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

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Abstract

Background

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

Methodology/Principal Findings

Embryonic gonadal tissue was dissected from Houbara bustard embryos at eight days post-incubation. Subseq 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 gPG with Houbara-specific primers and 31.3% (5/16) gonads collected from the injected chicken embryos showed th semen samples from 34 chimeric roosters were analyzed and eight were confirmed as germline chimeras. Sem artificially inseminate three female Houbara bustards. Subsequently, 45 Houbara eggs were obtained and incub male live born Houbara; the other was female but died before hatching. Genotyping confirmed that the male chi rooster.

Conclusion

This study demonstrates for the first time that Houbara gPGCs can migrate, differentiate and eventually give ris 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 bust *undulata*, *Chl. undulata macqueenii* and *Chl. undulata fuertaventurae* with *Chl. undulata macqueenii* being the r Houbara bustards in artificial environments has been attempted, but to date has not been successful in reviving

The domesticated chicken (*Gallus gallus domesticus*) belongs to the order *Galliformes*, and can, by contrast, pr under captive breeding conditions. For this reason, chicken is widely used as and agricultural animal and as an Houbara bustard breeding conservationists is to generate a means by which Houbara bustards could be produc 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 develop region [5], [6], until they enter the developing blood vessels in embryonic stage 10–12 [7]. Unlike mammalian P circulation 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, wh

Based on intra-species and inter-order chimera technology, derived progenies have been produced by transferr chicken using blastoderm cell transfer [15] and between Pheasant (*Phasianus colchicus*) and chicken [16]. So f an inter-order chimera as distantly related as Houbara bustard and chicken. The present study was undertaken 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 insen bustards for progeny testing were raised under the same conditions. White Leghorn chickens were maintained i 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 hum

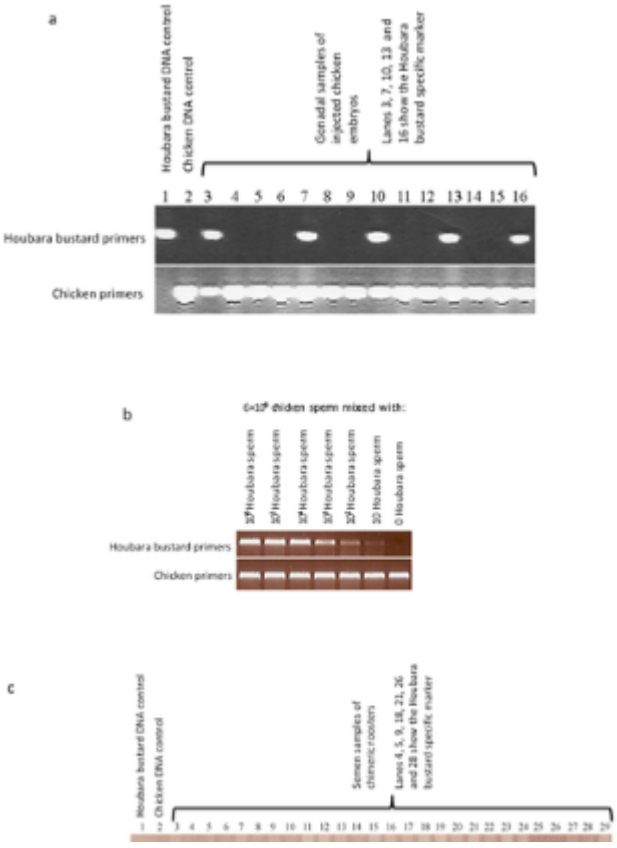
bustard embryos to determine the sex before dissecting the gonads, and male embryos were used as gonadal described below. The gonadal tissue was collected individually from Houbara bustard embryos under the stereo dissected into small pieces using the tip of 1ml syringes. Dissected tissues were then incubated in Trypsin (0.25 minutes at 37°C, and dissociated by pipetting with P200 pipetman until there were no obvious tissue clumps ob supplemented with 10% Fetal bovine serum (FBS) and antibiotics (L-Glutamine-Penicillin-Streptomycin solution 300g for 5 minutes to remove the supernatant and resuspended in 1ml DMEM (10% FBS). A total of 5µl of cell viability by the Trypan blue exclusion method. The cell concentration was adjusted to 4×10^6 cells/ml before tran

