and overlapping. Slides were fixed with 10% formalin and stained with Schiff's reagent. The number of holes per square millimetre overlying the GD was counted (at ×10) to assess fertility (Bramwell *et al.* 1995).

Fertility and hatchability rates

In all, 865 settable eggs (205, 229, 211 and 220 eggs per DA0, DA100, DA200 and DA300 group respectively) were collected, fumigated and then incubated for 21 days. Fertility (i.e. number

of fertile eggs divided by the total number of eggs set) and hatchability (i.e. number of chicks divided by the number of fertile eggs) rates were calculated at the end of the incubation period (Akhlaghi *et al.* 2014).

Blood sampling for hormonal analysis

Blood samples were drawn weekly from the brachial vein and placed into the EDTA anticoagulant tubes for the first 10 weeks. The tubes were centrifuged (1500*g*, 10 min, 15 °C) and the plasma was collected and stored at −20°C until assay. Serum testosterone concentrations were measured using a commercially available ELISA kit (Monobind). The intraassay CV and sensitivity were 6.08% and 0.0576 ng mL^{-1} respectively.

Tissue processing and histological analysis

Roosters were killed at the end of the experiment and both testicles were carefully removed, weighed and the testis index calculated (as testis weight/bodyweight; Sarabia Fragoso *et al.* 2013). The right testicle from each rooster was removed and cut into two sections; one section was snap frozen in liquid nitrogen and stored at −80°C for mRNA extraction and the other section was immersed in 10% buffered formalin (pH 7.4) and embedded in paraffin. Sections (4 μm) were cut and subsequently stained using the haematoxylin–eosin technique (Sarabia Fragoso *et al.* 2013). Morphometric data were generated from 20 photomicrographs randomly selected from four cross-sections of each testis at a magnification of ×10 using a light microscope (Zeiss) equipped with Dino-Eye Eyepiece Camera (AnMo Electronics) and analysed with ImageJ software (National Institutes of Health). The diameter of the seminiferous tubules, thickness of the seminiferous epithelium and number of spermatogonia (identified by their cytoplasmic and nuclear morphology) were determined in 20 randomly selected cross-sections of seminiferous tubules from each bird and averaged (Islam *et al.* 2010). The number of Leydig cells and blood vessels was also determined in each photomicrograph (0.37 mm^2) and averaged.

RNA extraction and real-time polymerase chain reaction

Total RNA was isolated using the RNeasy Mini Kit (Catalogue no. 74104; Qiagen) according to the manufacturer's protocol. Using the QuantiTec Reverse Transcription Kit (Catalogue no. 205311; Qiagen), cDNA was synthesised with the reaction conditions of 42°C for 30 min and 95°C for 3 min.

The nucleotide sequences of all genes investigated in the rooster, namely *StAR*, *P450scc*, androgen receptor (*AR*), LH receptor (*LHR*), *3BHSD*, proliferating cell nuclear antigen (*PCNA*), glutamate ionotropic receptor NMDA type subunit 1 (*GRIN1*) and glutamate ionotropic receptor NMDA type subunit 2B (*Gallus gallus*; *GRIN2B*) were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/, accessed 6 April 2016). Primers were designed using Primer3Plus online software (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi/, accessed 6 April 2016) and checked using OligoAnalyser (https://eu.idtdna.com/calc/analyzer, accessed 6 April 2016), OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html, accessed 6 April 2016) and PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed 6 April 2016; Table 2). Expression of both *GRIN1* and *GRIN2B* has been confirmed in rooster testis tissue (E-GEOD-14013). *GRIN2B* was selected as test gene in the present study due to its higher expression compared with *GRIN2A* in the testis. β-Actin (*ACTB*) was used as an internal control. Quantification of all transcripts was performed using a QuantiFast SYBR Green PCR Kit (Catalogue no. 204054; Qiagen) in a 20-μL reaction volume, containing 1 μL single-strand cDNA, 10 μL master mix, 0.5 μL forward and reverse primers and 8 μL distilled H_2O, using Rotor-Gene 6000 Real-Time PCR software (Corbett Research). The polymerase chain reaction (PCR) program consisted of a 5-min activation step at 95°C, followed by 40 cycles of 15 s at 95°C and 40 s at 60°C. At the end of each PCR, a melting curve analysis was performed at a rate of 0.1°C s^{-1} for all genes to check the specificity of the products. The efficiency of the assays was ≥95% and the standard curve R^2 was ≥0.999. The quantitative (q) PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Data were tested for normality using the Shapiro–Wilk test and arcsine transformation was performed when required. Single (histological and mRNA expression assay) and repeated-measurement (weekly semen and blood sampling) data were analysed using the general linear model (GLM) and mixed procedures respectively in SAS Version 9 (SAS Institute). Treatment as a fixed factor and bird as a random factor were considered in the equation model. Bird bodyweight and total number of spermatozoa were included as covariates for analysis of variance (ANOVA) and analysis of thiobarbituric acid-reactive substances (TBARS) data respectively. The statistical models for single- and repeated-measurement data were as follows:

