Access to pasture in an outdoor housing system affects welfare indicators and improves rooster sperm quality in two native Mediterranean breeds

J. Santiago-Moreno,[*,1] M. G. Gil,[†] S. G. Dávila,[†] J. L. Campo,[†] C. Castaño,[*] A. Toledano-Díaz,[*] M. T. Prieto,[*] and E. Blesbois[‡]

[*]*Department of Animal Reproduction, INIA, 28040 Madrid, Spain;* [†]*Department of Animal Genetic Improvement, INIA, 28040 Madrid, Spain; and* [‡]*INRA 0085 UMR PRC INRA-CNRS-University François Rabelais-Haras Nationaux, 37380 Nouzilly, France*

ABSTRACT The aim of the present work was to examine the influence of access to pasture in an outdoor housing system on rooster sperm quality and response to cryopreservation and to examine the possible correlation between values for sperm quality variables and welfare indicators. Two groups of Black-barred Andaluza and Red-barred Vasca roosters were housed in an outdoor system, with one group given daily access to a grazing area containing plant species that typically grow on uncultivated Mediterranean land. Semen was collected once per week from each group, and the following sperm quality variables were assessed: sperm volume, appearance, concentration, motility, membrane integrity, acrosome integrity, and morphological abnormalities. In addition, two welfare indicators were examined: the heterophil/lymphocyte (H/L) ratio, and the duration of tonic immobility (TI). Ejaculates from the birds with access to pasture had higher percentages of sperm showing progressive motility ($P = 0.019$), and returned a higher motility index ($P = 0.035$). Unexpectedly, the H/L ratio was also higher in these birds. Virtually no differences were seen between the treatment groups with respect to sperm quality after freezing-thawing, although the semen of the Red-barred Vasca birds with access to pasture did show a higher percentage of progressive motility ($P = 0.023$) than the birds of the same breed with no such access. Significant correlations were detected between the H/L ratio and sperm motility (r = 0.420, $P = 0.038$), the sperm motility index (r = 0.526, $P = 0.002$), and progressive motility (r = 0.467, $P = 0.003$). No differences were seen between the treatment groups with respect to the duration of TI. In conclusion, access to pasture improved fresh sperm motility.

Key words: free-range, welfare, sperm quality, sperm cryopreservation

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INTRODUCTION

Allowing access to pasture is seen as a way of improving the welfare of chickens. Indeed, interest in allowing such access is growing, the consequence of increasing consumer demand for more natural, wholesome food. Several studies have investigated the effects of different housing formats on chicken welfare (Campo et al., 2008; Lay et al., 2011, 2013), but more work will be needed if we are to understand the type and levels of stress associated with each. Interactions between the breed and the environment should also be taken into account when proposing alternative systems (Singh et al., 2009). The free range system, which is recommended for traditional breeds (Van de Weerd et al., 2009), and might also be the best for their *in situ* conservation, allows access to pasture. This is thought to have a positive influence on the birds' welfare, but little is known about its effect on sperm quality or sperm cryosurvival, and on the potential correlation between semen characteristics and behaviors.

Since the fertilization capacity of cryopreserved poultry sperm falls dramatically (Long, 2006), fresh semen of high quality is required. The effect of season in chicken breeds that are maintained outdoors year-round may affect the values of fresh semen variables (Santiago-Moreno et al., 2009a) and therefore influence the success of cryopreservation. Other factors that may affect sperm quality are the breed (Prieto et al., 2011), the social status (Cornwallis and Birkhead 2007), the presence of hens (Dávila et al., 2015), and the diet (Surai et al., 2000). The antioxidant capacity of sperm is low, and the presence of reactive oxygen species (ROS) is associated with male infertility (Surai et al., 2001). However, dietary supplementation with leafy plants, vegetables or fruit, all of which contain biologically active antioxidants, can enhance the anti-oxidative capacity

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[1]Corresponding author: moreno@inia.es
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of seminal plasma and improve sperm quality and characteristics (Ommati et al., 2013; Akhlaghi et al., 2014a,b; Borghei-Rad et al., 2017). Unfortunately, the type of vegetation on most free range land is either not specified or unknown; commonly it is just described as "grass" or "plants" (Walker and Gordon, 2003). It might be hypothesized, however, that allowing roosters access to grazing areas would provide them with natural antioxidants that favor sperm function, and therefore improve fertilization capacity. There is evidence for the production of ROS during freezing and thawing of sperm that may be a cause for the decrease in sperm function following cryopreservation (Chatterjee and Gagnon, 2001). Certainly, the use of antioxidants in sperm diluents provides some cryoprotection (O'Flaherty et al., 1997). Natural dietary antioxidants would also help prevent ROS-induced sperm death and loss of cell function during or following freezing-thawing.

Little is known about the behavior of roosters in free-range systems, but access to grazing areas might also increase the metabolic activity through the exploration of a more complex environment (Rivera-Ferre et al., 2007; Leone and Estevez 2008), the competition and even aggressive behaviors due to the new territory (Lay et al., 2011); all these factors might play a role in the behavioral patterns of birds and, consequently, affect their sexual activity and sperm quality (Cornwallis and Birkhead 2007).

Considering the aforementioned, it is here hypothesized that the access to pasture might improve the sperm quality and perhaps even the sperm cryoresistance. The aim of the present work was to examine the influence of access to pasture in an outdoor housing system on sperm quality and response to cryopreservation in Mediterranean chicken breeds traditionally reared in free range systems, and to seek possible correlations between values for sperm quality variables and welfare indicators.

