



# Primordial Germ Cell-Mediated Chimera Technology Pure-Line Houbara Bustard Offspring: Potential for Endangered Species

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## Abstract

### Background

The Houbara bustard (*Chlamydotis undulata*) is a wild seasonal breeding bird populating arid sandy semi-desert population has declined drastically during the last two decades and it is classified as vulnerable. Captive breeding and reviving population numbers and thus radical technological solutions are essential for the long term survival of the species. We investigate the use of primordial germ cell-mediated chimera technology to produce viable Houbara bustard offspring.

### Methodology/Principal Findings

Embryonic gonadal tissue was dissected from Houbara bustard embryos at eight days post-incubation. Subsequently, primordial germ cells (gPGCs) was injected into White Leghorn chicken (*Gallus gallus domesticus*) embryos, producing 83 chimeric roosters reached sexual maturity after 5 months. The incorporation and differentiation of Houbara gPGC with Houbara-specific primers and 31.3% (5/16) gonads collected from the injected chicken embryos showed that 8 of the 34 semen samples from 34 chimeric roosters were analyzed and eight were confirmed as germline chimeras. Seminal fluid was used to artificially inseminate three female Houbara bustards. Subsequently, 45 Houbara eggs were obtained and incubated. One male live born Houbara; the other was female but died before hatching. Genotyping confirmed that the male chimeric rooster.

### Conclusion

This study demonstrates for the first time that Houbara gPGCs can migrate, differentiate and eventually give rise to offspring. This approach may provide a promising tool for propagation and conservation of endangered avian species that can

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## Introduction

The Houbara bustard is classified as vulnerable on the IUCN Red List and is listed on Appendix I of CITES [1], belonging to the order *Gruiformes* and it is the only species of the genus *Chlamydotis* (*Chl.*). The Houbara bustard *undulata*, *Chl. undulata macqueenii* and *Chl. undulata fuertaventurae* with *Chl. undulata macqueenii* being the most common. The Houbara bustard breeding in artificial environments has been attempted, but to date has not been successful in reviving the species.

The domesticated chicken (*Gallus gallus domesticus*) belongs to the order *Galliformes*, and can, by contrast, prosper under captive breeding conditions. For this reason, chicken is widely used as an agricultural animal and as a model organism. Houbara bustard breeding conservationists are looking for a means by which Houbara bustards could be produced in a chicken background. Cell-mediated chimera technology is a promising approach with the potential to achieve this.

Avian primordial germ cells (PGCs), precursor of the germ cells, are epiblastic in origin [4] during early development in the dorsal region [5], [6], until they enter the developing blood vessels in embryonic stage 10–12 [7]. Unlike mammalian PGCs, they circulate to the developing embryonic gonad, which later develops into the testis or ovary. Circulating PGCs can be transferred into another chicken embryonic blood circulating system and can contribute a chimeric germ line, which can be used for chimera production.

Based on intra-species and inter-order chimera technology, derived progenies have been produced by transferring PGCs from chicken using blastoderm cell transfer [15] and between Pheasant (*Phasianus colchicus*) and chicken [16]. So far, no one has produced an inter-order chimera as distantly related as Houbara bustard and chicken. The present study was undertaken to determine if Houbara bustard gonadal PGCs can produce functional gametes when in a chicken background.

## Materials and Methods

### Animals

Houbara bustards (*Chlamydotis undulata undulata*) were raised and bred in the Houbara breeding center of the Dubai, United Arab Emirates (UAE). Fertilized Houbara bustard eggs were collected after being artificially inseminated. White Leghorn chickens were maintained in the same conditions. Chimeric chickens were raised under the same conditions.

### Preparation of donor Houbara bustard gonadal cells

Fertilized fresh Houbara bustard eggs were collected and incubated for 8 days at 37.8°C and 60% relative humidity.