$$Y_{ijk} = \mu + T_i + A_j + e_{ijk}$$

$$Y_{ijklm} = \mu + T_i + A_j + t_k + \beta x_1 + (T_i \times t_k) + e_{ijklm}$$

where Y_{ijk} and Y_{ijklm} are the parameters evaluated, μ is the mean of the population, T_i is the treatment effect, A_j is the random factor (bird), t_k is the time effect, βx_l is a covariate factor, $(T_i \times t_k)$ is the time and treatment interaction and e_{ijk} and e_{ijklm} are residual effects. Tukey's test was used to compare mean values. Data are presented as the least squares mean ±s.e.

Results

Sperm features

Table 3 summarises the effects of the treatments, time and their interactions on bodyweight, sperm quality parameters and plasma testosterone concentrations in roosters. Excluding abnormal sperm percentage, a significant effect was found for other features or measurements under the influence of time (bodyweight; $P < 0.01$), treatment (testosterone ($P < 0.01$) and malondialdehyde; $P < 0.01$ for both), treatment and time (ejaculate volume; $P < 0.01$) or treatment, time and their interactions (sperm total and forward motility, sperm plasma membrane integrity and functionality and sperm concentration; $P < 0.01$ for all).

Table 2. Primer sequences used for quantitative real-time polymerase chain reaction

ACTB, β-actin; *StAR*, steroidogenic acute regulatory protein; *P450scc*, cholesterol side-chain cleavage enzyme; *AR*, androgen receptor; *LHR*, LH receptor; *3BHSD*, 3β-hydroxysteroid dehydrogenase; *PCNA*, proliferating cell nuclear antigen; *GRIN1*, glutamate ionotropic receptor NMDA type subunit 1; *GRIN2B*, glutamate ionotropic receptor NMDA type subunit 2B

Gene	Sequence (5′–3′)	Product length (bp)	Accession no.
ACTB	Forward: ACGTCGCACTGGATTTCGAG Reverse: AAAGATGGCTGGAAGAGGGC	145	X00182
StAR	Forward: TTCAGCGAGATGGAGATGTCC Reverse: GGAACACCTTACCCACGTCC	160	NM_204686
P450scc	Forward: GTTGGGTGTCTACGAGAGCG Reverse: TTGCGGTAGTCACGGTATGC	126	NM_001001756
AR	Forward: AGTGCCAGCCCATCTTTCTC Reverse: CCTTTGCCCACTTGACGAC	159	NM_001040090
LHR	Forward: TCTTCAACGGCACTGGAGTC Reverse: TTTATCCAGAGGCGGCAGTC	105	NM_204936
3βHSD	Forward: GGGCAAGACTGAGGTGAAAATC Reverse: TGTGTGGATGACGAGCGAG	94	XM_015294370
PCNA	Forward: GGGCGTCAACCTAAACAGC Reverse: GCTTCAAACACTAGAGCCAACG	111	NM_204170
GRIN1	Forward: ATACTCTCACCAGGCCAACG Reverse: TTTCTCAGCCTTGGGTCCTC	214	XM_015279360
GRIN2B	Forward: GGAATCAGCGTCATGGTGTC Reverse: GCACCGATTATAGCCCACAG	168	XM_015289359

Table 3. Bodyweight, sperm features and plasma testosterone concentrations in broiler breeder roosters fed D-aspartate (DA)

Data are the least squares mean ± s.e. ($n = 8$ birds/group). Within rows, different superscript letters indicate significant differences ($P < 0.05$). DA0, 0 mg kg^{-1} day^{-1} DA; DA100, 100 mg kg^{-1} day^{-1} DA; DA200, 200 mg kg^{-1} day^{-1} DA; DA300, 300 mg kg^{-1} day^{-1} DA; MDA, malondialdehyde