MATERIAL AND METHODS

Bird Management and Sperm Collection

For this study, two native Mediterranean breeds, traditionally reared in free range systems, were used as model. Red-barred Vasca is a dual purpose chicken (mean 160 to 180 eggs/yr) and Black-barred Andaluza is a laying chicken (120 to 180 eggs/yr) (Campo 2010). The mean fertility and hatchability data in outdoor housing system are 91.1 and 84.7%, for Black-barred Andaluza and 89.2 and 83.3% for Red-barred Vasca (unpublished data), respectively. This work was performed at the El Encín Research Station (Alcalá de Henares, Madrid), where Black-barred Andaluza and Red-barred Vasca chickens are the subjects of a genetic resources conservation program that was started in 1975 (Campo and Orozco, 1982; Campo, 1998). The experimental roosters were reared together with hens in an all-litter floor pen (9 × 20 m) at a density of 10 birds/m^2 until 8 wk of age. Artificial light was provided only during the first week of age (23L:1D). The temperature was controlled with gas heaters (33 to 35°C during the first week after hatching, followed by a weekly 3°C reduction until reaching 18 to 20°C in the sixth week of life). From 8 to 24 wk of age, the birds were moved to another all-litter floor pen (13 × 20 m) at a density of 6 birds/m^2. During this period the lighting regime was 8L:16D. All birds were fed standard rearing diets containing 19% CP, 2,800 kcal ME/kg, 1% Ca, and 0.5% available *P*, until 8 wk of age, and 15% CP, 2,700 kcal ME/kg, 0.9% Ca, and 0.4% available *P*, until 24 wk. From 24 to 52 wk of age, the birds were kept in an all-litter floor pen at a density of 4 birds/m^2 and under natural photoperiod conditions.

A total of 40 roosters, all 1 yr old at the beginning of the experiment, were randomly selected to study the effect of access to pasture on sperm quality variables, sperm freezability, and welfare markers. In total, 20 roosters (10 of each breed) were randomly assigned to be further raised together in an outdoor housing with no access to pasture (O), whereas another 20 (10 of each breed) were similarly raised but with access to pasture from 9:00 am to 1:00 pm every day (OP). The outdoor areas in both treatments had a stocking density of 1 bird/4m^2; the associated indoor pen area had a stocking density of 4 birds/m^2. Each indoor area had a raised slatted floor (one-third of the total floor area) covering a dropping pit, with straw litter covering the rest of the floor. The pasture available to the OP birds contained plant species typical of uncultivated Mediterranean land grew: *Cichorium intybus* (chicory), *Diplotaxis erucoides* (white wall rocket), *Hordeum murinum* (mouse barley), *Lamium amplexicaule* (henbit deadnettle), *Malva sylvestris* (mallow), *Senecio vulgaris* (groundsel), and *Urtica urens* (dwarf nettle). All the birds had free access to water and were fed a diet composed of wheat, barley, soy, corn, and containing 16% CP, 2,700 kcal of ME/kg, 3.5% Ca, and 0.5% available *P* (Pasaranda, NUTER FEED S.A. (Unipersonal), Burgos Spain), accessible ad libitum over the entire experimental period. Feeders, drinkers and nest boxes were all located in the slatted floor area.

Semen was collected once per week through the experimental period, between 11 May and 29 June—a time of increasing photoperiod favorable to sperm production in roosters (Santiago-Moreno et al., 2009a)—using the massage technique of Burrows and Quinn (1937). A total of 8 semen samples from each animal were collected for fresh semen analysis (total, 320 samples). All samples were collected in 15 mL graduated centrifuge tubes (Bibby Sterilin Ltd., Stone, Staffs, UK). The ejaculate volume, its appearance, sperm concentration, sperm motility, viability, acrosome integrity and morphological abnormalities were examined on each individual semen sample. Two welfare indicators, the heterophil-to-lymphocyte (H/L) ratio and the

duration of tonic immobility (TI), were measured once weekly for all birds. During the last two weeks of the experimental period an additional semen sample was collected each week from each rooster (40 sperm samples per group (O and OP); total 80 samples) and frozen according to Santiago-Moreno et al. (2011) (see below). After 15 d, the straws were thawed for 30 s at 37°C in a water bath and the content of one straw per ejaculate poured into a polystyrene tube to assess sperm quality.

Assessment of Sperm Quality

After recording the semen volume, using a micropipette (Gilson, Villiers Le Bel, France), the ejaculates were immediately diluted 1:1 (vol/vol) at ambient temperature with Lake and Ravie medium, i.e., sodium glutamate (102.6 mM), glucose (40.4 mM), magnesium acetate $4H_2O$ (3.7 mM), potassium acetate (50.9 mM), polyvinylpyrrolidone (molecular mass = 10,000; 0.3 mM) (pH 7.08, final osmolarity 343 mOsm/kg) (Lake and Ravie, 1984). Tubes containing the diluents were kept warm in the hand of the operator to prevent temperature shock. The diluted semen was immediately cooled to 5°C, transported to the laboratory and examined within 45 min of collection. Semen appearance (color), sperm concentration, motility and viability, acrosome integrity and gross morphological abnormalities were then assessed. The semen appearance was given a numerical value: 0 transparent, 0.5 grey-transparent, 1 grey, 1.5 grey-white, 2 whitish, 2.5 milky-white, 3 white (Santiago-Moreno et al., 2009b). Total sperm concentrations were determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility was assessed by placing a small droplet of each sample, previously diluted to 1:20 (vol/vol) in the medium described above, on a warmed (37°C) glass slide. The percentage of motile spermatozoa was evaluated subjectively using a phase contrast microscope (Zeiss, Oberkochen, Germany) at 400 ×. The motility index was scored on a scale of 0 to 5, with 0 indicating no movement, 1 indicating tail movements but no progression, 2 only circular movements, 3 a large percentage of the spermatozoa showing progressive but not rectilinear movement, 4 a large percentage of spermatozoa showing rectilinear but not very vigorous movement, and 5 a large percentage of spermatozoa showing vigorous rectilinear, progressive movement (Santiago-Moreno et al., 2011). Sperm motility was determined objectively (Santiago-Moreno et al., 2012) using a computer-aided sperm analysis (CASA) system coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) and employing Sperm Class Analyzer ® v.4.0. software (Microptic S.L., Barcelona, Spain). Sperm samples were diluted 1:41 (v: v) in Lake and Ravie medium and loaded onto a warmed (38°C) 20 μm Leja® 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). A minimum of three fields and 500 sperm tracks were evaluated at 100× for each sample chamber (image acquisition rate 25 frames/s). The percentage of immotile spermatozoa, the percentage of spermatozoa showing non-progressive motility, and the percentage showing progressive motility were recorded.

