# Characterization of red-legged partridge (*Alectoris rufa*) sperm: Seasonal changes and influence of genetic purity

J. Santiago-Moreno,[*1,2] C. Castaño,[*] A. Toledano-Díaz,[*] M. C. Esteso,[*] A. López-Sebastián,[*] N. Gañán,[*] M. J. Hierro,[†] F. Marchal,[‡] J. L. Campo,[§] and E. Blesbois[**]

[*]*Dpto. Reproducción Animal, INIA, Spain;* [†]*Consejería Medio Ambiente y Ordenación del Territorio, Junta de Andalucía, Spain;* [‡]*Agencia de Medio Ambiente y Agua de Andalucía, Junta de Andalucía, Spain;* [§]*Dpto. Mejora Genética Animal, INIA, Spain; and* [**]*INRA 0085 UMR PRC INRA-CNRS-University François Rabelais-Haras Nationaux, 37380 Nouzilly, France*

**ABSTRACT** The general decline in wild Iberian populations of the red-legged partridge (*Alectoris rufa*) has been accompanied by an increase in game-farm facilities producing hybrids with chukar partridges (*Alectoris chukar*). Genetic introgression from chukar partridges is thought to modify male red-legged partridge reproductive indicators. The aim of the present study was to determine the effects of such genetic introgression on seasonal reproductive patterns by comparing the sperm and plasma testosterone concentrations of males from pure red-legged and hybrid red-legged/chukar populations. Semen was collected twice monthly over a 12-mo period using a massage technique. Both types of bird showed a clear seasonal pattern of spermatogenic activity. The proportion of males ejaculating sperm was higher ($P < 0.05$) among the pure red-legged birds. The greatest sperm production was recorded in March to May among the pure birds and April to May among the hybrids. Reproductive activity in both groups decreased in June, to reach a minimum in August to December among the hybrids and in September to December among the pure birds. Spermatogenic activity resumed in January in both groups. The sperm concentration produced by the pure birds was smaller than that of the hybrids ($P < 0.001$), but the percentage of motile sperm was higher in the pure birds ($P < 0.001$). The sperm of the hybrids showed greater straight-line velocity ($P < 0.05$), linearity ($P < 0.001$), straightness ($P < 0.001$), sperm wobble ($P < 0.05$), and beat-cross frequency values ($P < 0.001$). The length and area of the sperm head were smaller in the pure birds ($P < 0.05$). The seasonal plasma testosterone concentration pattern followed a trend roughly parallel to the ejaculatory response. The present results suggest that genetic introgression influences the reproductive variables of the red-legged partridge.

**Key words:** breeding activity, introgression, red-legged partridge, sperm morphometry, testosterone

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## INTRODUCTION

The red-legged partridge (*Alectoris rufa*) is a southwestern European game bird that inhabits farmland and open stony areas. The greater part of its global population is found within Spain, where it breeds naturally. Despite its ecological (biodiversity, food chain) and economic (game resource) importance, wild populations have declined sharply since the 1970s, the consequence of changes in agricultural practices and overhunting (Blanco-Aguiar et al., 2003; Casas and Viñuela, 2010; Díaz-Fernández et al., 2012). The general decline of wild populations in the Iberian Peninsula has been accompanied by an increase in game-farm facilities producing birds for release to maintain hunting quotas. It is estimated that at least 3 to 4 million farm-bred red-legged partridges are released every year in Spain (Garrido, 2002; Sánchez-García et al., 2009), but populations have failed to recover because these raised birds show reduced antipredator behaviour. Their mortality rate is therefore high. The release of hybrids produced by crossing the red-legged partridge and the chukar partridge (*Alectoris chukar*), a Eurasian species, is threatening the genetic integrity of wild Spanish birds. Indeed, at least 63% of supposedly red-legged partridges now raised on game farms, and 30 to 45% of birds living in the wild, possess chukar partridge genes (Blanco-Aguiar et al., 2008; Casas et al., 2013). Although the release of hybrid partridges has been forbidden under Spanish law, the competitiveness of markets has led to their continued, illegal, production. Indeed, *A. rufa* × *A. chukar* hybrids are preferred by game breeders because they are less sensitive to stress, and their productivity is better under captive


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[1]This work was funded by INIA grant RZ2012-00013-C02.
[2]Corresponding author: E-mail: moreno@inia.es; Tel: +34 91347 4020; Fax: +34 91347 4014

conditions (longer laying period and heavier body weight) (Potts, 1989). The preservation of the wild population of red-legged partridges is also affected by newly emerging infectious diseases, the propagation of which is facilitated by hybridization (Viñuela et al., 2013; Agüero et al., 2011).