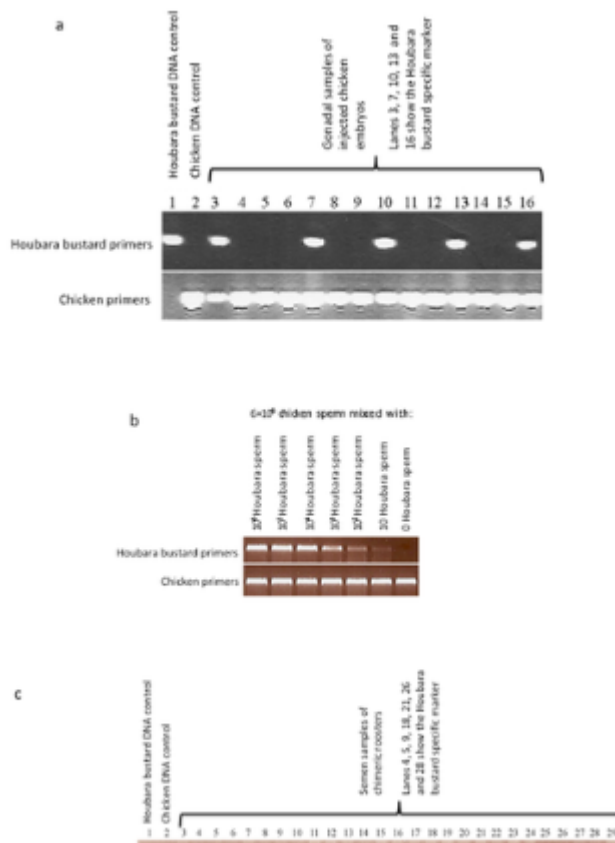
bustard embryos to determine the sex before dissecting the gonads, and male embryos were used as gonadal tissue as described below. The gonadal tissue was collected individually from Houbara bustard embryos under the stereomicroscope and dissected into small pieces using the tip of 1ml syringes. Dissected tissues were then incubated in Trypsin (0.25% for 30 minutes at 37°C, and dissociated by pipetting with P200 pipetman until there were no obvious tissue clumps observed. The suspension was supplemented with 10% Fetal bovine serum (FBS) and antibiotics (L-Glutamine-Penicillin-Streptomycin solution 100 units/ml for 5 minutes to remove the supernatant and resuspended in 1ml DMEM (10% FBS). A total of 5µl of cell suspension was used to determine cell viability by the Trypan blue exclusion method. The cell concentration was adjusted to  $4 \times 10^6$  cells/ml before transfer.

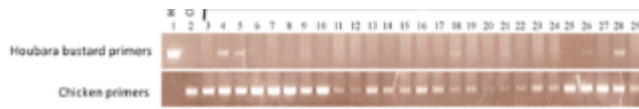
## Transfer of the Houbara bustard gonadal cells into White Leghorn chicken embryo

Fertilized White Leghorn chicken eggs were incubated with the “sharp end up” for 2.5 days until embryonic stage. A small window (about 10mm in diameter) was made into the shell to expose the embryo on the sharp end. A total of 100µl DMEM (10% FBS) was injected into dorsal aorta of each chicken embryo with a fine glass pipette. The injected embryo was fixed firmly by a heated surgical scalpel. All of the recipient eggs were incubated with the blunt end up until hatched. Gonadal tissues were collected from the embryos that died during the week before hatch. Histochemical staining and PCR species-specific primers as described below.

## Detection of Houbara bustard PGC-derived sperm from the semen of chimeric chickens

Chimeric chickens were raised to sexual maturity. Semen samples were collected from 34 chimeras weekly for 4 weeks and diluted 20 times in calcium and magnesium-free phosphate buffered saline (PBS) and 50 µl of diluted semen was used to identify sperm by a PCR species identification test as described below. The sensitivity of the PCR species identification test was determined by mixing Houbara bustard and chicken sperm with a graded ratio from one to 10 million sperm (Table 1, Figure 1b).