Propidium iodide (PI) and SYBR-14 were used as fluorochromes for the examination of sperm viability, counting 200 cells (Chalah and Brillard, 1998). For this, 4 μl of SYBR-14 and 10 μl of semen sample were placed in an Eppendorf tube containing 200 μl of HEPES medium (20 mM Hepes, 197 mM NaCl, 2.5 mM KOH, and 10 mM glucose; pH 7.0, osmolality 400 mOsm/kg), and incubated at 5°C for 10 min in the dark. Two microliters of PI were then added and the solution incubated for 2 min at 5°C. The samples were then examined by epifluorescence microscopy (400 ×; wavelength 450 to 490 nm) using a Nikon Eclipse E200 microscope (D-FL epifluorescence, C-SHG1 super-high-pressure mercury power supply, Nikon Instruments Inc., New York, USA). Sperm stained green (SYBR-14- positive) were deemed to be live, whereas red-colored sperm (PI-positive) and sperm showing both red and green colors were considered dead (any red colors means that the membrane is impaired and has lost its function) (Figure 1).

The percentage of spermatozoa with an intact acrosome was determined by phase contrast microscopy, examining 200 cells stained with aniline blue (Water Blue: Fluka, Buchs, Switzerland) (magnification 1,000×) following the procedure of Santiago-Moreno et al. (2009a) (Figure 1). The staining solution was prepared by adding 5 g of aniline blue to 100 mL PBS, filtering, and adjusting to a pH of 3.5 with a solution of 2% glacial acetic acid (Merck, Germany). Morphological abnormalities were assessed by bright field illumination microscopy (oil immersion; magnification 1,000 ×), examining 200 spermatozoa again stained with aniline blue. The different types of abnormality were recorded according to the method of Wakely and Kosin (1951). All semen variables were analyzed by the same technician.

Sperm motility, viability and acrosome integrity were assessed in thawed sperm samples as for fresh semen.

Sperm Cryopreservation

Cooled diluted semen was further diluted with Lake and Ravie medium to a concentration of 1,200 × 10^6 sperms/ml, and the cryoprotectant dimethylacetamide added to a final concentration of 6% (1,585 mOsm, pH = 6.9) (Santiago-Moreno et al., 2011). Samples were equilibrated at 5°C for 10 min, loaded into 0.25 ml French straws (Minitüb®, Landshut, Germany), and cooled from 5°C to −35°C at 7°C/min, and then from −35°C to −140°C at 60°C/min, before being plunged into liquid nitrogen (Santiago-Moreno et al., 2011). This

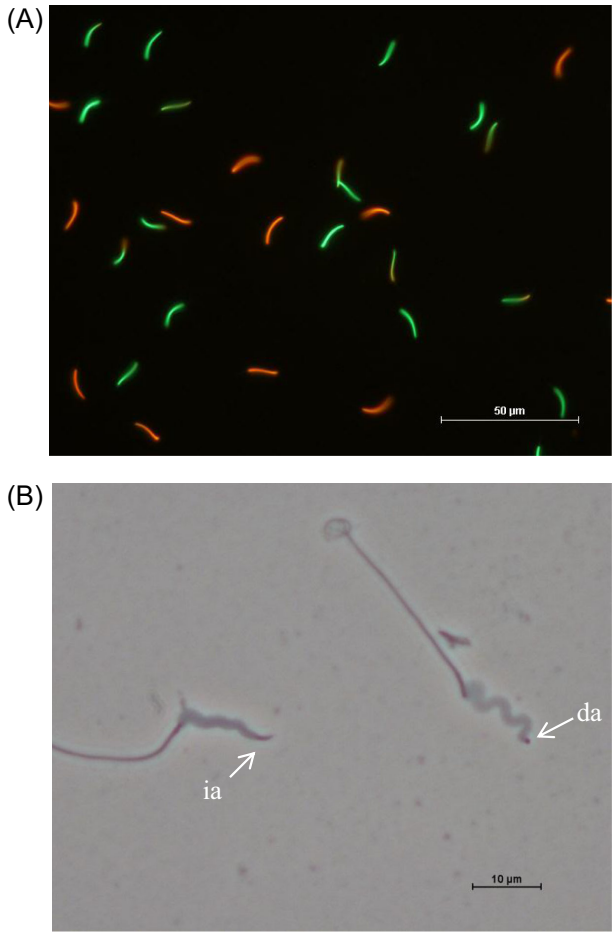

Figure 1. (A) Sperm viability evaluated with propidium iodide and SYBR-14; sperm cells stained green were deemed to be live, while red colored spermatozoa were considered dead (x400). (B) sperm stained with aniline blue with intact acrosome (ia) or damaged acrosome (da) (x1000)

freezing rate was achieved using a Computer Freezer-Icetube 1810 biological freezer unit (Minitüb, Tiefenbach, Germany).

Heterophil-to-Lymphocyte Ratio and Duration of Tonic Immobility

To determine the H/L ratio, the roosters were taken into another area and two drops of blood taken from a small puncture made in the comb; one drop was smeared on each of two glass slides. Following 2 to 4 h of fixation in methyl alcohol, the smears were stained with May-Grünwald and Giemsa stains (Lucas and Jamroz, 1961). One hundred leukocytes, including granular (heterophils, eosinophils, and basophils) and non-granular (lymphocytes and monocytes) cells, were counted on one slide for each bird (the other slide was supplementary), and the H/L ratio calculated.

TI was determined away from the birds' normal holding area on the day after blood sampling. Immediately after being caught, TI was induced by placing the birds on their back with the head hanging in a U-shaped wooden cradle for 10 s (Jones and Faure, 1981). The observer sat in full view of the bird, about 1 m away, fixing eyes on the bird (eye contact induces fear in roosters). If the bird remained immobile for 10 s after the experimenter removed his hands, a stopwatch was started to record the time elapsed until the bird righted itself. If the bird righted itself in under 10 s, it was deemed that TI had not been induced, and the restraining procedure was repeated (3 times maximum). If the bird showed no righting response over the 10 min test period, a maximum time of 600 s was recorded (thus TI ranged from 0 to 600 s).