It is here hypothesized that hybridization, and the artificial selection of productive features under farms conditions, might determine variations in sperm production in red-legged partridges and perhaps even modify the morphological and functional characteristics of their sperm. *A. chukar* is more promiscuous than *A. rufa*, which is monogamous (Vidal and Colominas, 2007), and it is well known that polyandry creates a background for sexual selection among males both before and after copulation (sperm competition) that influences a number of sperm variables (Pizzari et al., 2007; Parker and Pizzari, 2010; Collet et al., 2012), including the number of sperm produced, the percentage of sperm abnormalities, sperm cell size (Birkhead, 2000, Kleven et al., 2009), and swimming velocity variables (Kleven et al., 2009, Santiago-Moreno et al., 2014).

In this study, the reproductive indicators of pure red-legged and hybrid red-legged/chukar partridges were compared over a period of one year to examine the potential impact on the former species of chukar partridge genetic introgression.

## MATERIALS AND METHODS

### Animals

The studied birds were 19 pure red-legged partridges, all adult males, from the Lugar Nuevo red-legged partridge breeding facility (Andújar, Spain, 38°16′N; this facility belongs to the regional government of Andalusia and maintains a genetically pure red-legged partridge population [Dávila, 2009]), and 22 adult male red-legged partridges showing genetic introgression from *Alectoris chukar*. These birds were obtained from a commercial farm (Sequera, Segovia, Spain, 41°22′N), and genetic introgression by *A. chukar* is >35%. The genetic purity and *A. chukar* introgression shown by these two populations were tested according to official genetic analysis procedures recommended by the regional governments (Andalusian, Castilla y León) (FEDENCA, 2011). Genetic introgression was tested using mitochondrial cytochrome b gene (*mtDNA Cyt-b*), 7 species-specific nuclear DNA microsatellites for red-legged partridges (Aru1E45, Aru1E78, Aru1I68, Aru1F138, Aru1V16, Aru1H15, and Aru1F32), and 7 single nucleotide polymorphisms (**SNPs**) in mtDNA. The genetic testing was done by official regional government laboratories, using the techniques described by FEDENCA, 2011 (Andalusian, Castilla y León). The reports with results of analysis were provided to certify the purity or hybridization of birds. All birds used from the Lugar Nuevo red-legged partridge breeding facility were pure. Genetic assay was conducted in birds randomly selected from the commercial farm; the results revealed that 35% of sampled birds showed genetic introgression with *A. chukar*.

All the birds were 2-y-old at the beginning of the experiment. They were housed in outdoor cages (90 by 82 by 60 cm) in groups of three birds under natural photoperiod and temperature conditions at the El Encín Research Station (Alcalá de Henares, Spain, 40°31′N]). Naturally occurring variations in day length at that latitude were from 10 h 17 min to 16 h 3 min light/d (winter to summer solstices), including twilight. All birds were fed (ad libitum) a commercial feed containing 16% CP, 2,700 kcal of ME/kg, 3.5% Ca, and 0.5% available P over the entire experimental period, which lasted 12 mo. Periodically, they were supplemented with amino acids and vitamins (Aminolid, Intervet Schering Plough Animal Health, Barcelona, Spain) via their water. The birds were weighed using a 1000 g Pesola (Pesola light line, Oryx) precision scale (nearest 10 g) at the start of the experiment.

### Sperm Collection and Assessment of Sperm Variables

Sperm was collected twice per month using the massage technique of Burrows and Quinn (1937), as adapted to this species. Two people were required for this. One caught the male and held him firmly over a table, with one hand holding the legs and the other immobilizing the body and wings. The other person stimulated the bird, simultaneously stroking the back with the right hand and the abdomen with the left hand. The bird was then "milked" by making the copulatory organ protrude by mild stimulation and then gripping its base with the thumb and index finger of the right hand. Semen was recovered by capillarity using a microhaematocrit tube (GmbH + Co. KG, Wertheim, Germany) and immediately diluted 1:1 (v:v) at ambient temperature using a medium-containing sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate ($4H_2O$; 0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone ($M_r$ 10,000; 0.3 g) and 100 mL $H_2O$ (pH 7.08, osmolality 343 mOsm $kg^{-1}$; hereinafter referred to as Lake and Ravie medium) (Lake and Ravie, 1984). Diluted semen was immediately refrigerated at 5°C and transported to the laboratory. All individual sperm samples were examined within 45 min of collection, and ejaculate volume, sperm concentration, motility, and morphometric variables were recorded.