**Figure 1. PCR gels with species-specific primers showing the detection of Houbara bustard DNA.**  
 (a) Detection of bustard DNA in the gonadal tissue of chimeric chicken embryos. Lanes 3, 7, 10, 13, and 16 identification sensitivity test:  $6 \times 10^6$  chicken sperm mixed with decreasing quantities of bustard sperm; (c) De roosters. Lanes 4, 5, 9, 18, 21, 26 and 28 show the bustard DNA.  
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Birds ID	The number of samples collected	The number of positive samples	Percentage
bu029	12	1	8.5%
bu035	12	1	8.5%
bu036	15	1	13%
bu045	14	2	14.3%
bu066	12	1	8.5%
bu090	13	1	7.7%
bu429	9	1	11.1%
bu431	12	1	8.3%
<b>Total</b>	<b>99</b>	<b>8</b>	<b>8.2%</b>

**Table 1. Detection of donor cell-derived Houbara bustard sperm in the semen of chimeric roosters.**  
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### Progeny test

During the breeding season between January and May, semen samples were collected from 8 male chimeric ro samples previously. Fresh samples were re-checked by PCR for presence of Houbara bustard DNA. Doses of C inseminated twice a week into three virgin female Houbara bustards. The resulting eggs were collected and inci above. The remaining unhatched eggs were opened after 25 days incubation to examine the fertility and develo

Blood was collected from the resulting progeny; a piece of muscle tissue was dissected from the body of dead e genotyping and parentage verification tests were conducted with these samples by molecular analysis as descr

Season	Female Houbara ID	Total times of AI	The number of positive samples inseminated	The number of eggs	Fertility	Hatchability
1 <sup>st</sup> season	CO0109	36	4	7	0	0
	CO0114	36	5	8	0	0
	CO0142	36	5	5	0	0
2 <sup>nd</sup> season	CO0149	27	5	12	0	0
	CO0142	27	4	2	0	0
3 <sup>rd</sup> season	CO0149	18	6	1	0	0
	CO0154	18	6	16	23.2% (2/9)	52.3% (11/2)
<b>Total</b>		<b>198</b>	<b>35</b>	<b>45</b>	<b>4.4%</b>	<b>1.2%</b>

**Table 2. Progeny test of germline chimeric roosters by artificial insemination with female Houbara bu**

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## Molecular analysis

### DNA Extraction.

Pretreatment was done according to the sample type. a) Whole blood; 50µl of blood, collected in EDTA-vacutain K and 500µl of tissue lysis buffer, incubated at 56°C for 2 hrs. b) Tissue samples: about 25mg of the tissue was tissue lysis buffer, incubated at 56°C for 2 hrs. c) Semen samples: 25–50µl of semen was treated with 25µl of 0 followed by neutralization using 0.25M Tris-HCl (pH 8). Subsequently, any of the above lysed cells were mixed with Isoamylalcohol (25:24:1). The DNA was precipitated using 1/10 volume of 3M sodium acetate and 2.5 volumes 200ul of TE buffer (pH8), quantified by Nanodrop ND-1000 Spectrophotometer V3.5 (Nanodrop, Technologies Inc) reaction.

### Species Identification.

The specific primers CHN1F and CHN1R, BT2F and BT2R (Table 3) were used for identification of chicken and duck. The primers were designed using the sequence on chromosome 11 at the NCBI database, Trace/Gallus\_gallus\_WC are designed from the cytochrome b gene found in the mitochondria [19]. The Gene Bank accession number for expected product size and references are given in Table 3. Amplification was performed according to the following protocol: 94°C for 5 min, followed by 40 cycles of 94°C for 45sec, 58°C for 30sec and 72°C for 45 sec. The final extension was carried out at 72°C for 10 min.