Statistical Analysis

Differences between the groups in terms of sperm quality variables, the H/L ratio, and TI were examined by 3-way ANOVA (Sokal and Rohlf, 1981) following the model $x_{ijkl} = \mu + G_i + B_j + GB_{ij} + r_k + Gr_{ik} + Br_{jk} + GBr_{ijk} + \varepsilon_{ijkl}$, where x_{ijkl} = the analyzed measurement; μ = the overall mean; G_i = the effect of group ($i = 1 \ldots 2$); B_j = the effect of breed ($j = 1 \ldots 2$); r_k = the effect of replicate ($k = 1 \ldots 8$ for fresh semen analysis, and $k = 1 \ldots 2$ for frozen-thawed semen analysis); GB_{ij}, Gr_{ik}, Br_{jk}, and GBr_{ijk} = the interactions; and ε_{ijkl} = the residual ($l = 1 \ldots 10$). Group and breed were considered fixed effects, with replicates assumed to be random. The results showed that replicates by group, replicates by breed, and replicates by group-breed interactions were not significant in either experiment, and they were pooled with the residual to give a two-way factorial model of treatment and breed effects (xijk = μ+ Gi + Bj + GBij + εijk). Correlation ($\mathbf{r}_{xx\prime}$) between the welfare indicators ($\mathbf{x}$) and each of the sperm quality variables ($\mathbf{x}'$) was determined by ANCOVA i.e., calculated from the residual variances ($\mathbf{var}_x$ and $\mathbf{var}_{x\prime}$, respectively) and covariance ($\mathbf{cov}_{xx\prime}$): $r_{xx\prime} = cov_{xx\prime}/(var_x var_{x\prime})^{0.5}$. The significances of differences among breeds and groups were determined using the Student–Newman–Keuls multiple range test (Snedecor and Cochran, 1980). The TI and the H/L ratio showed non-normal distributions; values were therefore, respectively, transformed to logarithms and square roots before analysis. The sperm variable results were also square root-transformed before analysis.

RESULTS

Sperm Quality: Fresh Samples

The semen from the OP birds showed a significantly higher ($P < 0.05$) percentage of sperm demonstrating progressive motility (41.99%), and a higher motility index (3.90), than did that of the O group birds (26.14% and 3.00) (Table 1). No differences were seen with respect to any other sperm quality variable (Tables 1 and 2). Significant differences were also

Table 1. Values (means) for fresh semen motility variables in roosters raised with (OP) or without (O) access to pasture. Sperm motility (%), immotile sperm (%), non-progressive motility (%), and progressive motility were analyzed by CASA system. Sperm motility index was evaluated subjectively; n = 320 sperm samples.

Effect	Sperm motility (%)	Sperm motility index	Immotile sperm (%)	Non-progressive motility (%)	Progressive motility (%)
Group effect					
OP	70.03	3.90[a]	28.59	31.55	41.99[a]
O	57.47	3.00[b]	39.06	36.54	26.14[b]
Breed effect					
Red-barred Vasca	60.85	3.20[b]	36.46	34.11	33.34
Black-barred Andaluza	68.80	3.70[a]	30.55	34.34	33.93
Error mean square	600.86	0.74	543.26	164.45	641.7

[a,b]Means for the same effect and variable with no common superscript differ significantly ($P < 0.05$).

Table 2. Values (means) for fresh sperm quality variables in roosters raised with (OP) or without (O) access to pasture; n = 320 sperm samples.

Effects	Ejaculate volume (mL)	Sperm appearance (color)	Sperm viability (%)	Sperm concentration (billion/mL)	Acrosome integrity (%)	Morphologically abnormal sperm (%)
Group effect						
OP	0.17	1.90	43.09	2.99	94.07	31.46
O	0.15	1.70	34.96	3.01	94.60	39.54
Breed effect						
Red-barred Vasca	0.18	1.50[b]	37.61	2.13[b]	93.17	34.90
Black-barred Andaluza	0.13	2.40[a]	40.92	4.39[a]	96.42	37.51
Error mean square	14.26	1.22	438.32	5,549.18	114.48	422.29

[a,b]Means for the same effect and variable with no common superscript differ significantly ($P < 0.05$).

detected between breeds (Tables 1 and 2) for sperm appearance (2.40 vs 1.50, $P < 0.01$), motility index (3.70 vs 3.20, $P < 0.001$) and concentration (4.39 billion/mL vs 2.13 billion/mL, $P < 0.001$), with the Black-barred Andaluza breed's values always higher than Red-barred Vasca, independent of the O/OP treatment. No differences were seen between breeds for any other sperm quality variable.

Sperm Quality: Frozen-Thawed Samples

Table 3 shows the sperm quality values for frozen-thawed sperm samples. No significant differences were seen between the O and OP groups for any sperm quality variable. However, the Black-barred Andaluza roosters returned higher frozen-thawed sperm motility values than the Red-barred Vasca birds (24.23 vs 11.70%, $P < 0.01$), independent of O/OP treatment. The interaction *group* × *breed* had a significant ($P < 0.05$) effect on the percentage of sperm showing progressive motility (Table 4), with the Black-barred Andaluza OP birds returning lower values (7.45%) than their Red-barred Vasca counterparts (18.27%). Semen of the Red-barred Vasca birds with access to pasture did show a higher percentage of progressive motility than did that of the birds of the same breed with no such access (18.27 vs 6.65%, $P < 0.05$).

Welfare Indicators

Table 5 shows the effect of access to pasture on the H/L ratio and TI. The OP roosters had significantly more ($P < 0.01$) heterophils (41.52) and fewer ($P < 0.05$) lymphocytes (53.26) than the O birds (34.35 and 57.80, respectively); the H/L ratio was therefore higher ($P < 0.01$) in the OP birds (0.78) than in the O birds (0.61). No differences were seen between the O and OP groups in terms of TI (410.15 and 381.74, respectively), although it was significantly shorter ($P < 0.05$) in the Red-barred Vasca (326.05) than in the Black-barred Andaluza birds (470.26).