	Treatment				*P*-value		
	DA0	DA100	DA200	DA300	Treatment	Time	Treatment × time
Bodyweight (g)	5901.91 ± 30.62	5874.96 ± 35.27	5795.52 ± 40.37	5823.53 ± 43.72	0.64	<0.01	0.26
Total motility (%)	81.14 ± 0.36^{c}	82.98 ± 0.39^{b}	87.09 ± 0.63^{a}	86.12 ± 0.37^{a}	<0.01	<0.01	<0.01
Forward motility (%)	70.15 ± 0.36^{d}	71.69 ± 0.38^{c}	77.87 ± 0.67^{a}	75.91 ± 0.56^{b}	<0.01	<0.01	<0.01
Abnormality (%)	4.99 ± 0.14	4.54 ± 0.16	4.76 ± 0.14	4.71 ± 0.14	0.39	0.44	0.08
Plasma membrane functionality (%)	62.69 ± 0.34^{b}	63.96 ± 0.45^{b}	69.5 ± 0.50^{a}	68.28 ± 0.39^{a}	<0.01	<0.01	<0.01
Plasma membrane integrity (%)	81.64 ± 0.57^{d}	84.06 ± 0.45^{c}	90.78 ± 0.67^{a}	88.59 ± 0.57^{b}	<0.01	<0.01	<0.01
Ejaculate volume (mL)	0.40 ± 0.01^{b}	0.41 ± 0.01^{b}	0.46 ± 0.01^{a}	0.45 ± 0.01^{a}	<0.01	<0.01	0.08
Sperm concentration (×10^{9} cells mL^{-1})	4.11 ± 0.01^{c}	4.75 ± 0.03^{a}	4.76 ± 0.04^{a}	4.55 ± 0.03^{b}	<0.01	<0.01	<0.01
MDA (nM mL^{-1})	0.48 ± 0.01^{b}	0.48 ± 0.01^{b}	0.56 ± 0.02^{b}	0.78 ± 0.02^{a}	<0.01	0.11	0.07
Testosterone (ng mL^{-1})	4.2± 0.1^{c}	4.64 ± 0.09^{b}	5.04 ± 0.08^{a}	5.2 ± 0.1^{a}	<0.01	0.94	0.13

Regardless of dose, DA feeding significantly ($P < 0.01$) improved sperm total and forward motility, with the values of both these parameters highest in the DA200 treatment group (87.09 ± 0.63% and 77.87 ± 0.67% respectively; $P < 0.01$; Table 3). The interactive effect of treatment and time on sperm total and forward motility revealed a relatively steady trend for both the DA0 and DA100 groups (Fig. 1*a*, *b*). However, the DA200 and DA300 treatment groups showed a progressive increase in total motility through Week 6 before reaching a plateau (Fig. 1*a*, *b*). Interestingly, forward motility in the DA200 group did not peak until Week 8 (Fig. 1*b*).

Sperm plasma membrane functionality was significantly ($P < 0.01$) higher in the DA200 and DA300 groups compared with the DA100 and DA0 groups (69.50 ± 0.50% and 68.28 ± 0.39% vs 63.96 ± 0.45% and 62.69 ± 0.34% respectively). The interactive effect of time and treatment on sperm plasma membrane functionality is shown in Fig. 1*c*. With the exception of the DA100 group during the first 3 weeks of the study, other DA-treated groups had an increasing trend for sperm membrane functionality that plateaued near the end of the experiment.

In contrast with this trend, membrane functionality in the control group (DA0) exhibited a relatively fluctuating trend that reached a peak in the Week 6 (64.53 ± 0.34%) and decreased thereafter. DA significantly ($P < 0.05$) improved sperm plasma membrane integrity regardless of the dose administered