No.	Primer	Primer sequence (5'-3')	Tm (°C)	Product Size (bp)	Method
1	CHN1F	CCT CCC AGT CCC AGT AAG AAG TAG	58	221	Species identification [19]
	CHN1R	CAA CAT GAT GGG CGA GTS CT			
2	BT2F	GGC TCC SFA KSA GFC CTS TTC TTA	58	318	Species identification [19]
	BT2R	GGG TST AGT CTT CAT TCT TTS GTT			
3	USP1	CTA TCC CTA CGA CAA/CCT TCC TAT TTS C	58	380	Bird Sexing [20]
	USP3	AAC TGG ATG/CT TCA GAT/TC/TA CAT CTT CT			
4	MYO INT1	AAC CCT GGA GGA TCC ATT GG	56	280	Bird Sexing [20]
	MYO INT2	CAG TGA GGT CTA GTA TCC AAG G			
5	F2	AGA TAT TCC GGA TCT GAT AGT GA	52	110	Bird Sexing [21]
	R2	TCT GGA TGG CTA AAT CCT TT			

**Table 3. Primer list for species identification test and molecular sexing.**

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### Sex Determination.

Sex identification was performed according to a) [20]; primers USP1 and USP3 were used to determine the sex control primers (Table 3). The cycling conditions were as follows: an initial denaturation at 95°C for 5 min was followed by annealing at 50°C for 30 sec and extension at 72°C for 1 min and a final extension at 72°C for 10 min. b) primer fragment that was restriction digested using the enzyme HaeIII. The thermal cycling conditions included a 5 min 95°C 30sec, 55°C for 15 seconds and 72°C for 15 seconds. This was followed by a 1 minute annealing at 56°C amplified PCR product was digested with HaeIII at 37°C for 1 hour. The digested and the undigested products were analyzed on agarose gel to determine sex and make sure all the samples had been amplified.

### Microsatellite analysis.

The genome DNA samples were extracted from blood of the live progeny and related adult birds, and subjected to PCR amplification.

verification. The list of primers, their sequences and references are shown in Table 4. PCR was performed in a and Fast Start Taq Polymerase (Roche Diagnostics, USA). M13 F or R tailed primers were used and grouped in analyzed by running on ABI 3730 XL DNA Analyzer and the genotypes were analyzed using the Genemapper \

No.	Primer name	Primer sequence
1	BuA2F-RR	CAQ GAA ACA GCT ATG ACC GCA GCA AAG AGA AOC AAA G
	BuA2R	CAA GCT CCT GTA GGG ATC A
2	BuA10F-RR	CAQ GAA ACA GCT ATG ACC GCT GAA TCT TGG CTT AGA TG
	BuA10R	AAQ GAA CAG AAA GGT TCT CTG
3	BuA10F-MF	TGT AAA ACG ACG GGC AGT CTC GCA TTT CAG TGG CTT C
	BuA10R	GGC ACG GCA GAA CAG ATC
4	BuA22F-RR	TGT AAA ACG ACG GGC AGT ACA GCT ATG CAC GCA CAT C
	BuA22R	TGC AAG GGG TTA ATG CTG T
5	BuA20F-RR	CAQ GAA ACA GCT ATG ACC GAG AAG GAA AGA CAC ACG TA
	BuA20R	AAA TTA CTG GAG AGT CAG G
6	BuA120F-RR	CAQ GAA ACA GCT ATG ACC GAA GAA GAA TGC AAC AAG T
	BuA120R	ACA TTA AAG TAC ACC CAG AA
7	BuA200F-RR	TGT AAA ACG ACG GGC AGT GCA TTT CAG TAA CTT CTC C
	BuA200R	TTT GCT GGT GGC ABA TTC
8	BuA200F-MF	TGT AAA ACG ACG GGC AGT GCT AAG ATG CAA AAC CAA AAC T
	BuA200R	CAT GCA ATG TGG AAT GAC T
9	BuA210F-MF	TGT AAA ACG ACG GGC AGT CTC CAT TTT CAA GCA ATC TTC
	BuA210R	GGC CTC TTT TAA TAA CTC AAA
10	BuA210F-RR	TGT AAA ACG ACG GGC AGT GCA GCT TAA AGG ATG TGA A
	BuA210R	TGA TGA AAT GGC AAG TAA ATG
11	BuA210F-MF	TGT AAA ACG ACG GGC AGT GCT CCT GAA ACC AGT CTC
	BuA210R	GGC AGA CAG AAA CAG AAG G
12	BuA210F-RR	CAQ GAA ACA GCT ATG ACC AGA AAC CTG GGG TAA TGA
	BuA210R	AAT GGC TAC CTC TTC CTT S
13	BuA210F-RR	CAQ GAA ACA GCT ATG ACC ACT CAG CTC TGG GAA AGT TAT G
	BuA210R	TTG TCT TTA TGG ATC CTC AAT G