Correlation between Sperm Quality Variables and Welfare Indicators

The H/L ratio correlated significantly with sperm motility (r = 0.420; $P < 0.05$), the sperm motility index (r = 0.526; $P < 0.01$), the percentage of sperm showing immotility (r = −0.373; $P < 0.05$), and the percentage showing progressive motility (r = 0.467; $P < 0.01$). A significant correlation was also detected between TI

Table 3. Values (means) for frozen-thawed sperm quality variables in roosters raised with (OP) or without (O) access to pasture; n = 80 sperm samples.

Effects	Sperm motility (%)	Sperm motility index	Sperm viability (%)	Immotile sperm (%)	Non-progressive motility (%)	Progressive motility (%)	Intact acrosome (%)
Group effect							
OP	19.23	2.40	36.33	64.77	22.80	12.44	12.38
O	18.20	2.25	24.10	73.47	16.84	9.61	10.00
Breed effect							
Red-barred Vasca	11.70[b]	2.40	34.00	67.03	19.35	13.62	13.90
Black-barred Andaluza	24.23[a]	2.28	28.54	69.72	20.87	9.36	9.38
Error mean square	60.72	0.23	578.80	285.97	123.98	62.77	29.99

[a,b]Means for the same effect and variable with no common superscript differ significantly ($P < 0.05$).

Table 4. Values (means) for frozen-thawed sperm progressive motility (%) in Black-barred Andaluza and Red-barred Vasca roosters raised with (OP) or without (O) access to pasture; n = 80 sperm samples.

Group	Breed	
	Red-barred Vasca	Black-barred Andaluza
OP	18.27[a,x]	7.45[y]
O	6.65 [b]	11.58

[a,b]Means within the same column with no common superscript differ significantly ($P < 0.05$).
[x, y]Means within the same row with no common superscript differ significantly ($P < 0.05$).

and sperm viability (r = −0.37; $P < 0.05$). No other sperm quality variables correlated with TI.

DISCUSSION

The present results show that the outdoor housing system with access to pasture improved the motility of fresh sperm in roosters but had no overall effect on sperm cryoresistance. The H/L ratio correlated significantly with sperm motility variables.

Forage intake is known to positively affect breeding in free range chickens, the consequence of an increased intake of bioactive substances such as tocopherols, carotenoids, and α-linolenic acid (Dal Bosco et al., 2012). The antioxidant activity of many such compounds may improve the values recorded for sperm quality variables. Although natural antioxidants are present in poultry semen (Surai 1999), the relatively small amount of cytoplasm in sperm cells means they possess little antioxidant capacity and are therefore prone to ROS-induced injuries. The enhanced production of ROS by sperm leads to the functional demise of these cells as a consequence of an attack on the unsaturated fatty acids that abound in the sperm plasma membrane. Sperm contain high levels of unsaturated fatty acids that renders the sperm plasma membrane particularly susceptible to oxidative attack. Certainly, semen quality depends on the capacity to limit the harm caused by lipid peroxidation (Donoghue and Donoghue, 1997). Lipid peroxidation involves a change in the physical properties of plasma membrane, characterized by a loss of fluidity that produces a loss of fertilizing potential. The intake of antioxidants in the diet may block the chain reaction that enhances the formation of lipid radicals, and thus improving semen quality. In our study, a number of plants of the pasture have known antioxidant properties, which could explain the improved sperm quality in birds with access to pasture.

Among the plants making up the present pasture community, *C. intybus* and *U. urens* may contribute towards an increased intake of carotenoids (Guil et al., 2003; Horsted et al., 2006), whereas *M. sylvestris* and *U. urens* may improve the α-linolenic acid intake (Guil et al., 1996; Guil et al., 2003). *C. intybus* has antibacterial, anti-inflammatory and anti-hypercholesteolemic

Table 5. Mean heterophil number (H), lymphocyte number (L), H/L ratio and duration of tonic immobility (TI) in roosters raised with (OP) or without (O) access to pasture; n = 320.

Effect	H/L	H	L	TI
Group effect				
OP	0.78[a]	41.52[a]	53.26[b]	381.74
O	0.61[b]	34.35[b]	57.80[a]	410.15
Breed effect				
Red-barred Vasca	0.66	36.65	57.05	326.05[b]
Black-barred Andaluza	0.73	39.10	54.05	470.26[a]
Error mean square	0.02	44.54	31.17	35,032.53

[a,b]Means for the same effect and variable with no common superscript differ significantly ($P < 0.05$). ($P < 0.05$).

properties (Saeed et al., 2015), the consequence of its containing a number of beneficial compounds (Shad et al., 2013). This plant species has been shown to provide a protective effect against heat stress in broiler chickens by generally increasing the concentration of antioxidant enzymes (Khodadadi et al., 2016). A similar mechanism might be expected to reduce ROS production, in turn improving sperm motility. Moreover, *D. erucoides* is very rich in antioxidants, including carotenoids, flavonoids and vitamin C, rendering it a food with strong antioxidant potential (Salvatore et al., 2005) with a likely beneficial impact on reproductive function. Similarly, *L. amplexicaule* contains flavonoids that scavenge free radicals (Nugroho et al., 2009). *M. sylvestris* is widely used in traditional Mediterranean and European medicine, as well as in the ethnoveterinary treatment of various diseases (Varadyova et al., 2017). Indeed, *M. sylvestris* extract has been shown to have a beneficial antioxidative effect in different organs, including the testes (Ben Saad et al., 2017). *U. urens* is also a sources of antioxidants (Jaradat et al., 2016). Certainly, members of the genus *Urtica* have been reported to prevent the adverse effects of nicotine on sperm variables (Jalili et al., 2014).

The first question of this study was what component of the OP system resulted in the improvement of sperm motility variables, the most logical contributors being plants, and even invertebrates (worms and insects; Rivera-Ferre et al., 2007) as sources of carotenoids. The period of access to grazing (4 h a day for 7 wk) seems enough to get sufficient herbage intake (Rivera-Ferre et al., 2007) and to exert an effect during spermatogenesis, which last 17 d, and during sperm passage to deferent ducts (24 to 72 h) (Etches, 1996). In addition, OP system might also improve the sperm variables though an indirect way related to behavioral and endocrine interactions in a new enriched environment (see below).

