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Primordial Germ Cell-Mediated Chimera Technology
Pure-Line Houbara Bustard Offspring: Potential for Repopulating an 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 reviving population numbers and thus radical technological solutions are essential for the long term survival of this species. In this study, 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, germ cells (gPGCs) was injected into White Leghorn chicken (Gallus gallus domesticus) embryos, producing 83% (12/14) chimeric roosters reached sexual maturity after 5 months. The incorporation and differentiation of Houbara gPGCs with Houbara-specific primers and 31.3% (5/16) gonads collected from the injected chicken embryos showed the presence of Houbara germ cells. Semen samples from 34 chimeric roosters were analyzed and eight were confirmed as germline chimeras. Semen was used for artificial insemination of three female Houbara bustards. Subsequently, 45 Houbara eggs were obtained and incubated. One male live born Houbara rooster was produced; the other was female but died before hatching. Genotyping confirmed that the male chick was indeed a chimera.

Conclusion

This study demonstrates for the first time that Houbara gPGCs can migrate, differentiate and eventually give rise to viable offspring. This approach may provide a promising tool for propagation and conservation of endangered avian species that cannot be endangered by recent radical technological solutions.
Introduction

The Houbara bustard is classified as vulnerable on the IUCN Red List and is listed on Appendix I of CITES [1], [2] 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 species. Houbara bustards in artificial environments has been attempted, but to date has not been successful in reviving wild populations.

The domesticated chicken (Gallus gallus domesticus) belongs to the order Galliformes, and can, by contrast, produce offspring under captive breeding conditions. For this reason, chicken is widely used as an agricultural animal and as an alternative to wild systems. Houbara bustard breeding conservationists is to generate a means by which Houbara bustards could be produced using avian techniques.

Avian primordial germ cells (PGCs), precursor of the germ cells, are epiblastic in origin [4] during early development. They circulate to the developing embryonic gonad, which later develops into the testis or ovary. Circulating PGCs [8] transferred into another chicken embryonic blood circulating system and can contribute a chimeric germ line, which can then develop into functional gametes.

Based on intra-species and inter-order chimera technology, derived progenies have been produced by transferring chicken using blastoderm cell transfer [15] and between Pheasant (Phasianus colchicus) and chicken [16]. So far, an inter-order chimera as distantly related as Houbara bustard and chicken. The present study was undertaken to determine whether 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 with semen from Houbara bustards for progeny testing were raised under the same conditions. White Leghorn chickens were maintained in the same facility and were collected after artificial insemination (AI). 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 donors 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 tissue was then cultured in Medium (supplemented with 10% Fetal bovine serum (FBS) and antibiotics (L-Glutamine-Penicillin-Streptomycin solution)) for 5 minutes to remove the supernatant and resuspended in 1ml DMEM (10% FBS). A total of 5µl of cell suspension was withdrawn with a P200 pipetman, and viability by the Trypan blue exclusion method. The cell concentration was adjusted to 4×10^6 cells/ml before transfer.

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

Fertilized White Leghorn chicken eggs were incubated with the “sharp end up” for 2.5 days until embryonic stage 30. A small window (about 10mm in diameter) was made into the shell to expose the embryo on the sharp end. A total of 5µl of Medium (10% FBS) was injected into dorsal aorta of each chicken embryo with a fine glass pipette. The injected eggs were fixed firmly by a heated surgical scalpel. All of the recipient eggs were incubated with the blunt end up until hatch. Gonadal tissues were collected from the embryos that died during the week before hatch. Houbara bustard species-specific primers as described below.

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

Chimeric chickens were raised to sexual maturity. Semen samples were collected from 34 chimeras weekly for 1 month. Semen was diluted 20 times in calcium and magnesium-free phosphate buffered saline (PBS) and 50 µl of diluted semen with sperm by a PCR species identification test as described below. The sensitivity of the PCR species identification test was measured with 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 show identification sensitivity test: 6 × 10^6 chicken sperm mixed with decreasing quantities of bustard sperm; (c) Detection of bustard DNA in the semen of chimeric roosters. Lanes 4, 5, 9, 18, 21, 26 and 28 show the bustard DNA. doi:10.1371/journal.pone.0015824.g001

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<th>Sample ID</th>
<th>The number of samples collected</th>
<th>The number of positive samples</th>
<th>Percentage</th>
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<td>33%</td>
</tr>
<tr>
<td>Proc.</td>
<td>T2</td>
<td>1</td>
<td>33%</td>
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<td>66%</td>
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<td>Proc.</td>
<td>T6</td>
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<tr>
<td>Proc.</td>
<td>T16</td>
<td>1</td>
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</tbody>
</table>

Table 1. Detection of donor cell-derived Houbara bustard sperm in the semen of chimeric roosters. doi:10.1371/journal.pone.0015824.t001

Progeny test

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

Blood was collected from the resulting progeny; a piece of muscle tissue was dissected from the body of dead embryos. Genotyping and parentage verification tests were conducted with these samples by molecular analysis as described below.