Sperm volume was calculated by measuring the length of the semen column in the microcapillary tube using a plastic ruler (accuracy ± 1 mm) and calculating the equivalent value in volume units ($\mu$L).

Sperm concentration and motility were assayed as previously described (Santiago-Moreno et al., 2012) using a computer-aided sperm analyses (CASA) system coupled to a phase contrast microscope (Nikon Eclipse model 50i, Nikon Instruments Europe B.V., Izasa S.A.; negative contrast) and employing Sperm Class

Analyzer (SCA, Barcelona, Spain) v.4.0. software (Microptic S.L., Barcelona, Spain). For motility analysis, sperm samples were diluted to a concentration of approximately 40 million sperm/mL and loaded onto warmed (38°C) 20 $\mu$m Leja 8-chamber slides (Leja Products B.V., Nieuw-Vennep, The Netherlands). The percentage of motile spermatozoa and the percentage showing progressive motility (spermatozoa swimming forward quickly in a straight line) were recorded. Sperm movement characteristics—curvilinear velocity (**VCL**), straight-line velocity (**VSL**), average path velocity (**VAP**), amplitude of lateral head displacement (**ALH**), and beat-cross frequency (**BCF**)—were also recorded. Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity of forward progression (LIN = VSL/VCL × 100), straightness (STR = VSL/VAP × 100), and wobble (WOB = VAP/VCL × 100). Mean values of VSL, VCL, VAP, ALH, and BCF parameters describe the vigor of spermatozoa, while LIN, STR and WOB indicate progressiveness. A minimum of 3 fields and 500 sperm tracks were evaluated at a magnification of 10× for each sample (image acquisition rate 25 frames/s).

Sperm head morphometry was assessed in 16 sperm smears (one for each individual bird) per experimental group. A 5 $\mu$L drop of each diluted sperm sample was spread on a glass slide and allowed to dry. These smears were then fixed at room temperature in buffered 2% glutaraldehyde in PBS for 30 min and air-dried. The slides were then stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH = 3.5) for 5 min, washed with distilled water, and air-dried once more. The staining solution was prepared by adding 5 g of aniline blue (Water Blue, Fluka, Sigma-Aldrich, St. Louis, MO) to 100 mL PBS, filtering, and adjusting to a pH of 3.5 with a solution of 2% glacial acetic acid (Merck, Darmstadt, Germany). The samples stained with aniline blue were then used to perform a computerized morphometric analysis using a computer-aided cell analysis system (Software Motic Image Advanced version 3.0, Motic Spain, S.L.U. Barcelona, Spain) coupled to a Motic BA 210 optical microscope (Motic Spain, S.L.U. Barcelona, Spain) (Figure 1). The smears were examined using a 100× oil immersion objective (bright field). The video signal was acquired using a Moticam camera (1SP 1.3 MP, Motic Spain S.L, Barcelona, Spain) attached to the microscope and connected to a computer. Twenty-five spermatozoa per sample were randomly captured under the program's manual acquisition mode. Each sperm head was measured for length (including the acrosome), width, and area. The area was calculated as the sum of the pixelated area within the head boundary. The length and width were measured using the virtual callipers provided by the program. The system detected the boundary of sperm heads, and their outlines were displayed as green overlays superimposed on the video image. Head boundary detections were traced manually by the operator using an editing tool provided by the system. Aniline blue staining clearly separated the end of the head from the midpiece and the start of the flagellum; precise measurements of the sperm head were therefore guaranteed.

## Blood Collection and Testosterone Analysis

Blood samples (0.2 mL) were recovered monthly from the brachial vein of 10 representative birds, chosen randomly, from each group (10 pure birds and 10 hybrid birds). This sampling regimen was considered less stressful for the birds than more frequent sampling. The samples from each group were then separately pooled to increase the accuracy of the mean concentrations recorded; marked fluctuations in peripheral plasma testosterone concentrations can occur over relatively short periods due to intermittent secretion. Each blood pool was centrifuged at 1,500 × $g$ for 20 min. The plasma was immediately separated and stored at –20°C until hormone analysis was undertaken. Plasma testosterone was determined by radioimmunoassay as previously described (Fargallo et al., 2007) (detection limit 0.05 ng/mL). The samples were analyzed in a single assay. The intra-assay coefficient of variation was 10.7% (n = 10), and the mean extraction recovery was 74.5% (n = 10). Low and high testosterone control samples were included at frequent intervals in these assays.

