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# Watching sexy displays improves hatching success and offspring growth through maternal allocation

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Male attractiveness can have tremendous effects on the fitness of his offspring via good genes, but also via enhanced maternal allocation of resources. Yet the proximate mechanisms influencing differential maternal allocation in relation to male sexiness are poorly known. Here, we studied the importance of visual stimulation for maternal allocation in the Houbara bustard, a vulnerable bird species bred in captivity to support wild populations. Artificial insemination allowed controlling for potential confounding factors, such as a male's territory quality, social interactions or sperm quality/quantity, probably linked to mate attractiveness. We show that artificially inseminated females stimulated by highly displaying males increased their hatching success, owing to increased fertilization success. The females also increased the allocation of maternal androgens in their eggs, leading to an increase of circulating testosterone and growth rate in chicks. Hence, visual stimulation of the females can promote differential maternal allocation and favour offspring fitness. Our results further suggest that using artificial insemination for species conservation without appropriate stimulation of the breeding females probably has negative impacts on their breeding performance and therefore on population viability.

**Keywords:** maternal investment; reproduction; fertility; mate choice; conservation biology

## 1. INTRODUCTION

Maternal effects—the mother's non-genetic contributions to her offspring's phenotype—can be viewed as a general mechanism for adaptive response to environmental conditions (Fox *et al.* 1997; Fox & Mousseau 1998; Mousseau & Fox 1998). In particular, variations in the energy and resources females invest in reproduction, known as differential maternal allocation, have been shown to have dramatic impacts on offspring number and/or quality (Cunningham & Russell 2000; Sheldon 2000; Groothuis & Schwabl 2008). Given the fitness consequences of differential maternal allocation, most of the studies published during the last decade in this area deal with ultimate questions, whereas insights about the proximate mechanisms are lacking at the moment (Sheldon 2000; Groothuis & Schwabl 2008).

Recent works investigating components transferred by the mother to her embryos focus on maternal transfer of androgens, such as testosterone and its precursors (e.g. androstenedione). Maternal androgens have been found to be major determinants of offspring development both before and after birth (reviewed by Carere & Balthazart 2007).

Among internal and external factors affecting maternal allocation, mate attractiveness is the least understood. Given that reproduction is costly, females are expected to adjust the level of their investment into current reproduction according to her expected fitness return (Trivers 1972).

Females indeed produce more eggs, allocate more testosterone and sometimes produce more sons when mated with more attractive males compared with less attractive ones (Burley 1988; Ellegren *et al.* 1996; Gil *et al.* 1999; Sheldon 2000; Loyau *et al.* 2007; Szigeti *et al.* 2007). However, the underlying mechanisms remain unknown, and how the signal is perceived and processed is especially unclear (Groothuis & Schwabl 2008). Maternal differential allocation could arise through a purely sensory pathway, with females being directly and proportionally stimulated by male phenotypic quality, even in the absence of mating. In this case, any kind of signal involved in mate choice (i.e. a fixed morphological trait or a flexible behavioural trait) is expected to affect maternal allocation. However, one cannot discard the possibility that maternal allocation could be determined by correlates of male attractiveness such as a male–male interaction, territory quality or sperm quality/quantity. Most studies investigating maternal differential allocation experimentally manipulated male traits involved in mate choice to decouple the attractiveness perceived by the female and the intrinsic quality of the male (e.g. Saino *et al.* 2002; Williamson *et al.* 2006). Unfortunately, these manipulations are likely to affect other components of the parental environment such as male–male interactions and the male's perception of his own attractiveness, which would thus modify the male's behaviour (Cuthill *et al.* 1997). Another unstudied area is female reproductive allocation when information on the attractiveness of the male she is paired with is lacking. In the absence of information on mate quality, investment might be a 'baseline' allocation because stimulation is necessary to trigger higher allocation.