During cryopreservation, avian sperm is subjected to a succession of thermal, osmotic and mechanical stresses (Blesbois and Brillard, 2007), the effects of which might differ in roosters raised under OP and O conditions. The Red-barred Vasca OP birds showed a higher percentage of frozen-thawed sperms demonstrating progressive motility than did the corresponding O birds. Feeding on the plant species typical of uncultivated Mediterranean land thus appeared to exert a positive, if in some respects breed dependent, effect on sperm kinetic activity. Freezing-thawing processes damage components of the antioxidant system; thus, antioxidants activity is decreased in seminal plasma after sperm cryopreservation (Partyka et al., 2012). The imbalance between ROS production and sperm antioxidant activity during freezing-thawing is a major cause of cryodamage (Chatterjee and Gagnon 2001; Partyka et al., 2012). ROS generation during cryopreservation can result in DNA damage (Lopes et al., 1998), cytoskeletal alterations (Hinshaw et al., 1986), and have negative effects on the sperm axoneme (de Lamirade and Gagnon 1992) and mitochondrial activity (Partyka et al., 2012) that lead to loss of motility. Despite the improvement in fresh semen quality via the putative contributions of dietary antioxidants, in the present work the free radical scavenging activity of the cells was still insufficient to protect from oxidative damage during cryopreservation. Many investigators have focused on the use of antioxidants in cryomedia to reduce the adverse effects of ROS on sperm cells with varying results (Amini et al., 2015; Moghbeli et al., 2016; Safa et al., 2016). However, no studies have focused on assessing the intake of plants as a possible source of antioxidants to reduce cryodamage. The present data suggest that antioxidants provided by the diet may appear in semen, but that their influence wanes through dilution and during the course of cryopreservation. However, pasture access did lead to an improvement in the frozen-thawed sperm motility in the Red-barred Vasca birds, suggesting that, at least in some breeds, it may play some cryoprotective role.

Fear is a powerful stressor seriously detrimental to animal welfare. The H/L ratio is usually greater in fearful than in less fearful birds (Jones, 1989), and as such may be used as an indicator of the stress response in chickens (Gross and Siegel, 1983; Campo and Dávila, 2002). Unexpectedly, the H/L ratio was higher in the OP birds. This might be explained in that access to pasture leaves roosters at an increased risk of certain infections and other diseases, of which a high H/L ratio is a likely physiological signs. Furthermore, unlike the O roosters, the OP roosters showed increased aggressiveness and did not undertake dust bathing. Similar results were reported by Campo et al., (2013) in chickens of different Spanish breeds. However, it should be remembered that each type of housing system presents unique challenges associated with different types of behavior (e.g., feather pecking or competition for certain types of plant) that may result in stress. Gross and Siegel (1993) suggested that H/L ratios of about 0.2, 0.5, and 0.8 are characteristic of low, moderate, and high degrees of stress, respectively. However, some authors suggest a value of 0.8 may be considered to reflect moderate stress (Campo and Dávila, 2002). Our data suggest that H/L seems reflect a context of increased aggressiveness in the OP system rather a stress condition proper. In fact, the TI, a well-established stress marker (Gallup 1979), was no different in the OP and O birds. Note, however, that TI was shorter in the Red-barred Vasca than in the Black-barred Andaluza roosters, in agreement with that reported by other authors (Campo and Alvarez, 1991). In the present work, the H/L ratio correlated with sperm motility. The competition and aggressive behaviors in the OP system might have an underlying endocrine modulation by androgens. The testosterone has a dual effect, enhancing the aggressive behavior and sperm kinetic function of males but depressing their immune response (Lehmann, et al., 1988; Folstad and Karter, 1992), that might explain its

influence on increasing heterophils and decreasing of lymphocytes and the positive correlation between H/L ratio and sperm motility.

In conclusion, access to pasture improves fresh sperm motility. Although overall offered no cryoprotective benefits, in the Red-barred Vasca birds the access to pasture appears to have a certain protective role during freezing-thawing process. The unexpected increase of H/L ratio in the OP birds may be due to behavioral and immunological factors associated with this management system.

REFERENCES

Akhlaghi, A., Y. J. Ahangari, M. Zhandi, and E. Peeble. 2014. Reproductive performance, semen quality, and fatty acid profile of spermatozoa in senescent broiler breeder roosters as enhanced by the long-term feeding of dried apple pomace. Anim. Reprod. Sci. 147:64–73.

Akhlaghi, A., Y. J. Ahangari, B. Navidshad, Z. A. Pirsaraei, M. Zhandi, H. Deldar, M. R. Rezvani, M. Dadpasand, S. R. Hashemi, R. Poureslami, and E. D. Peebles. 2014b. Improvements in semen quality, sperm fatty acids, and reproductive performance in aged Cobb 500 breeder roosters fed diets containing dried ginger rhizomes (*Zingiber officinale*). Poult. Sci. 93:1236–1244.

Amini, M. R., H. Kohram, A. Zare-Shahaneh, M. Zhandi, H. Sharideh, and M. M. Nabi. 2015. The effects of different levels of catalase and superoxide dismutase in modified Beltsville extender on rooster post-thawed sperm quality. Cryobiology 70:226–232.

Ben Saad, A., I. Rjeibi, H. Alimi, S. Ncib, S. Amani, N. Zouari, and L. Zourgui. 2017. Lithium induced, oxidative stress and related damages in testes and heart in male rats: the protective effects of Malva sylvestris extract. Biomed. Pharmacother. 86:127–135.

Blesbois, E., and J. P. Brillard. 2007. Specific features of in vivo and in vitro sperm storage in birds. Animal 1:1472–1481.

Borghei-Rad, S.M., S. Zeinoaldini, M. Zhandi, H. Moravej, and M. Ansar. 2017. Feeding rosemary leaves powder ameliorates rooster age-related subfertility. Theriogenology 101:35–43.

Burrows, W. H., and J. P. Quinn. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poult. Sci. 16:19–24.

Campo, J. L. 1998. Conservation and genetic study of Spanish chicken breeds (28). Pages 155–158 in Proc. 6th World Congr. Genet. Appl. Livestock Prod. Univ. New England, Armindale, New South Wales, Australia.