**Table 4. Primer list of genotyping analysis using Houbara bustard microsatellites (STR) markers [26]**  
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## Results

### Harvest of donor Houbara bustard gonadal cells

Chicken embryos developed to embryonic stages 26, 28 and 30 after 5, 6 and 7 days incubation, while Houbara slower development in the same age, approximately 1–2 days delay, and showed significant individual variance period (22–24 days). The total number of gonadal cells in 8dpi Houbara bustard embryos was  $102.7 \pm 21.2 \times 10^3$  embryos, and  $114.8 \pm 20.5 \times 10^3$  cells ( $n = 23$ ) and  $96.9 \pm 1.2\%$  in male embryos.

The morphological characteristics of Houbara bustard gPGCs were similar to that of chicken. They can easily be larger in size (12–15  $\mu\text{m}$  in diameter) as well as richer in granules in the cytoplasm than somatic cells.

### Production of chimeric chickens

In total, 138 chicken embryos were injected with Houbara bustard gonadal cells from individual male embryos, and incubation with a hatchability of 60.1% (83/138). All of the hatched putative chimeric chicks had a typical White under normal conditions, and 35 male and 35 female birds reached to sexual maturity after 5 months. Houbara 31.3% (5/16) gonadal tissues of the injected chicken embryos pre-hatch (Figure 1a). These results suggested that and survived in chimeric chicken gonads, even across the considerable phylogenetic distance.

### Molecular analysis of chimeric embryos and semen samples from adult chimeric

Houbara bustard species-specific primers have been developed and used to detect Houbara bustard sperm pro

identification PCR test was determined with the mixed samples of Houbara bustard and chicken sperm. Certain Houbara bustard sperm was mixed with six million chicken sperm. Results showed that Houbara bustard sperm 10 Houbara bustard sperm in chicken semen containing 6 million sperm (Figure 1b).

A total of 302 semen samples were collected from 34 chimeric roosters. Houbara bustard species-specific DNA 23.5% (8/34) birds. Since the semen does not contain somatic cells, these results indicated that eight chimeric roosters and therefore considered as germline chimeras. Further 95 semen samples were collected from these 8 confirmed as Houbara bustard-DNA positive (Figure 1c; Table 1). These results suggested that Houbara bustard might be able to differentiate into sperms in the testis of chimeric rooster.

## Progeny test

Three female Houbara bustards were artificially inseminated 198 times with the semen samples collected from roosters. Three samples inseminated were confirmed containing Houbara bustard sperms by molecular analysis. Subsequently incubated, of which two eggs were found to be fertile. One successfully hatched after 22 days of incubation, with the chick hatching (Figure 2a–d, Table 2). The chick and the dead embryo showed typical Houbara bustard phenotype, a





**Figure 2. Parents, offspring and progeny tests.**

(a) Germline chimeric rooster; (b) Female Houbara bustard (HB020154); (c) Houbara bustard chick generated by sperm produced by chimeric rooster (d) Dead Houbara bustard embryo from chimeric rooster; (e) species identification PCR of the dead Houbara embryo and the live Houbara chick.