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We investigated the proximate pathway of maternal hormone allocation in the Houbara bustard (*Chlamydotis undulata undulata*), a vulnerable lekking species (IUCN 2010). Lekking species are particularly appropriate for studies of differential allocation because male contribution to reproduction is limited to genes and mating is heavily skewed towards a few (presumably attractive) males (Höglund & Alatalo 1995). A captive breeding programme to support wild populations of the Houbara bustard is currently under way at Emirates Center for Wildlife Propagation (ECWP) in Missour, Morocco. Successful captive breeding only occurs through artificial reproduction in this species (Saint Jalme *et al.* 1994, 1996). Because males and females are (individually) housed in different areas, a female can spend her entire life without once seeing a male. In this species, artificial insemination has been found to be associated with a 15 per cent decrease in hatching success (Saint Jalme *et al.* 1996), which may be in part accounted for by maternal differential allocation.

We wished to investigate proximate factors influencing maternal hormone allocation, while controlling for potentially confounding factors. We experimentally stimulated females by exposing them to signals of variable attractiveness. We partitioned captive females into three groups: 'poorly stimulated' females ( $D^-$ ) were placed in front of poorly displaying males; 'highly stimulated' females ( $D^+$ ) were placed in front of highly displaying males; and females of the control group (C) were placed in aviaries facing other females, and were therefore not stimulated by any male. Given that display frequency honestly signals male quality (Chargé *et al.* in press), we hypothesized that display activity could stimulate maternal allocation. We used artificial insemination to assess the consequences of maternal allocation in terms of offspring fitness, while controlling for male ejaculate effects (Sheldon 2000). Given that reproduction is costly, we predicted that females would invest less in the absence of stimulation or with weak stimulation. We further predicted that appropriate stimulation of a female should increase her maternal allocation and should have a positive effect on offspring fitness.

Our aims were to (i) examine whether female stimulation by male display promotes differential maternal hormone allocation, (ii) investigate whether maternal hormone allocation is disrupted by artificial insemination, and (iii) explore a way to maintain high maternal hormone allocation, in captivity, for breeding programmes and species conservation.

## 2. MATERIAL AND METHODS

### (a) *Experimental procedure*

The study was carried out at ECWP (Eastern Morocco, 33°00' N, 04°06' W, 965 m altitude) during the 2007 breeding season. Experimental breeders were individually housed in 4 × 4 × 2 m outdoor aviaries. Individuals are visually separated from the neighbours but could see the individuals placed in front of them. Ninety females of comparable age, condition (body weight/tarsus length), level of inbreeding and number of eggs per year ( $F_{2,87}$ , all  $p > 0.05$ ) were randomly assigned to one of the three following treatments: housed in front of females as traditionally done (C), in front of 30 poorly displaying males ( $D^-$ ) or in front of

30 highly displaying males ( $D^+$ ). The 30 aviaries of a given experimental group of females were organized along one line and all three lines of the three experimental groups were parallel to each other (see electronic supplementary material, figure S1). The facing aviaries were similar and parallel, at 5 m distance, meaning that a female was able to see the bird in the facing aviary and his or her two neighbours through the wire mesh. Female individual level of inbreeding was estimated from pedigree analysis.

Males were assigned to the  $D^-$  and the  $D^+$  group on the basis of their display behaviour frequency in 2006. In addition, between 13 February and 17 April 2007, males were observed during the daily peak of display behaviour (i.e. during the 3 h after dawn). Ten males were observed altogether during a sequence of one hour, for a total of 138 h. We recorded the frequency and length of male courtship displays and counted the number of days a given male displayed during the overall breeding season. Male Houbara courtship display behaviour is characterized by the erection of feathers on the neck and head, and running either straight or in a circular pattern (Gaucher *et al.* 1996).  $D^+$  and  $D^-$  males differed only in courtship behaviours (ANOVA, display frequency:  $F_{1,58} = 17.42$ ,  $p = 0.0001$ ; display length:  $F_{1,58} = 21.77$ ,  $p < 0.0001$ ; number of displaying days:  $F_{1,58} = 41.71$ ,  $p < 0.0001$ ; circulating testosterone, tarsus length, body mass, condition: all  $p > 0.05$ ). We did not measure every trait that could be linked to male attractiveness; therefore we cannot rule out the possibility that other sexual traits (such as plumage and/or vocalizations) also differ between  $D^+$  and  $D^-$  males and contributed to female stimulation.