Table 2. Progeny test of germline chimeric roosters by artificial insemination with female Houbara bustards.
Molecular analysis

DNA Extraction.

Pretreatment was done according to the sample type. a) Whole blood: 50µl of blood, collected in EDTA-vacutainer 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 0.25M 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 of 200ul of TE buffer (pH8), quantified by Nanodrop ND-1000 Spectrophotometer V3.5 (Nanodrop, Technologies Inc, Wilmington, DE, USA).

Species Identification.

The specific primers CHN1F and CHN1R, BTD2F and BTD2R (Table 3) were used for identification of chicken and the expected product size and references are given in Table 3. Amplification was performed according to the following conditions: 35 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 7 min. The obtained PCR products were separated on 2.5% agarose gel and stained with ethidium bromide.

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

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 30 cycles of 95°C for 45sec, 55°C for 15 seconds and 72°C for 15 seconds. The final extension was carried out at 72°C for 10 min. b) primers W1 and W2 were used to amplify PCR product that was restriction digested using the enzyme HaeIII. The thermal cycling conditions included a 5 min annealing at 56°C and a final extension at 72°C for 10 min. The digested and the undigested products were separated on 2.5% agarose gel and stained with ethidium bromide.

Microsatellite analysis.

The genome DNA samples were extracted from blood of the live progeny and related adult birds, and subjected to microsatellite analysis.
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 ir
analyzed by running on ABI 3730 XL DNA Analyzer and the genotypes were analyzed using the Genemapper V4

Table 4. Primer list of genotyping analysis using Houbara bustard microsatellites (STR) markers [26].
doi:10.1371/journal.pone.0015824.t004

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 b
slower development in the same age, approximately 1–2 days delay, and showed significant individual variance a
period (22–24 days). The total number of gonadal cells in 8dpi Houbara bustard embryos was 102.7±21.2
× 10^3 cells (n = 23) and 114.8±20.5 × 10^3 cells (n = 23) and 96.9±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 µm 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, ou
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 bu
31.3% (5/16) gonadal tissues of the injected chicken embryos pre-hatch (Figure 1a). These results suggested tha
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 prc
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 sperm 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 the 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, while hatching (Figure 2a–d, Table 2). The chick and the dead embryo showed typical Houbara bustard phenotype, all
Species identification and parentage test of the resulting progenies

A Houbara bustard species-specific DNA fragment was amplified from both offspring, however chicken species-providing strong evidence that the resulting offspring were genetically pure Houbara bustard. The hatched chick 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 genotypes with the parental (donor) genotype. Unfortunately no sample was kept from the donor animal and thus parents. At the same time one can predict the donor's genotype from its offspring and their mother. By comparing donor's genotype as described in Table 5.

Table 5. Genotyping analysis of the Houbara bustard family using Houbara bustard microsatellites (STR) markers

<table>
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<tr>
<th>No.</th>
<th>Hound</th>
<th>Mother Houbara</th>
<th>Live Houbara chick</th>
<th>Dead Houbara embryo</th>
<th>Proven donor</th>
<th>Proven mother</th>
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Table 5. Genotyping analysis of the Houbara bustard family using Houbara bustard microsatellites (STR) markers

Discussion

In the present study, a viable Houbara bustard was successfully hatched between a male chimeric rooster and a female Houbara bustard. The 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, providing hormonal systems, might be widely conserved in different avian species and orders. This might help us 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 identification 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 confirmed in the present study, it was confirmed that Houbara bustard sperm could be detected, indicating that donor germ cell differentiation occurs in the 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 Houbara semen sample, but no specific pattern was observed. However, donor DNA was detectable from the host semen negative result on donor sperm in the host semen is due to the PCR sensitivity. Furthermore, Houbara bustard I seasonal. It is also not clear if the donor sperm was generated and released in the host testis by its own spermatogonia. 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 breeding pattern and the chance to produce even more Houbara bustards using the domestic male chicken reproduction system annually.

The efficiency of the progeny production is still very low, only one live chick and one dead before hatching. It is important to note that the Houbara bustard, not hybrid, and not from a parthenogenic development. Furthermore we provided strong evidence that 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 understood. Chimeric rooster semen is fluctuating. To increase the number of donor derived sperm few efforts need to be made by purification and culture in vitro [24], [25], 2) reduce the endogenous chicken PGCs using mechanic or chemical methods.

Furthermore, production of female chimeras between chicken and other domestic avian species by PGCs transplanted. PGCs derived offspring has not been achieved from the chimeric hens [27]. If female PGCs could differentiate into post-bursa 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 breeding pattern and the chance to produce even more Houbara bustards using the domestic male chicken reproduction system annually.

The authors are grateful to the International Foundation for Conservation & Development of Wildlife (IFCDW) in Aouda, Libya, for support and providing facilities. Many thanks go to Dr. Jürgen Sasse, Mr. Jahabar Ali, Mr. Lahcen Ben Kamel, Mr. Chris Dumatol and Dr. Fatima, for their support.

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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