Transfer of the Houbara bustard gonadal cells into White Leghorn chicken embry

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

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

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



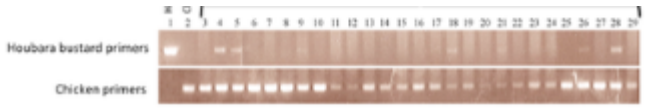


Figure 1. PCR gels with species-specific primers showing the detection of Houbara bustard DNA.
(**a**) Detection of bustard DNA in the gonadal tissue of chimeric chicken embryos. Lanes 3, 7, 10, 13, and 16
identification sensitivity test: 6×10^6 chicken sperm mixed with decreasing quantities of bustard 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
hv339	12	1	8.3%
hv335	12	1	8.3%
hv336	10	1	10%
hv345	14	2	14.3%
hv366	12	1	8.3%
hv390	13	1	7.7%
hv429	9	1	11.1%
hv432	12	1	7.7%
Total	**95**	**9**	**9.5%**

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 (
inseminated twice a week into three virgin female Houbara bustards. The resulting eggs were collected and inc
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

	Female Houbara ID	Total times of AI	The number of positive samples inseminated	The number of eggs	Fertility	Hatchability
1st season	008148	36	4	7	0	0
	028154	36	5	8	0	0
	018242	36	5	5	0	0
2nd season	008148	27	5	12	0	0
	018242	27	4	2	0	0
3rd season	008148	18	6	1	0	0
	028154	18	6	10	20.0% (2/10)	50.0% (1/2)
Total		150	35	45	4.4%	2.2%

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

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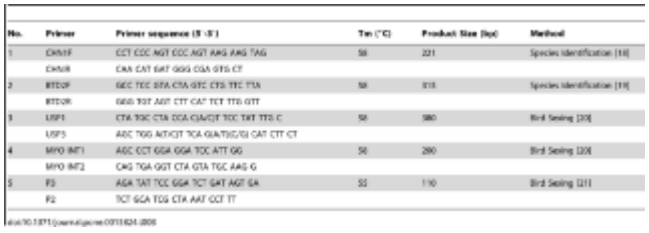
Molecular analysis

DNA Extraction.

Pretreatment was done according to the sample type. a) Whole blood; 50µl of blood, collected in EDTA-vacutai 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 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 I reaction.

Species Identification.

The specific primers CHN1F and CHN1R, BTD2F and BTD2R (Table 3) were used for identification of chicken a primers were designed using the sequence on chromosome 11 at the NCBI database, Trace/Gallus_gallus_WG are designed from the cytochrome b gene found in the mitochondria [19]. The Gene Bank accession number fo expected product size and references are given in Table 3. Amplification was performed according to the followi min, followed by 40 cycles of 94°C for 45sec, 58°C for 30sec and 72°C for 45 sec. The final extension was carri



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 [18]
	CHN1R	CAA CAT GAT GGG CGA GTG CT			
2	BTD2F	GCC TCC DTA CTA GTC CTG TTC TTA	58	818	Species identification [19]
	BTD2R	GGG TGT AGT CTT CAT TCT TTG GTT			
3	USP1	CTA TGC CTA CCA CIA/GT TCC TAT TTG C	58	580	Bird Sexing [20]
	USP3	AGC TGG ACT/CT TCA GIA/TG/G) CAT CTT CT			
4	MYO INT1	AGC CCT GGA GGA TCC ATT GG	58	280	Bird Sexing [20]
	MYO INT2	CAG TGA GGT CTA GTA TGC AAG G			
5	F3	AGA TAT TCC GGA TCT GAT AGT GA	53	110	Bird Sexing [21]
	F2	TCT GCA TCG CTA AAT CCT TT			

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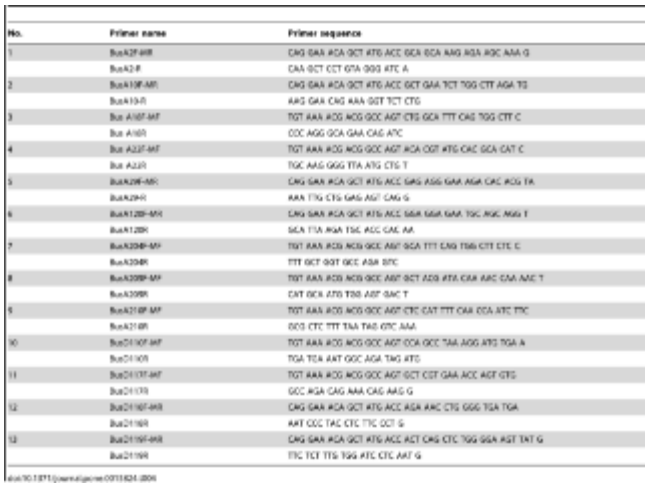
Sex Determination.