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### Species identification and parentage test of the resulting progenies

A Houbara bustard species-specific DNA fragment was amplified from both offspring, however chicken species-specific fragments were also present, providing strong evidence that the resulting offspring were genetically pure Houbara bustard. The hatched chick was a female (Figure 2f). The genotyping analysis using Houbara bustard and chicken microsatellites (STR) markers provided independent confirmation that the resulting offspring were genetically pure Houbara bustard, not chick produced through parthenogenesis, as their genotypes are not fully derived from their mother. Moreover, it verified the genotypes with the parental (donor) genotype. Unfortunately no sample was kept from the donor animal and the parents. At the same time one can predict the donor's genotype from its offspring and their mother. By comparing the donor's genotype as described in Table 5.

No.	Markers	Mother Houbara	Live Houbara chick	Dead Houbara embryo	Predicted donor	Grand mother	Grand father
1	BusA2	155/165	155/160*	155	155/155	155/160	155/159
2	BusA10	162	162	162	162	162	162
3	BusA13	144/145	148/146*	144/146*	146/7	146/148	146
4	BusA22	158	158	158	158	158	158
5	BusA29	158/164	158	166/160*	158/160	160/164	158/160
6	BusA139	202/200	202/200*	202/200*	200/7	200/200	200
7	BusA204	176/180	188/176*	176/176*	176	176/180	176
8	BusA205	216	216	216	216	216	216
9	BusA212	146/152	146/142*	132/142*	142/7	132/152	142/146
10	BusD112	161/165	161	161/157*	157/167	161/165	161/155
11	BusD117	168/172	172	168/172	172/176	176	172
12	BusD118	274/278	274	274/280*	280/276	276/278	274/266
13	BusD119	260/268	268	260	260/268	268	260

\*alleles from paternal genome.  
doi:10.1371/journal.pone.0015824.t005

**Table 5. Genotyping analysis of the Houbara bustard family using Houbara bustard microsatellites (S)**  
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### Discussion

In the present study, a viable Houbara bustard was successfully hatched between a male chimeric rooster and a female Houbara bustard. This study demonstrates that PGCs can be harvested from embryos with a high viability and that germ cells are able



and differentiate into functional sperm alongside the endogenous chicken sperm. In other words, the male chick spermatogonial development in the testis. The chimeric rooster thus served as a surrogate father of the chick, including hormonal systems, might be widely conserved in different avian species and orders. This might help u particularly the male spermatogenesis process.

Already pheasant PGCs derived progenies were successfully produced from chimeric roosters [16]. Also donor chicken-quail chimera; however, no progeny was obtained [22]. Highly sensitive molecular sexing and species i were developed as a result of this research, also providing strong molecular tools for Houbara bustard research sperm, and the tracing of the donor cells in the chimeric embryo or rooster.

The Houbara bustard reaches sexual maturity at about two years (one year at the earliest) [23]; this was confirm sexual maturity in about five months. In the present study, it was confirmed that Houbara bustard sperm could b maturity, indicating that donor germ cell differentiation occurs in same time that the recipient spermatozoa forms rooster gonads supports the development of host germ cell as well as of the donor germ cells. The donor Houb; semen sample, but no specific pattern was observed. However, donor DNA was detectable from the host seme negative result on donor sperm in the host semen is due to the PCR sensitivity. Furthermore, Houbara bustard I season. It is also not clear if the donor sperm was generated and released in the host testis by its own spermat Houbara bustard spermatogenesis in the host seminiferous tubules needs to be investigated further. Unlike the differentiation pattern, these observations suggest that the Houbara bustard PGCs follow the non-seasonal bree the chance to produce even more Houbara bustards using the domestic male chicken reproduction system all y

The efficiency of the progeny production is still very low, only one live chick and one dead before hatching. It is i pure Houbara bustard, not hybrid, and not from a parthenogenic development. Furthermore we provided strong of the chimeric rooster and the Houbara bustard by the parentage test, which is a strong molecular tool, for the kind of competition between the donor and recipient germ cells in the chimeric body, but still it is not well unders chimeric rooster semen is fluctuating. To increase the number of donor derived sperm few efforts need to be ma by purification and culture in vitro [24], [25], 2) reduce the endogenous chicken PGCs using mechanic or chemi

Furthermore, production of female chimeras between chicken and other domestic avian species by PGCs trans PGCs derived offspring has not been achieved from the chimeric hens [27]. If female PGCs could differentiate ii bustards could be reproduced through male and female chimeric chicken. This will greatly increase the Houbar; from hunting wild birds, and discourage people from trading or smuggling Houbara bustards. In the end, this tec other endangered avian species that cannot be bred in captivity.