## Statistical Analysis

Sperm variables that were not normally distributed, as determined by the Shapiro-Wilk test, were arcsine-transformed before statistical analysis. All sperm variables, except the morphometric variables, were normally distributed after arcsine transformation. Therefore, differences in these sperm variables were assessed by the parametric repeated-measures ANOVA following the statistical model:

$$x_{ijk} = m + A_i + a_{j(i)} + B_k + AB_{ik} + e_{ijk}$$

Where:
$x_{ijk}$ = Measured sperm variable
m = Overall mean of variable $x$
$A_i$ = Effect of group (i = 1 to 2 groups)
$a_{j(i)}$ = Effect of a bird within a group
$B_k$ = Effect of month or season (k = 1 to 9 for months; j = 1 to 3 for seasons)
$AB_{ik}$ = Interaction between A and B
$e_{ijk}$ = Residual (confounded with interaction between bird [$a_{j(i)}$] and month/season [$B_k$])

In the analysis, only the seasons of spring (April, May, June), summer (July, August, September), and winter (January, February, March) were taken into account; autumn was not included because virtually no ejaculates were obtained during this time (just from a few birds in October).

Even after arcsine transformation, the morphometric variables of the sperm from both the pure and

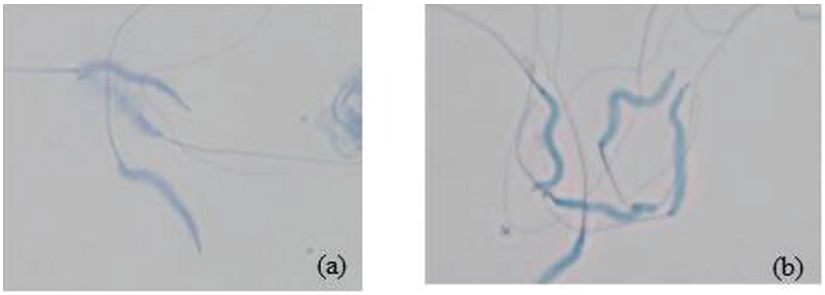


**Figure 1.** Sperm from pure (a) and hybrid (b) red-legged partridges stained with aniline blue and analyzed using a computer-aided cell analysis system (Software Motic Image Advanced version 3.0).

hybrid birds were not normally distributed, as determined by the Shapiro-Wilk test. The comparison of sperm head measures was therefore undertaken using the nonparametric Mann-Whitney U Test for unmatched samples. The statistical model used was:

$$x_{ij} = m + A_i + e_{ij}$$

Where:
$x_{ij}$ = Measured sperm head variable
m = Overall mean of variable $x$
$A_i$ = Effect of group (i = 1 to 2 groups)
$e_{ij}$ = Residual (j = 1 to 16).

The duration of reproductive activity in the two groups was compared using the Student $t$-test. The chi-square test was used to determine whether the percentage of birds that ejaculated, and the presence of spermatozoa, was associated with genetic purity. Linear Pearson correlations were calculated to assess the significance of the association between seasonal variations in plasma testosterone concentration and the percentage of birds showing an ejaculatory response and the presence of sperm. The results are presented as mean ± SEM. All statistical calculations were made using Statistica software for Windows v.12 (StatSoft Inc., Tulsa, OK).

## RESULTS

The BW of the hybrids was greater than that of the pure birds (463.6 ± 7.5 g vs 427.9 ± 16.9 g; $P < 0.05$). Figure 2 shows the percentage of pure and hybrid birds showing an ejaculatory response to massage and the presence of spermatozoa. Both groups of birds showed a clear seasonal pattern in reproductive activity. Spermatogenic activity began in January in both groups, but the proportion of males ejaculating sperm was higher ($P < 0.05$) in the pure species than in the hybrids (Figure 2). In the pure birds, the strongest ejaculatory response with presence of spermatozoa was recorded from March to May (over 75% of birds), whereas in the hybrids, the maximum ejaculatory response was recorded from April to May (again 75%). In both groups, the reproductive response declined in June and reached a minimum, with no response, from September to December in the pure birds (8 mo of semen production) and from August to December in the hybrids (7 mo of semen production). The mean duration of the sperm-producing period was very variable between individuals, at 2 to 8 mo (4.9 ± 0.3 mo) in the pure birds and 3 to 6 mo (4.1 ± 0.3 mo) in the hybrids (no significant difference). In both groups, a residual reproductive response (<8% of birds), accompanied by very low semen volumes (<3 $\mu$L), was observed in October.