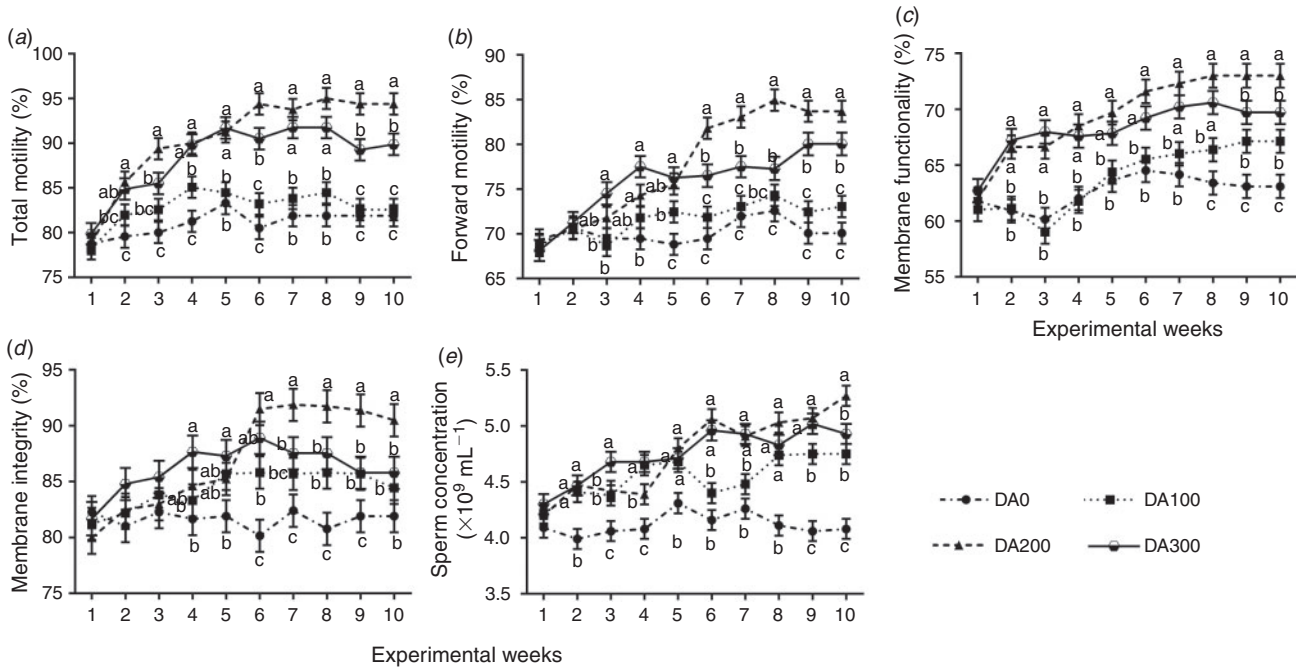

Fig. 1. Weekly variations in sperm (*a*) total motility, (*b*) forward motility, (*c*) membrane functionality, (*d*) membrane integrity and (*e*) sperm concentration in roosters (eight birds per group) administered different doses of D-aspartate (DA; 0, 100, 200 or 300 mg kg^{-1} day^{-1}, p.o. (DA0, DA100, DA200 and DA300 respectively)). Data are the least squares mean ± s.e. At each time point, different letters indicate significant differences among groups ($P < 0.05$).

(Table 3) and, like forward motility, plasma membrane integrity was significantly ($P < 0.05$) improved in the DA200 group compared with the other DA-treated groups. The interaction of time and treatment for plasma membrane integrity is shown in Fig. 1*d*. A relatively steady trend was observed for both the DA0 and DA100 groups during the present study, but in the DA200 and DA300 groups plasma membrane integrity exhibited an increasing trend during the first 6 weeks and plateaued thereafter.

The sperm concentration in semen samples from the DA100 and DA200 groups was significantly ($P < 0.01$) higher than in the DA0 and DA300 groups (4.75 ± 0.03 and 4.76 ± 0.04 vs 4.11 ± 0.01 and 4.55 ± 0.03 ×10^9 spermatozoa mL^{-1} respectively). Fig. 1*e* depicts the time and treatment interaction for sperm concentration. A relative increasing trend was observed for all DA-treated groups in the initial weeks of the study; however, this continued through to the end of the in the DA200 only. Daily administration of 300 mg kg^{-1} BW^{-1}DA was the only treatment that resulted in a significant increase in *N*-methyl-D-aspartate (NMDA) levels (Table 3).

Blood testosterone concentrations

Administration of DA significantly increased circulating testosterone concentrations in the present study (Table 3). Among the DA treatments, birds in the DA200 and DA300 groups had significantly ($P < 0.01$) higher serum testosterone concentrations than those in the DA100 group (5.04 ± 0.08 and 5.2 ± 0. vs 4.64 ± 0.09 ng mL^{-1} respectively).

Fertility and hatchability rates

Data related to sperm penetration of eggs, fertility and hatchability rates are given in Table 4. Although the mean number of sperm penetration holes was increased in the DA300 group, birds that received 200 mg kg^{-1} day^{-1} DA had a significantly ($P = 0.04$) higher number of sperm penetration holes than in the control group (148.2 ± 20.7 vs 69.4 ± 20.7 respectively). The fertility rate in both the DA200 and DA300 groups was significantly ($P < 0.01$) higher than in the DA100 and DA0 groups (Table 4). Hatchability rate was significantly ($P = 0.03$) higher in the DA-treated groups than in the control group (Table 4).

Testis index and histological parameters

Testis index and morphology data are presented in Table 5. Although there were no significant differences in testis index ($P = 0.25$), number of Leydig cells ($P = 0.98$) and the number of blood vessels ($P = 0.96$) among the DA-treated groups, the number of spermatogonia ($P = 0.01$), seminiferous tubule diameter ($P < 0.01$) and seminiferous epithelium thickness ($P < 0.01$) were significantly higher in the DA100 and DA200 groups compared with the control group (Table 5; Fig. 2).

Relative mRNA levels for test genes in the testes

Transcript levels of some of the genes involved in the mechanism of action of DA in rooster testes are shown in Fig. 3. Oral administration of DA significantly increased mRNA levels of StAR ($P < 0.01$), P450scc ($P < 0.01$), 3BHSD ($P = 0.01$), AR ($P < 0.01$), LHR ($P = 0.01$), PCNA ($P < 0.01$),

Table 4. Fertility, hatchability and sperm penetration rates of roosters administered D-aspartate (DA)
Data are the least squares mean ± s.e. ($n = 5$ birds/group). Within rows, different superscript letters indicate significant differences ($P < 0.05$). DA0, 0 mg kg^{-1} day^{-1} DA; DA100, 100 mg kg^{-1} day^{-1} DA; DA200, 200 mg kg^{-1} day^{-1} DA; DA300, 300 mg kg^{-1} day^{-1} DA; GD, germinal disc