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## Author Contributions

Conceived and designed the experiments: UW CL I-KC. Performed the experiments: CL VB ZG KAK SS I-KC. / Contributed reagents/materials/analysis tools: UW KAK RW CL. Wrote the paper: UW CL KAK JK I-KC DKG.

## References

1. IUCN Red List of Threatened Species website. Available: <http://www.iucnredlist.org>. Accessed 2010.
2. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) website. Available: <http://www.cites.org/>
3. Emirates Center for Wildlife Propagation website. Available: <http://www.ecwp.org/vo/index.php>. Accessed 2010.
4. Eyal-Giladi H, Ginsburg M, Farbarov A (1981) Avian primordial germ cells are of epiblast origin. *J Embol* 1: 1–10. CrossRef • PubMed/NCBI • Google Scholar
5. Swift CH (1914) Origin and early history of the primordial germ cells in the chick. *Am J Anat* 15: 483–511. CrossRef • PubMed/NCBI • Google Scholar
6. Ginsburg M, Eyal-Giladi H (1986) Temporal and spatial aspects of the gradual migration of primordial germ cells in the avian embryo. *J Emb Exp Morphol* 95: 53–71. CrossRef • PubMed/NCBI • Google Scholar
7. Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. *J Morphol* 88: 295–311. CrossRef • PubMed/NCBI • Google Scholar
8. Yasuda Y, Tajima A, Fujimoto T, Kuwana T (1992) A method to obtain avian germ line chimeras using microinjection. *Dev Biol* 150: 521–528. doi: 10.1530/jrf.0.0960521. CrossRef • PubMed/NCBI • Google Scholar
9. Tajima A, Naito M, Yasuda Y, Kuwana T (1993) Production of germ line chimera by transfer of primordial germ cells (*domesticus*). *Theriogenology* 40: 509–519. doi: 10.1016/0093-691X(93)90404-S. CrossRef • PubMed/NCBI • Google Scholar
10. Chang IK, Done KJ, Hong YH, Park TS, Moon YK, et al. (1997) Production of germline chimeric chicker. *Int J Anim Reprod Biotechnol* 21: 495–499. doi: 10.1006/cbir.1997.0173. CrossRef • PubMed/NCBI • Google Scholar
11. Tajima A, Naito M, Yasuda Y, Kuwana T (1998) Production of germline chimeras by transfer of cryopreserved primordial germ cells of chicken. *J Exp Zool* 280: 265–267. doi: 10.1002/(SICI)1097-010X(19980215)280:3<265::AID-JEZ8>3.3.CO;2-3. CrossRef • PubMed/NCBI • Google Scholar
12. Shin SS, Kim TM, Kim SY, Kim TW, Seo HW, et al. (2008) Generation of transgenic quail through germ line chimera. *Transgenic Res* 17: 2435–2444. doi: 10.1096/fj.07-101485. CrossRef • PubMed/NCBI • Google Scholar
13. Park TS, Kim MA, Lim JM, Han JY (2008) Production of quail (*Coturnix japonica*) germline chimeras derived from primordial germ cells. *Mol Reprod Devel* 75: 274–281. doi: 10.1002/mrd.20821. CrossRef • PubMed/NCBI • Google Scholar
14. Kim JN, Park TS, Park SH, Park KJ, Kim TM, et al. (2010) Migration and proliferation of intact and gene edited primordial germ cells in a transgenic chicken. *Biol Reprod* 82: 257–262. doi: 10.1095/biolreprod.108.069989. CrossRef • PubMed/NCBI • Google Scholar
15. Li ZD, Deng H, Liu CH, Song YH, Sha J, et al. (2002) Production of Duck-chicken chimeras by transfer of duck primordial germ cells into chicken. *Transgenic Res* 11: 109–115. CrossRef • PubMed/NCBI • Google Scholar
16. Kang SJ, Choi JW, Kim SY, Park KJ, Kim TM, et al. (2008) Reproduction of wild birds via interspecies germ line chimera. *Transgenic Res* 17: 2435–2444. doi: 10.1095/biolreprod.108.069989. CrossRef • PubMed/NCBI • Google Scholar
17. Chang IK, Tajima A, Chikamune T, Ohno T (1995) Proliferation of chick primordial germ cells cultured in vitro. *Int J Anim Reprod Biotechnol* 19: 143–149. doi: 10.1006/cbir.1995.1055. CrossRef • PubMed/NCBI • Google Scholar
18. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* 7: 403–431. doi: 10.1089/104296700753131274. CrossRef • PubMed/NCBI • Google Scholar