A given female was able to see three males (the facing male and his two neighbours). We hypothesized that she would pay more attention to the more attractive one. We therefore took into account the maximal display activity perceived by a female.

To control for semen quality, experimental females were artificially inseminated with semen randomly chosen with respect to the experiment. Procedures of semen collection and artificial insemination in this species have been precisely described in a previous study (Saint Jalme *et al.* 1994). Briefly, semen collection was achieved using a Petri dish held between a copulating male and a dummy female. Semen was immediately transferred in a 2 ml Eppendorf vial and diluted (v/v) with an extender (Lake 7.1; Lake & Ravie 1982) within 15 min after collection to keep spermatozoa alive longer. The female was inseminated within 1 h with a dose of 9.4 million spermatozoa. This dose corresponds to one-third of the original ejaculate collected, meaning that one male can inseminate several females in a given day if the ejaculate contains enough spermatozoa. Each female received two to three homospermic inseminations between two egg depositions according to their inter-egg deposition length. Male and female pairing was chosen on the basis of pedigrees to minimize inbreeding.

One control female died during the course of the experiment (although not owing to the experiment) and was removed from the statistical analyses. The experimental females laid 1178 eggs and produced 750 chicks. All females laid eggs except one female from the  $D^-$  group. We collected eggs daily. Egg pulling promoted up to 10 egg depositions. We used the first egg of each of the first 10 females laid in each experimental group (i.e. 30 eggs) to assess hormone

levels. These eggs were immediately frozen after laying. The remaining eggs were incubated in an artificial incubator at 37.7°C and humidity was adjusted to allow a theoretical mass loss of 15 to 17 per cent during incubation. Non-hatched eggs were dissected to record whether an embryo developed.

In addition, 88 eggs from 35 nests were collected in the wild, to have an indication of maternal allocation in the wild. Each wild female laid  $2.51 \pm 0.11$  eggs per nest. Once a wild nest was discovered, and the female identified, eggs were collected to promote a replacement clutch without knowing when they were laid. They were transported to the ECWP and incubated there. Four wild eggs, laid by four different females, failed to develop an embryo (suggesting that they were of poorer than average quality) and were used to obtain an indication of maternal allocation in the wild. We assume that maternal allocation is underestimated in these eggs (but see Rutstein *et al.* 2004). The chicks were raised under standardized conditions, blindly with respect to the experiment. They were weighed (to the nearest gram) every day until around one month old, and then every 5 or 10 days until 200 days, to record growth rate. At the age of 15 days, we took 200  $\mu$ l of blood from wild chicks ( $n = 22$ ) and the first three chicks of each experimental female ( $n = 243$ ) to measure levels of circulating testosterone. We centrifuged blood for 5 min at 3000 r.p.m. and stored the plasma at  $-20^\circ\text{C}$  until extraction. Obviously, the treatment of wild and captive eggs differed to some extent and the sample size of wild eggs was small. Despite these limitations, we believe a comparison is necessary when undergoing a captive breeding programme to be able to better mimic natural conditions.

Hormone assays were performed using Enzyme Immunoassay Kit (Neogen, Lexington, USA; for more details see electronic supplementary material, appendix S1).

### (b) Data analysis

We investigated the effect of the experimental stimulation on number of eggs per female, hatching success, fertilization success, testosterone levels, chick sex ratio and chick growth using SAS 9.1.3 (Cary, USA; SAS 2001). The experimental stimulation was included in the model either as a group effect (treatment effect: C, D<sup>+</sup>, D<sup>-</sup>; we gave value 0, 1 and 2 to C, D<sup>-</sup> and D<sup>+</sup> females, respectively, to account for the stimulation's hierarchy between the three groups) or as a linear effect (maximal display frequency observed by a given female). To avoid the inclusion of highly correlated variables in the same model, treatment effect and maximal display activity were never entered together in a model.