	Treatment				*P*-value
	DA0	DA100	DA200	DA300	
Sperm penetration (holes mm^{-2} GD)	69.4 ± 20.7^{b}	60.8 ± 21.3^{b}	148.2 ± 20.7^{a}	116.2 ± 25.5^{ab}	0.04
Fertility rate (%)	76 ± 1^{c}	76.48 ± 0.73^{c}	86.58 ± 1.42^{a}	81.84 ± 0.86^{b}	<0.01
Hatchability of fertile eggs (%)	77 ± 1^{b}	82.6 ± 1.1^{a}	84 ± 2^{a}	83.8 ± 2.1^{a}	0.03

Table 5. Testicular parameters of roosters administered D-aspartate (DA)
Data are the least squares mean ± s.e. ($n = 8$ birds/group). Within rows, different superscript letters indicate significant differences ($P < 0.05$). DA0, 0 mg kg^{-1} day^{-1} DA; DA100, 100 mg kg^{-1} day^{-1} DA; DA200, 200 mg kg^{-1} day^{-1} DA; DA300, 300 mg kg^{-1} day^{-1} DA

	Treatment				*P*-value
	DA0	DA100	DA200	DA300	
Testis index[A]	5.74 ± 0.35	6.96 ± 0.57	6.88 ± 0.39	6.86 ± 0.56	0.25
Seminiferous tubule diameter[B] (μm)	295 ± 5^{b}	389 ± 12^{a}	380 ± 7^{a}	298 ± 9^{b}	<0.01
Seminiferous epithelium thickness[B] (μm)	76.5 ± 1.4^{b}	97.54 ± 2.86^{a}	101.41 ± 7.78^{a}	80.0 ± 5.2^{b}	<0.01
No. spermatogonia[B]	196.85 ± 1.93^{c}	229.8 ± 6.7^{a}	218.25 ± 8.24^{ab}	201.3 ± 9.4^{bc}	0.01
No. Leydig cells[C]	25.09 ± 2.76	26.9 ± 2.9	26.0 ± 3.6	25.92 ± 3.95	0.98
No. blood vessels[C]	1.72 ± 0.29	1.66 ± 0.22	1.8 ± 0.2	1.68 ± 0.22	0.96

[A]Calculated as testis weight (g)/bodyweight (kg).
[B]Calculated based on 20 random cross-sections of seminiferous tubules in each bird and averaged.
[C]Calculated based on 20 micrographs (0.37 mm^2) and averaged.

GRIN1 ($P < 0.01$) and GRIN2B ($P < 0.01$) compared with their respective controls (Fig. 3). A dose-response relationship was noted between StAR and 3BHSD mRNA levels and DA concentrations (Fig. 3*a*, *c*). Testicular P450scc, AR and GRIN1A transcript levels in roosters fed 200 mg kg^{-1} day^{-1} DA were significantly elevated compared with levels in the other groups (Fig. 3*b*, *d*, *g*). However, transcript levels of PCNA and GRIN2B were significantly higher in the DA100 group compared with the other groups (Fig. 3*f*, *h*).

Discussion

DA has been shown to improve male reproductive fitness in several species (Raucci *et al.* 2004, 2014; Macchia *et al.* 2010; D'Aniello *et al.* 2012); accordingly, it was proposed that DA could be used to improve age-related subfertility in broiler breeder roosters. In the present study, DA treatment improved the fertility rate by 10% in birds provided with 200 mg kg^{-1} day^{-1} DA. This increased fertility following DA treatment was the result of significant enhancement of several reproductive performance parameters, including sperm quality and quantity, mRNA levels of key molecules involved in steroidogenesis and spermatogenesis, blood testosterone concentrations and histological parameters of testis (i.e. seminiferous tubule diameter, seminiferous epithelium thickness and number of spermatogonia). These results are consistent with previous *in vivo* studies regarding sperm quality and quantity parameters, fertility and blood testosterone concentrations (Macchia *et al.* 2010; D'Aniello *et al.* 2012), as well as steroidogenic enzymes (Raucci *et al.* 2014). In the present study, the beneficial effect of DA started in Week 2 of treatment, which coincides with the 14-day period from the onset of meiosis to the end of spermiogenesis in the rooster (De Reviers 1968). The increased testosterone concentrations resulting from DA treatment may have improved both sperm concentration and sperm mobility. For example, low fertility in aging roosters has been attributed to the retention of spermatozoa in the seminiferous tubules because of low testosterone (Rosenstrauch *et al.* 1994; Weil *et al.* 1999; Smith and Walker 2014) and an optimal level of testosterone is associated with improved sperm mobility (Meeker *et al.* 2007).