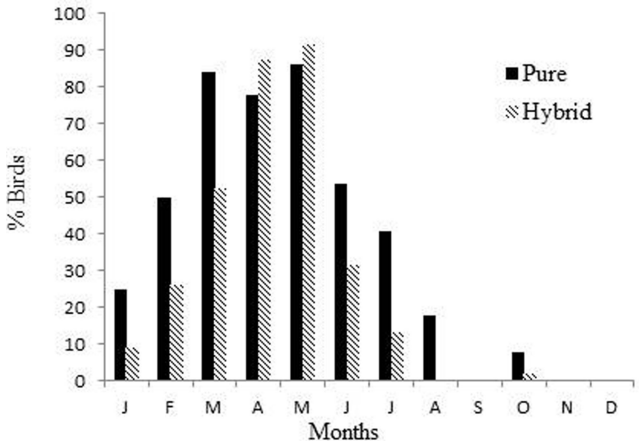


**Figure 2.** Percentage of birds showing an ejaculatory response to massage, and the presence of spermatozoa.

Sperm concentration was the only sperm variable affected by season in either group (Table 1), with higher sperm concentrations recorded in spring than in winter and summer ($P < 0.001$). The hybrids, however, always yielded higher mean sperm concentrations. Although the remaining variables were not affected by season, some representative sperm parameters in pure and hybrid birds during different seasons are illustrated in Figure 3. Tables 2 and 3 compare the quantitative and motility variables recorded for the two groups of birds. The percentage of motile sperm was higher in the pure birds, but no differences were seen in the progressive sperm motility. Straight-line velocity (**VSL**), linearity of forward progression (**LIN**), straightness (**STR**), wobble (**WOB**), and beat-cross frequency (**BCF**) were higher in the hybrid birds (Table 3). The length and the area of the sperm head were smaller in the pure than in the hybrid birds (Table 4).

Figure 4 shows that the seasonal plasma testosterone concentration pattern followed a trend roughly

**Table 1.** Effect of season on sperm concentration in pure red-legged partridge (*Alectoris rufa,* n = 19) and hybrid birds (*A. rufa* × *A. chukar;* n = 22).

| Sperm concentration | Winter | Spring | Summer | *P* |
|---|---|---|---|---|
| Pure birds ($\times 10^6$sperm/mL) | $60.9 \pm 25.7^{b}$ | $749.7 \pm 144.6^{a}$ | $49.7 \pm 20.0^{c}$ | 0.00 |
| Hybrids ($\times 10^6$sperm/mL) | $524.25 \pm 152.12^{ab}$ | $1480.2 \pm 88.5^{a}$ | $180.75 \pm 104.0^{b}$ | 0.00 |

Different letters within rows indicate significant differences between seasons within each bird group.