Campo, J. L. 2010. Razas Españolas de Gallinas. El Programa de Conservación del INIA (1975-2010). J. L. Campo, ed. INIA, Madrid, Spain.

Campo, J. L., and F. Orozco. 1982. Conservation and genetical study of Spain chicken breeds (6). Pages 88–93 in Proc. 2nd World Congr. Genet. Appl. Livestock Prod.. Editorial Garsi, Madrid, Spain.

Campo, J. L., and C. Alvarez. 1991. Tonic immobility of several Spanish breeds of chickens. Archiv Geflügelkunde 55:19–22.

Campo, J. L., and S. G. Dávila. 2002. Estimation of heritability for heterophil:lymphocyte ratio in chickens by restricted maximum likelihood. Effects of age, sex, and crossing. Poult. Sci. 81:1448–1453.

Campo, J. L., M. T. Prieto, and S. G. Dávila. 2008. Effects of housing system and cold stress on heterophil-to-lymphocyte ratio, fluctuating asymmetry, and tonic immobility duration of chickens. Poult. Sci. 87:621–626.

Campo, JL, R Cabezas, O Torres, IG Briones, and C Alonso. 2013. Egg quality and welfare of white-, tinted-, and brown-shell egg layers in three different non-cage housing systems. Archiv Fur Geflugelkunde 77:179–188.

Chalah, T., and J. P. Brillard. 1998. Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual fluorescence (SYBR-14/PI). Theriogenology 50:487–493.

Chatterjee, S., and C. Gagnon. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev. 59:451–458.

Cornwallis, C. K., and T. R. Birkhead. 2007. Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. Am. Nat. 170:758–770.

Dal Bosco, A., C. Mugnai, S. Ruggeri, S. Mattioli, and C. Castellini. 2012. Fatty acid composition of meat and estimated indices of lipid metabolism in different poultry genotypes reared under organic system. Poult. Sci. 91:2039–2045.

Dávila, S. G., J. L. Campo, M. G. Gil, C. Castaño, and J. Santiago-Moreno. 2015. Effect of the presence of hens on roosters sperm variables. Poult. Sci. 94:1645–1649.

Donoghue, A. M., and D. J. Donoghue. 1997. Effects of water- and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. Poult. Sci. 76:1440–1445.

Etches, R. J. 1996. Reproduction in Poultry. CABI International, Wallingford, UK.

Folstad, I., and A. J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am. Nat. 139:603–622

Gallup, G. G. 1979. Tonic immobility as a measure of fear in domestic fowl. Anim. Behav. 27:316–317.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Dis. 27:972–979.

Gross, W. B., and P. B. Siegel. 1993. General principles of stress and welfare. Pages 21–34 in Livestock, Handling and Transport. T. Grandin, ed. CABI, Wallingford, UK.

Guil, J. L., M. E. Torija, J. J. Giménez, and I. Rodríguez. 1996. Identification of fatty acids in edible wild plants by gas chromatography. J. Chromatogr. A 719:229–235.

Guil, J. L., M. M. Rebolloso, and M. E. Torija. 2003. Fatty acids and carotenoids from stinging nettle (*Urtica dioica*). J. Food Compos. Anal. 16:111–119.

Hinshaw, D. B., L. A. Sklar, B. Bohl, I. Schraufstatter, P.A. Hyslop, M.W. Rossi, R. Spragg, and C. Cochrane. 1986. Cytoskeletal and morphologic impact of cellular oxidant injury. Am. J. Pathol. 123:454–464.

Horsted, K., M. Hammershoj, and J. E. Hermansen. 2006. Short-term effects on productivity and egg quality in nutrient-restricted versus non-restricted organic layers with access to different forage crops. Acta Agric. Scand. A 56:42–54.

Jalili, C., M. R. Salahshoor, and A. Naseri. 2014. Protective effect of Urtica dioica L against nicotineinduced damage on sperm parameters, testosterone and testis tissue in mice. Iran. J. Reprod. Med. 12:401–408.

Jaradat, N. A., B. Damiri, and M. N. Abualhasan. 2016. Antioxidant evaluation for *Urtica urens*, *Rumex cyprius* and *Borago officinalis* edible wild plants in Palestine. Pak. J. Pharm. Sci. 29 (1 Suppl):325–330.

Jones, R. B. 1989. Chronic stressors, tonic immobility and leucocytic responses in the domestic fowl. Physiol. Behav. 46:439–442.

Jones, R. B., and J. M. Faure. 1981. Sex and strain comparisons of tonic immobility ("Righting time") in the domestic fowl and the effects of various methods of induction. Behav. Processes. 6:47–55.

Khodadadi, M., S. S. Mousavinasab, F. Khamesipour, and S. Katsande. 2016. The effect of *Cichorium intybus* L. ethanol extraction on the pathological and biomedical indexes of the liver and kidney of broilers reared under heat stress. Rev. Bras. Cienc. Avic. 18:407–412.

Lake, P. E., and O. Ravie. 1984. An exploration of cryoprotective compounds for fowl spermatozoa. Br. Poult. Sci. 25:145–150.

de Lamirade, E., and C. Gagnon. 1992. Reactive oxygen species and human spermatozoa. I. effects on the motility of intact spermatozoa and on sperm axonemes. J. Androl. 13:368–378.

Lay, D. C., R. M. Fulton, P. Y. Hester, D. M. Karcher, J. B. Kjaer, J. A. Mench, B. A. Mullens, R. C. Newberry, C. J. Nicol, N. P. O'Sullivan, and R. E. Porter. 2011. Hen welfare in different housing systems. Poult. Sci. 90:278–294.

Leone, E. H., and I. Estevez. 2008. Use of space in the domestic fowl: separating the effects of enclosure size, group size and density. Anim. Behav. 76:1673–1682

Lehmann, D., K. Siebold, and L. R. Emmons. 1988. Androgens inhibit proliferation of human peripheral blood lymphocytes in vitro. Clin. Immunol. Immunopathol. 46:122–128.

Lopes, S., A. Jurisicova, J. Sun, and R. F. Casper. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum. Reprod. 13:896–900.