It seems that the role of DA treatment in spermatogenesis and steroidogenesis is mediated through the NMDA receptor in spermatogonia, Sertoli and Leydig cells (Di Fiore *et al.* 2016). This receptor is a heterotetramer composed of one obligatory subunit (GRIN1) and four modulatory subunits (GRIN2A, GRIN2B, GRIN2C and GRIN2D), each encoded by a separate gene. Both GRIN1 and GRIN2A mRNA levels were upregulated in rats after oral administration of DA (Santillo *et al.* 2014). In the present study, DA significantly enhanced both GRIN1 and GRIN2B mRNA levels in rooster testes. The translocation of cholesterol from the cytoplasm into mitochondria is a rate-limiting step mediated by StAR in Leydig cells. Within the

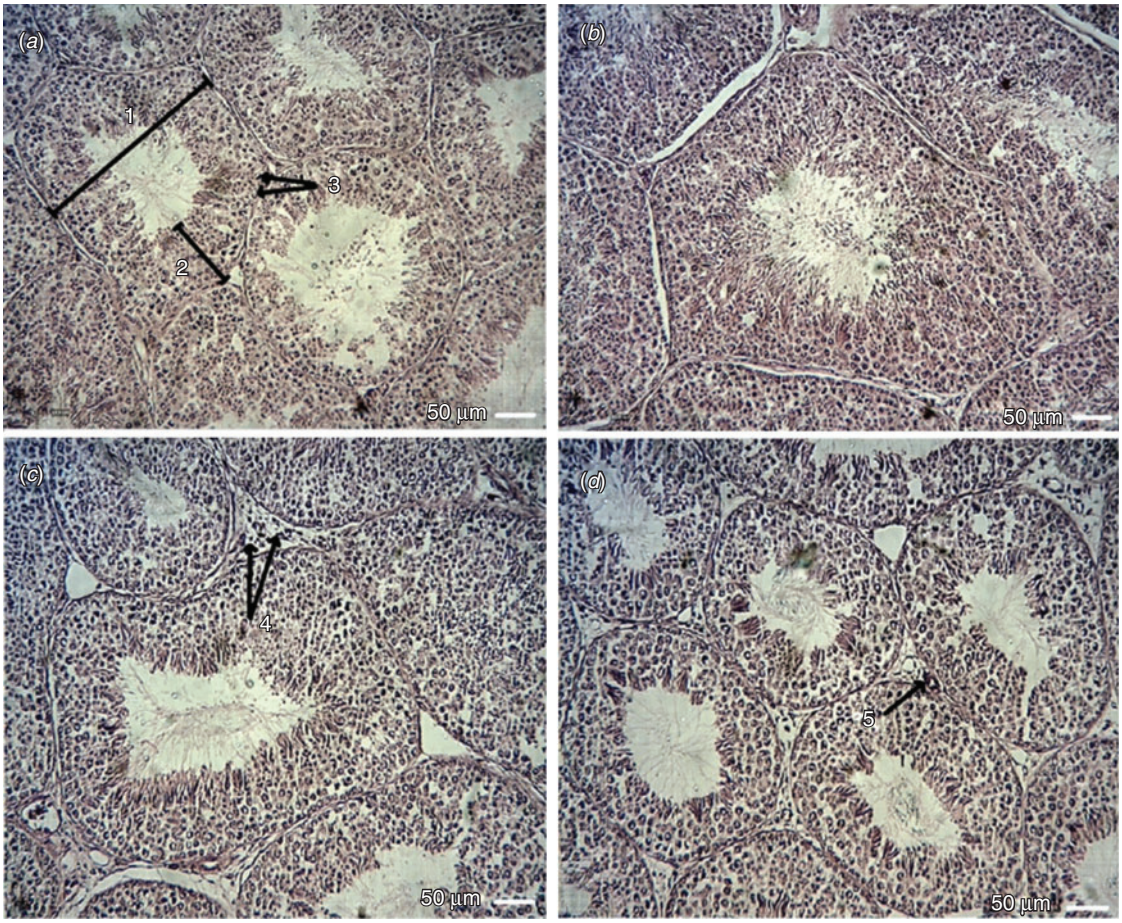

Fig. 2. Photomicrographs of cross-sections of testes from rooster administered different doses of D-aspartate (DA) for 12 successive weeks: (*a*) 0 mg kg^{-1} day^{-1} DA; (*b*) 100 mg kg^{-1} day^{-1} DA; (*c*) 200 mg kg^{-1} day^{-1} DA; (*d*) 300 mg kg^{-1} day^{-1} DA. 1, seminiferous tubule diameter; 2, seminiferous epithelium thickness; 3, spermatogonia; 4, Leydig cells; 5, blood vessel.