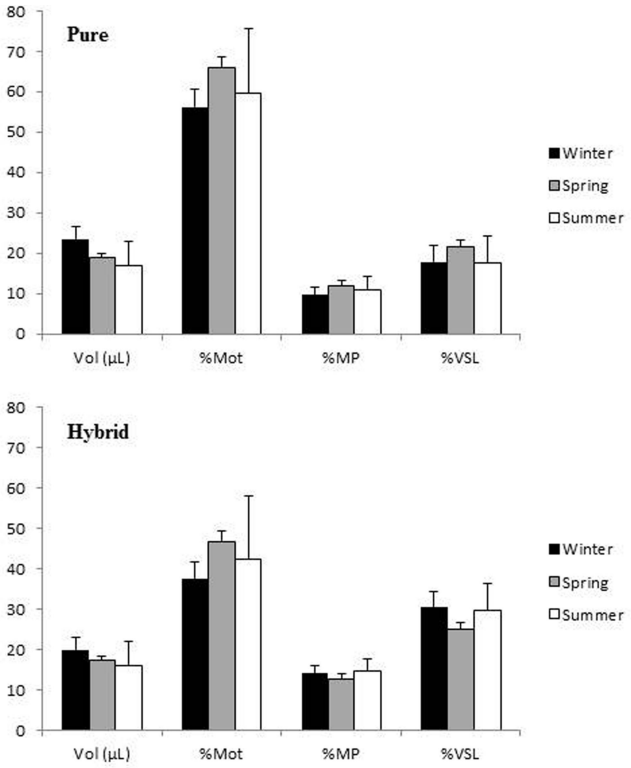


**Figure 3.** Representative sperm parameters in pure and hybrid birds during different seasons.

parallel to the ejaculatory response. The ejaculatory response peak was temporally slightly shifted from the testosterone level peak. Testosterone concentrations increased in January in both groups of birds, reaching maximum values in March and April, before falling to basal levels again from August to December. The plasma testosterone concentration correlated significantly with the reproductive response in both the pure ($R^2 = 0.69$, $P < 0.001$) and hybrid birds ($R^2 = 0.59$, $P < 0.01$).

**Table 3.** Sperm motility variables for the pure birds (*Alectoris rufa,* n = 19) and hybrids (*A. rufa* × *A. chukar;* n = 22), as determined by CASA.

| Sperm motility variables | Pure | Hybrid | *P* |
|---|---|---|---|
| Motile sperm (%) | $61.9 \pm 2.5^{a}$ | $44.8 \pm 1.3^{b}$ | 0.00 |
| Progressive motility (%) | $11.7 \pm 1.3^{a}$ | $13.1 \pm 1.0^{a}$ | 0.48 |
| VCL ($\mu$m/s) | $41.3 \pm 2.8^{a}$ | $44.1 \pm 1.8^{a}$ | 0.42 |
| VSL ($\mu$m/s) | $19.8 \pm 2.1^{b}$ | $27.3 \pm 1.5^{a}$ | 0.01 |
| VAP ($\mu$m/s) | $28.8 \pm 2.2^{a}$ | $33.7 \pm 1.7^{a}$ | 0.12 |
| LIN (%) | $45.6 \pm 2.4^{b}$ | $56.7 \pm 1.6^{a}$ | 0.00 |
| STR (%) | $64.9 \pm 2.3^{b}$ | $76.0 \pm 1.4^{a}$ | 0.00 |
| WOB (%) | $67.3 \pm 2.1^{b}$ | $72.2 \pm 1.3^{a}$ | 0.04 |
| ALH ($\mu$m) | $2.8 \pm 0.2^{a}$ | $2.8 \pm 0.6^{a}$ | 0.98 |
| BCF (Hz) | $5.6 \pm 0.5^{b}$ | $7.8 \pm 0.3^{a}$ | 0.00 |

Different letters within rows indicate significant differences between pure and hybrid birds for a given variable.

## DISCUSSION

This is the first report of sperm variable values for pure red-legged partridge and *Alectoris rufa* × *Alectoris chukar* hybrid birds. Both the pure and hybrid groups showed a clear seasonal pattern in terms of their ejaculatory (% of birds) and endocrine (circulating testosterone) responses. In both groups, spermatogenic activity started in January and reached a peak during winter to spring (increasing photoperiod), followed by a photorefractive period in summer and autumn (shortening days) when activity fell, before the establishment of a new annual cycle. The strongest reproductive response (ejaculatory response and the presence of sperm) lasted longer in the pure birds. Lengthening of the semen collection period to August might be helpful in conservation programs. The hybrids produced larger numbers of sperm cells at any given time, and the sperm heads were bigger, but the pure birds returned higher proportions of motile sperm.

As in other *Phasianidae* species, successful ejaculations occurred during times of increasing day length but lasted significantly longer (even until July and August in some birds). For example, in grey partridges (*Pedix*

**Table 2.** Sperm quantitative variables for pure red-legged partridge (*Alectoris rufa,* n = 19) and hybrid birds (*A. rufa* × *A. chuckar;* n = 22).

| Sperm quantitative variable | Pure | Hybrid | *P* |
|---|---|---|---|
| Volume ($\mu$L) | $19.8 \pm 1.8^{a}$ | $17.6 \pm 1.1^{a}$ | 0.30 |
| Concentration ($\times 10^6$ sperm/mL) | $722.1 \pm 123.9^{b}$ | $1359.0 \pm 80.7^{a}$ | 0.00 |
| Mean number of sperms/ejaculate ($\times 10^6$) | $12.5 \pm 2.4^{a}$ | $21.2 \pm 2.4^{a}$ | 0.07 |

Different letters within rows indicate significant differences between pure and hybrid birds for a given variable.