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 fo 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 v 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

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 \



No.	Primer name	Primer sequence
1	BusA2F-M13	CAG GAA ACA GCT ATG ACC GCA GCA AAG AGA AGC AAA G
	BusA2-R	CAA GCT CCT GTA GGG ATC A
2	BusA10F-M1	CAG GAA ACA GCT ATG ACC GCT GAA TCT TGG CTT AGA TG
	BusA10-R	AAG GAA CAG AAA GGT TCT CTG
3	Bus A10F-M1	TGT AAA ACG ACG GCC AGT CTG GCA TTT CAC TGG CTT C
	Bus A10R	CCC AGG GCA GAA CAG ATC
4	Bus A22F-M1	TGT AAA ACG ACG GCC AGT ACA CGT ATG CAC GCA CAT C
	Bus A22R	TGC AAG GGG TTA ATG CTG T
5	BusA29F-M1	CAG GAA ACA GCT ATG ACC GAG AGG GAA AGA CAC ACG TA
	BusA29R	AAA TTG CTG GAG AGT CAG G
6	BusA12BF-M1	CAG GAA ACA GCT ATG ACC GGA GGA GAA TGC AGC AAG T
	BusA12BR	GCA TTA AGA TGC AGC CAC AA
7	BusA20HP-M1	TGT AAA ACG ACG GCC AGT GCA TTT CAG TGG CTT CTC C
	BusA20HR	TTT GCT GGT GCC AGA GTC
8	BusA20BP-M1	TGT AAA ACG ACG GCC AGT GCT ACG ATA CAA AAC CAA AAC T
	BusA20BR	CAT GCA ATG TGG AGT GAC T
9	BusA21BF-M1	TGT AAA ACG ACG GCC AGT CTC CAT TTT CAA CCA ATC TTC
	BusA21BR	GCG CTC TTT TAA TAG GTC AAA
10	BusD19CF-M1	TGT AAA ACG ACG GCC AGT CCA GCC TAA AGG ATG TGA A
	BusD19CR	TGA TCA AAT GCC AGA TAG ATG
11	BusD13T-M1	TGT AAA ACG ACG GCC AGT CCT GAA ACC AGT GTG
	BusD13TR	GCC AGA CAG AAA CAG AAG G
12	BusD19CF-M13	CAG GAA ACA GCT ATG ACC AGA AAC CTG GGG TGA TGA
	BusD19CR	AAT CCC TAC CTC TTC CCT G
13	BusD19CF-M13	CAG GAA ACA GCT ATG ACC ACT CAG CTC TGG GGA AGT TAT G
	BusD19CR	TTC TCT TTG TGG ATC CTC AAT G

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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±21.2×10^3 embryos, 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 b 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, 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 tl 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 bustar 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 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, wh 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 generat sperm produced by chimeric rooster (**d**) Dead Houbara bustard embryo from chimeric rooster; (**e**) species id 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- 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 a 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 verif genotypes with the parental (donor) genotype. Unfortunately no sample was kept from the donor animal and thu parents. At the same time one can predict the donor's genotype from its offspring and their mother. By comparir 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	BuuA2	155/165	155/165*	155	155/155	155/165	155/189
2	Bu A10	162	162	162	162	162	162
3	BuuA18	144/146	146/146*	144/146*	146/?	146/148	146
4	BuuA22	158	158	158	158	158	158
5	Bu A29	158/164	158	164/180*	158/160	160/164	158/160
6	Bu A120	280/280	280/280*	280/280*	280/?	280/290	280
7	Bu A204	176/180	180/176*	176/176*	176	176/180	176
8	Bu A205	216	216	216	216	216	216
9	Bu A210	146/150	146/142*	150/142*	142/?	150/152	142/146
10	Bu D118	161/165	161	161/161*	161/161	161/161	161/165
11	BuuD127	168/172	172	168/172	172/176	176	172
12	Bu D118	274/278	274	278/286*	286/274	278/278	274/286
13	BuuD129	260/268	260	260	260/268	268	260

*alleles from paternal genome.
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Table 5. Genotyping analysis of the Houbara bustard family using Houbara bustard microsatellites (S

Discussion

In the present study, a viable Houbara bustard was successfully hatched between a male chimeric rooster and f 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, p 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 confirn 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 l season. It is also not clear if the donor sperm was generated and released in the host testis by its own spermato 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 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 i bustards could be reproduced through male and female chimeric chicken. This will greatly increase the Houbara 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. A Contributed reagents/materials/analysis tools: UW KAK RW CL. Wrote the paper: UW CL KAK JK I-KC DKG.

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