- CrossRef • PubMed/NCBI • Google Scholar
19. Pitra C, Lieckfeldt D, Alonso JC (2000) Population subdivision in Europe's great bustard inferred from mtDNA. *Ecol* 9: 1165–1170. doi: 10.1046/j.1365-294x.2000.00983.x.  
CrossRef • PubMed/NCBI • Google Scholar
  20. Ogawa A, Solovei I, Hutchison N, Saitoh Y, Ikeda JE, et al. (1997) Molecular characterization and cytology of the W chromosome of chicken and its use as a universal probe for sexing carinatae birds. *Chrom Res* 5: 105–112. doi: 10.1007/s10545-97-00000-0.  
CrossRef • PubMed/NCBI • Google Scholar
  21. Griffiths R, Daan S, Dijkstra C (1996) Sex identification in birds using two CHD genes. *Proc Roy Soc Lond B Biol Sci* 263: 681–685. doi: 10.1098/rspb.1996.0081.  
CrossRef • PubMed/NCBI • Google Scholar
  22. Li HC, Zhao L, Kagami H, Matsui K, Ono T (2001) Identification of transferred chicken germ cells in quail embryos by PCR products. *J Poult Sci* 38: 308–316. doi: 10.2141/jpsa.38.308.  
CrossRef • PubMed/NCBI • Google Scholar
  23. Saint-Jalme M, Van Heezik Y (1995) Propagation of the Houbara bustard. London: Keegan Paul International.
  24. van de Lavoie MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, et al. (2006) Germline transmission of a transgene in a wild bird. *Nature* 441: 766–769. doi: 10.1038/nature04831.  
CrossRef • PubMed/NCBI • Google Scholar
  25. Yamamoto Y, Usui F, Nakamura Y, Ito Y, Tagami T, et al. (2007) A novel method to isolate primordial germ cells in chicken. *Biol Reprod* 77: 115–119. doi: 10.1095/biolreprod.107.061200.  
CrossRef • PubMed/NCBI • Google Scholar
  26. Nakamura Y, Yamamoto Y, Usui F, Atsumi Y, Ito Y, et al. (2008) Increased proportion of donor primordial germ cells in recipient embryos using busulfan sustained-release emulsion in chickens. *Reprod Fert Dev* 20: 900–908. doi: 10.1071/rfd07119.  
CrossRef • PubMed/NCBI • Google Scholar
  27. Liu CH, Chang IK, Sasse J, Dumatoiu CJ, Basker JV, et al. (2007) Xenogenic oogenesis of chicken (*Gallus gallus*) chimeric quail (*Coturnix japonica*) ovary. *Anim Reprod Sci* 101: 344–350. doi: 10.1016/j.anireprosci.2007.05.005.  
CrossRef • PubMed/NCBI • Google Scholar