**Table 4.** Values of sperm head morphometric variables (mean ± SE) for pure (*Alectoris rufa*, n = 16) and hybrid (*A. rufa* × *A. chukar;* n = 16) birds. Values in brackets are the CV. Twenty-five spermatozoa per sample were randomly captured and subjected to computerized morphometric analysis.

| Sperm morphometric variables | Pure | Hybrid | *P* |
|---|---|---|---|
| Length ($\mu$m) | $14.37 \pm 0.18^{b}$ (24.38%) | $15.03 \pm 0.13^{a}$ (17.15%) | 0.00 |
| Width ($\mu$m) | $1.35 \pm 0.02^{a}$ (33.53%) | $1.28 \pm 0.02^{a}$ (25.63%) | 0.36 |
| Area ($\mu m^2$) | $14.72 \pm 0.33^{b}$ (43.65%) | $14.83 \pm 0.26^{a}$ (35.28%) | 0.01 |
| N (total sperm measured) | 400 | 400 | |

Different letter within rows indicate significant differences between pure and hybrid birds for a given variable.

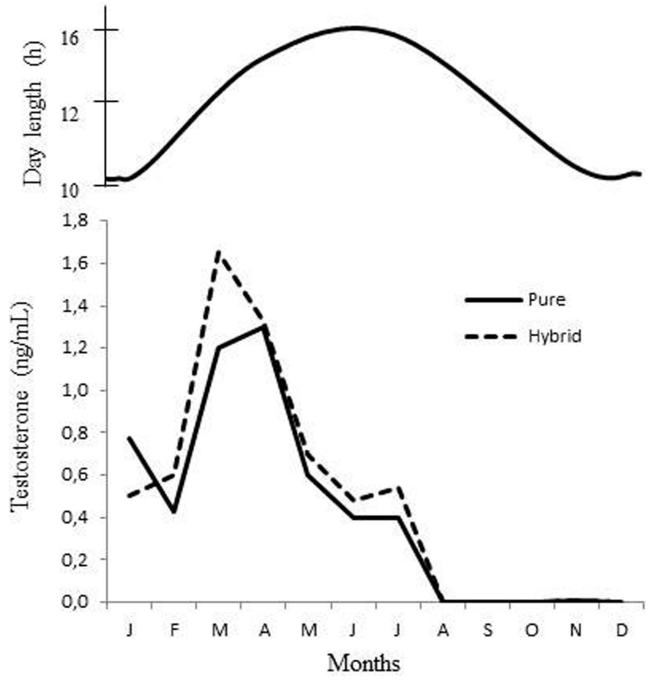


**Figure 4.** Changes in day length and plasma testosterone concentration (ng/mL) over the year, as measured in monthly pooled blood samples from pure and hybrid birds.

*perdix*) from northern Italy (about 45°N), spermatozoa are detectable from April to May. In Poland (about 51°N), the period of successful ejaculations in the capercaillie (*Tetrao urogallus*) is restricted from March to May (Lukaszewicz et al., 2011). The influence of latitude on reproductive activity is well known (Dawson, 2013), and this might determine that Mediterranean partridges have a longer breeding season than other *Phasianidae* species from more northerly latitudes. In the wild, red-legged partridges establish mating pairs in January, and laying runs from March to May (Durango et al., 2003). In the Barbary partridge (*Alectoris barbara*), another species of the Mediterranean area, the reproductive season lasts from March to June (Madeddu et al., 2010). Under farm conditions, the reproductive season of the red-legged partridge is determined as the period between the first and the last egg laid and usually extends (under the natural photoperiod) from March to July (Gaudioso et al., 2002; Mourao et al., 2010). If artificial lighting is added from December, laying may begin in early January (González-Redondo, 2006). Unfortunately, none of these farm-bird studies provide data on the purity of the partridges examined. In any event, the present results suggest that the period of sperm production is longer than the laying period. This should be taken into account when attempting to define the breeding season in this species. Stretching the sperm production period into the summer might be a reproductive strategy that allows for a replacement brood should chicks raised earlier in the season not survive.