mitochondria, cholesterol is converted to pregnenolone by P450scc. Then, pregnenolone, a precursor for the synthesis of all steroid hormones, is converted to dehydroepiandrosterone and then androstenedione by 3β-HSD in the smooth endoplasmic reticulum. Finally, the conversion of androstenedione to testosterone is catalysed by 17β-HSD (Heng *et al.* 2017). Steroidogenesis is under the strict control of LH. The binding of LH to its receptor (LHR) has both a chronic and acute effect on Leydig cells. In the acute stimulatory phase, cholesterol is transported across the mitochondrial membrane, whereas the chronic effect of LH exposure enhances the expression of steroidogenic enzymes (Midzak *et al.* 2009). Interestingly, Di Nisio *et al.* (2016) reported DA delays LHR internalisation in murine Leydig cells incubated with human chorionic gonadotrophin, resulting in prolonged stimulation of steroidogenesis. Aside from the pivotal role of LHR in reproduction, mice lacking AR in Leydig cells were infertile due to the arrest of spermatogenesis at the spermatid stage and lower serum testosterone concentrations, leading to the reduction of several key steroidogenic enzymes, including 17β-HSD, 3β-HSD and P450scc, rather than changes in the number o f Sertoli cells (Xu *et al.* 2007). Moreover, previous studies have reported an age-associated decrease in the mRNA expression of these key steroidogenic enzymes (Luo *et al.* 1996, 2005). In the present study, DA enhanced mRNA levels of three key enzymes involved in steroidogenesis (StAR, 3BHSD and P450scc), probably by increasing LHR and AR transcript levels and/or via the adenylyl cyclase/cAMP/protein kinase A and mitogen-activated protein kinase (MAPK) pathways as proposed by Di Fiore *et al.* (2016). The effects of DA on *StAR*, *3BHSD* and *P450scc* expression in the present study are in agreement with the findings of Raucci *et al.* (2014), who, using *in vivo* and *in vitro* experiments, confirmed the effectiveness of exogenous DA in increasing StAR, P450scc and 3BHSD mRNA levels in rat testes and immature Leydig cells. The hypothalamic–pituitary–gonadal axis is the primary axis regulating reproduction. Coincubation of the hypothalamus and pituitary in the presence of exogenous DA induced the synthesis and release of GnRH and LH (D'Aniello *et al.* 2000).

The improvement in sperm total and forward motility, plasma membrane integrity and functionality in the present study may be mediated by DA binding to its receptor, NMDA, which is permeable to extracellular Ca^{2+}. This cation plays a