Long, J. A. 2006. Avian semen cryopreservation: What are the biological challenges? Poult. Sci. 85:232–236.

Lucas, A. M., and C. Jamroz. 1961. Atlas of Avian Hematology. Agriculture Monograf 25. USDA, Washington DC.

Moghbeli, M., H. Kohram, A. Zare-Shahaneh, M. Zhandi, M. Sharafi, M. M. Nabi, V. Zahedi, and H. Sharideh. 2016. Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? Cryobiology 72:264–268.

Nugroho, A., J. K. Choi, J. H. Park, K. T. Lee, B. C. Cha, and H. J. Park. 2009. Two New flavonol glycosides from *Lamium amplexicaule* L. and their in vitro free radical scavenging and tyrosinase inhibitory activities. Planta Med. 75:364–366.

O'Flaherty, C., M. Beconi, and N. Beorlegui. 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia 29:269–275.

Ommati, M., M. J. Zamiri, A. Akhlaghi, H. Atashi, M. R. Jafarzadeh, M. R. Rezvani, and F. Saemi. 2013. Seminal characteristics, sperm fatty acids, and blood biochemical attributes in breeder roosters orally administered with sage (*Salvia officinalis*) extract. Anim. Prod. Sci. 53:548–554.

Partyka, A., E. Łukaszewicz, and W. Nizanski. 2012. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. Theriogenology 77:1497–1504.

Prieto, M. T., J. L. Campo, and J. Santiago-Moreno. 2011. Relationship among fluctuating asymmetry, morphological traits, and sperm quality in layers. Poult. Sci. 90:2845–2854.

Rivera-Ferre, M.G., E. A. Lantinga, and R. P. Kwakkel. 2007. Herbage intake and use of outdoor area by organic broilers: effects of vegetation type and shelter addition. NJAS-Wagening. J. Life Sci. 54:279–291.

Saeed, M., A. R. Baloch, M. Wang, R. N. Soomro, A. M. Baloch, B. A. Bux, M. A. Arian, S. S. Faraz, and H. M. Zakriya. 2015. Use of *Cichorium intybus* leaf extract as growth promoter, hepatoprotectant and immune modulent in broilers. J. Anim. Prod. Adv. 5:585–591.

Safa, S., G. Moghaddam, R. J. Jozani, H. Daghigh Kia, and H. Janmohammadi. 2016. Effect of vitamin E and selenium nanoparticles on post-thaw variables and oxidative status of rooster semen. Anim. Reprod. Sci. 174:100–106.

Salvatore, S., N. Pellegrini, O. V. Brenna, D. Del Rio, G. Frasca, F. Brighenti, and R. Tumino. 2005. Antioxidant characterization of some Sicilian edible wild Greens. J. Agric. Food Chem. 53:9465–9471

Santiago-Moreno, J., C. Castaño, M. A. Coloma, A. Gómez-Brunet, A. Toledano-Díaz, A. López-Sebastián, and J. L. Campo. 2009a. Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry. Poult. Sci. 88:2661–2669.

Santiago-Moreno, J., A. López-Sebastián, C. Castaño, M. A. Coloma, A. Gómez-Brunet, A. Toledano-Díaz, M. T. Prieto, and J. L. Campo. 2009b. Sperm variables as predictors of fertility in Black Castellana roosters: use in the selection of sperm donors for genome resource banking purposes. Span. J. Agric. Res. 7:555–562.

Santiago-Moreno, J., C. Castaño, A. Toledano-Díaz, M. A. Coloma, A. López-Sebastian, M. T. Prieto, and J. L. Campo. 2011. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: optimization of freezing rate and equilibration time. Poult. Sci. 90:2047–2053.

Santiago-Moreno, J., C. Castaño, A. Toledano-Díaz, M. A. Coloma, A. López-Sebastián, M. T. Prieto, and J. L. Campo. 2012. Influence of season on the freezability of free-range poultry semen. Reprod. Dom. Anim. 47:578–583.

Shad, M. A, H. Nawaz, T. Rehman, and N. Ikram. 2013. Determination of some biochemicals, phytochemicals and antioxidant properties of different parts of *Cichorium intybus* L.: a comparative study. J. Anim. Plant Sci. 23:1060–1066.

Singh, R., K. M. Cheng, and F. G. Silversides. 2009. Production performance and egg quality of four strains of laying hens kept in conventional cages and floor pens. Poult. Sci. 88:256–264.

Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. 7th ed. Iowa State University Press, Ames, IA.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry. Freeman and Co, London U.K.

Surai, P. F. 1999. Vitamin E in avian reproduction. Avian Poult. Biol. Rev. 10:1–60

Surai, P. F., R. C. Noble, N. H. C. Sparks, and B. K. Speake. 2000. Effect of long-term supplementation with arachidonic or docosahexaenoic acids on sperm production in the broiler chicken. J. Reprod. Fertil. 120:257–264.

Surai, P. F., N. Fujihara, B. K. Speake, J. P. Brillard, G. J. Wishart, and N. H. C. Sparks. 2001. Polyunsaturated fatty acids, lipid peroxidation and antioxidant protection in avian semen—Review. Asian Australas. J. Anim. Sci 14:1024–1050.

Van de Weerd, H. A., R. Keatinge, and S. Roderick. 2009. A review of key health-related welfare issues in organic poultry production. Worlds Poult. Sci. J. 65:649–684.

Varadyova, Z., S. Kisidayova, K. Cobanova, E. Gresakova, M. Babjak, A. Konigova, M. U. Dolinska, and M. Varady. 2017. The impact of a mixture of medicinal herbs on ruminal fermentation, parasitological status and hematological parameters of the lambs experimentally infected with *Haemonchus contortus*. Small Rumin. Res. 151:124–132.

Wakely, W. J., and I. L. Kosin. 1951. A study of the morphology of the turkey spermatozoa with special reference to a seasonal prevalence of abnormal types. Am. J. Vet. Res. 12:240–245.

Walker, A., and S. Gordon. 2003. Intake of nutrients from pasture by poultry. Proc. Nutr. Soc. 62:253–256.