The percentage of motile sperm produced by the pure and hybrid birds was similar to that seen in wild species such as the Canada goose (*Branta Canadensis*; mean sperm motility 50%) (Kowalczyk and Lukaszewicz, 2012) and similar to viable sperm recorded in domestic species such as the turkey (69%) and guinea fowl (64%) (Blesbois et al., 2005). In the domestic chicken, mean sperm motility is higher (85%) (Blesbois et al., 2008) but varies widely between different lines, showing that this variable can be selected for in breeding programs (Froman et al., 1997).

The proportion of motile sperm was greater in the pure birds than in the hybrids. This might be useful in future ex situ red-legged partridge conservation programs involving the cryopreservation of semen. Certainly, the percentage of motile sperm is one of the characteristics predictive of the success of semen freezing in chickens (Blesbois et al., 2008). The examination of quantitative and qualitative variables such as the semen volume, sperm cell concentration, sperm motility, sperm viability, morphological abnormalities, and acrosome integrity may provide a valuable way of predicting fertilization potential (Chalah et al., 1999; Santiago-Moreno et al., 2009). Therefore, these differences in sperm variables, between pure and hybrids birds, might produce a variable fertility rate depending on genetic purity. However, several other factors should be taken into account. For example, person-to-person differences and daily/individual stress also influence the collection success and sperm qualitative and quantitative variables.

The greater BW of the hybrids is in line with the fact that chukar partridges are larger than red-legged partridges (Cramp and Simmons, 1980). This observation disagrees with the report of Casas et al. (2013),

which indicates pure males to be heavier. The weight of the testes, and hence sperm production, increases with BW (Etches, 1996). The larger body weight of the hybrid birds may, therefore, at least partly explain their higher sperm concentrations, although this trait may have been unknowingly selected for during farm breeding. Further, this sperm variable may also be influenced by the grade of polyandry shown by the females (Collet et al., 2012).

When one female copulates with several males, factors such as sperm count and quality are fundamental in determining which male will fertilize an egg. Sperm competition is a strong selective force that promotes, among other things, larger sperm size and a faster swimming velocity. Although larger sperm heads might be thought a handicap to rapid swimming (Lüpold et al., 2009), the present data show that sperms with longer heads may swim faster (see straight-line velocity [**VSL**] values), as reported by other authors (Gomendio and Roldan, 1991; Fitzpatrick et al., 2009). Several authors indicate that males of species with polyandrous females have longer and faster sperms than species with sexually monogamous females (Briskie et al., 1997; Birkhead, 2000; Kleven et al., 2009). Because *A. chukar* is more promiscuous than *A. rufa* (Vidal and Colominas, 2007), more polyandry might be expected in the hybrids than in the pure birds. The present findings support this hypothesis.

The sperm head of the pure birds was shorter. Further, the CV for all variables examined was higher in the pure than in the hybrid partridges. At least in passerine birds, smaller intraspecific variation in sperm morphometry is related to an increased risk of sperm competition (Immler et al., 2012).

The present data show that the increase in testosterone secretion in January and February, and spermatogenic activity, occur before the laying period, coinciding with the establishment of breeding pairs in the wild (Perez,Garrido, 2006). This rise in testosterone secretion, which occurs about two months before the first ovulation in females, might stimulate competition between males for the establishment of dominance and the defense of territory. High testosterone levels would allow the onset of spermatogenesis cycles and the passage of spermatozoa through the epididymis. Successful reproduction upon the first ovulations in March should therefore be guaranteed. This time lag between the onset of male reproductive activity and laying in females agrees with previous studies in the grey partridge. The latter species pairs in January, coinciding with the first peak in androgen concentration, while laying does not start until April (Fraissinet et al., 1987). In the present work, no difference was seen in the testosterone profiles of the two groups despite the difference in BW (greater in the hybrids). Although the blood sampling regime may have been insufficient to provide conclusive results, artificial selection determining body conformation might also affect endocrine function and thus influence reproductive activity and behavior, as seen in chickens (McGary et al., 2002, 2003). Indeed, it cannot be ruled out that the shorter period of spermatogenic activity and less motile sperm seen in the hybrids are consequences of artificial selection under farm conditions.

In conclusion, the present work shows that the introgression of *A. chukar* genetic material (which in itself may have been affected by artificial selection pressures) into the red-legged partridge may influence the latter species's reproductive variables. Higher BW, sperm concentrations, and velocity sperm parameters, together with a larger head size, would favor reproduction of hybrid birds in a competitive scene (polyandry) with pure birds in the wild. The present results regarding time of sperm collection, and higher proportion of motile sperm in the pure birds, may of use in future red-legged partridge conservation programs.

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