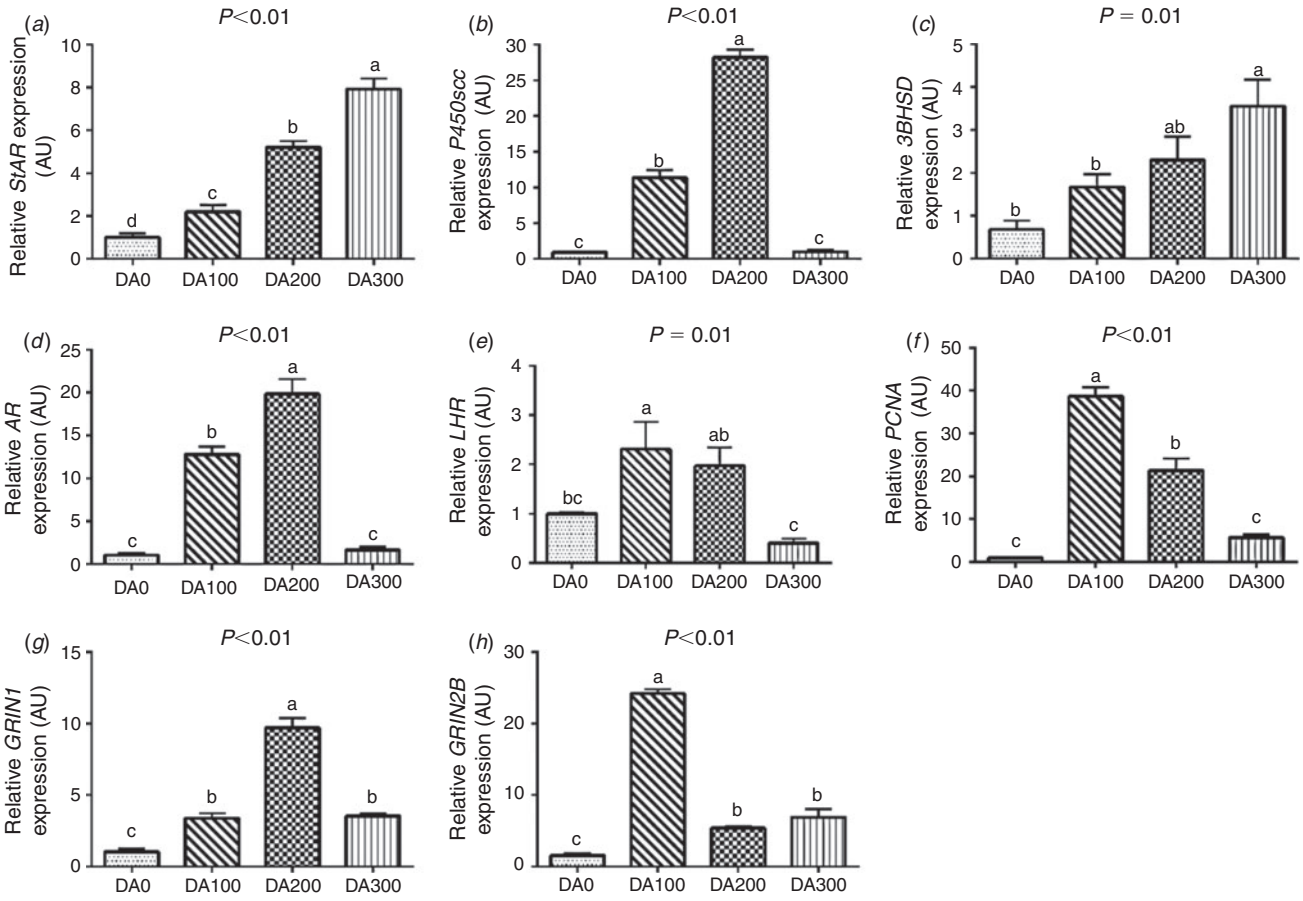

Fig. 3. Relative mRNA expression of (*a*) steroidogenic acute regulatory protein (StAR), (*b*) cholesterol side-chain cleavage enzyme (P450scc), (*c*) 3β-hydroxysteroid dehydrogenase (3BHSD), (*d*) androgen receptor (AR), (*e*) LH receptor (LHR), (*f*) proliferating cell nuclear antigen (PCNA), (*g*) glutamate ionotropic receptor NMDA type subunit 1 (GRIN1) and (*h*) glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B*) in rooster testis ($n = 5$ birds per group) administered different doses of D-aspartate (DA; 0, 100, 200 or 300 mg kg^{-1} day^{-1}, p.o. (DA0, DA100, DA200 and DA300 respectively)) for 12 successive weeks. Expression of the test genes was normalised against that of β-actin (*ACTB*), used as an internal control. Data are the mean ± s.e. Different letters indicate significant differences among groups ($P < 0.05$). AU, arbitrary units.

synergistic role with HCO_3 in increasing sperm flagella beating frequency, and Ca^{2+} presence is required to maitain the HCO_3-promoting effect (Macchia *et al.* 2010). Inclusion of DA in the medium of spermatogonial GC-1 cells induced phosphorylation of MAPK and AKT proteins and upregulated protein levels of PCNA and aurora kinase B (AURKB), stimulating cellular proliferation and survival pathways (Santillo *et al.* 2016). Age-related decreases in seminiferous tubule diameter and epithelium thickening reported by Sarabia Fragoso *et al.* (2013) were significantly alleviated by enhanced PCNA mRNA levels, and, in the present study, enhanced PCNA mRNA levels were associated with improved morphology and morphometric properties of testis in the current study.

AI was used in the present study to assess fertility in order to remove the effect of male weight and mating preferences. In addition, young laying females were used in the present study to minimise age-related contributions to subfertility of hens. A promising outcome of the present study was the improvement in the fertility rate of ~10% in DA200 roosters. Although the detailed mechanism of sperm transport in the reproductive tract of hens is not fully understood, greater plasma membrane integrity and motility are necessary for sperm uptake into sperm storage tubules (SSTs; Sasanami *et al.* 2013). This greater forward motility and plasma membrane integrity resulting from DA treatment likely increased the population of functional spermatozoa in the SSTs and thus improved fertility. Although all DA treatments resulted in significant improvement in fertility, motility and plasma membrane integrity compared with control, the moderate level of treatment (DA200) was significantly better when compared with the other DA treatments. The diminished return from increasing the dose of DA can be explained by the fact that the DA concentration is controlled by DA oxidase in Sertoli cells, which catalyses DA degradation to oxaloacetate, ammonium and hydrogen peroxide (Tomita *et al.* 2016). The resulting increase in hydrogen peroxide production in the group treated with the higher dose (300 mg kg^{-1} day^{-1}) in the present study may have attenuated some of the positive effects of DA on the investigated traits.

Conclusion

Oral administration of DA to senescent roosters improved their reproductive performance, including sperm quality parameters, fertility and hatchability rates, as well as some morphological and morphometric properties of the testes, via induction of steroidogenesis and spermatogenesis pathways. Taking all the data into account, providing 200 mg kg^{-1} day^{-1} DA was the most successful treatment in improving the reproductive fitness of senescent roosters. Fertility is a key determinant of profitability in commercial broiler breeder operations, and remains a considerable limiting factor in broiler productivity. The present study shows that supplementing rooster feed with 200 mg kg^{-1} day^{-1} DA is a practical strategy that can mitigate the age-associated decline in broiler breeder fertility. This proposed dietary approach could be easily implemented as an alternative to the conventional practice of managing flock fertility through male replacement ('spiking') without compromising biosecurity and negatively affecting bird welfare.

Conflicts of interest

The authors declare no conflicts of interest.

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