We examined the effect of the stimulation on number of eggs per female using generalized linear models (GLM; Proc GENMOD; distribution of error terms: Poisson; link function: log). The effect of the stimulation on fertilization success and hatching success using generalized linear mixed models (GLMM; Proc GLIMMIX) was investigated with a binomial distribution of error terms (0/1 = non-fertilized/fertilized or non-hatched/hatched; link function: logit). We included the treatment effect as a fixed effect, and the mother's identity as a random factor, which accounts for repeated sampling (SAS 2001). We examined the effect of the stimulation on egg testosterone levels using GLM (Proc GENMOD; Poisson error; link: log) with the treatment effect as a fixed effect. We investigated the effect of the

stimulation on chick sex ratio with a GLMM (Proc GLIMMIX; binomial error; link: logit). We tested the effect of the stimulation on chick growth by modelling chick body mass as a function of age, squared age, sex, birth date and group with a GLMM (Proc GLIMMIX; normal error; link: identity). Chick identity nested within the mother's identity was set as the random factor.

The explanatory variables were the treatment effect, display activity perceived by the female (maximal display activity), mother's condition, age, inbreeding rate, father's identity (to control for a potential genetic effect on offspring development), number of eggs per female, egg rank (except for testosterone models as eggs were all first eggs) and standardized variables ( $z$ -scores) for laying date and egg mass. They were entered in the candidate models as independent fixed variables. When needed and when possible, we adjusted the degrees of freedom using the Satterthwaite correction, which can result in fractional degrees of freedom.

We selected minimum adequate multivariate models using a maximum-likelihood model selection based on Akaike's information criterion for small sample size (AIC<sub>c</sub>; Burnham & Anderson 2002; Johnson & Omland 2004). We evaluated a set of candidate models obtained by any combination of the explanatory variables. Models were ranked using a freely available spreadsheet (Brian R. Mitchell software; <http://www.uvm.edu/~bmitchel/software.html>) according to their AIC<sub>c</sub>. Among this set of candidate models, we retained models with the smallest AIC<sub>c</sub> (and the highest cumulative model weight  $w_i$ ; all  $w_i$  were  $>0.75$ ). The four best models explaining each independent variable are presented in electronic supplementary material, table S2. To explain circulating testosterone levels in chicks, we ran a model including testosterone level of sacrificed eggs (which reduced sample size) and a model including all sampled chicks. Post hoc simple comparisons between groups (C versus D<sup>-</sup>, C versus D<sup>+</sup> and D<sup>-</sup> versus D<sup>+</sup>) were computed for any overall significant treatment effect. Given the small sample size, we did not use statistics to compare maternal allocation in the wild and in captivity.

## 3. RESULTS

### (a) Hatching success and fertilization success

Our treatment did not increase the number of eggs laid per female over the breeding season (GLM, treatment:  $F_{1,84} = 0.00$ ,  $p = 0.9569$ ; GLM, maximal display frequency observed:  $F_{1,57} = 1.32$ ,  $p = 0.2557$ ; table 1). Wild eggs had higher fertilization and hatching rates than eggs laid by C, D<sup>-</sup> and D<sup>+</sup> females (electronic supplementary material, figure S2). Stimulation of the females had a positive effect on the overall hatching success. When we considered the maximal display frequency of the three males observed by a given female, hatching success increased with display frequency (GLMM, treatment:  $F_{1,72.66} = 4.62$ ,  $p = 0.0349$ ; GLMM, maximal display frequency:  $F_{1,72.7} = 4.09$ ,  $p = 0.0467$ ; table 1 and figure 1). The higher hatching success was the result of a higher fertilization success and/or early embryo development and not the result of lower late embryo death (GLMM, treatment:  $F_{1,74.5} = 4.72$ ,  $p = 0.0330$ ; GLMM, maximal display frequency:  $F_{1,77.2} = 5.64$ ,  $p = 0.0201$ ; table 1). When including only fertilized eggs in the model, display frequency did

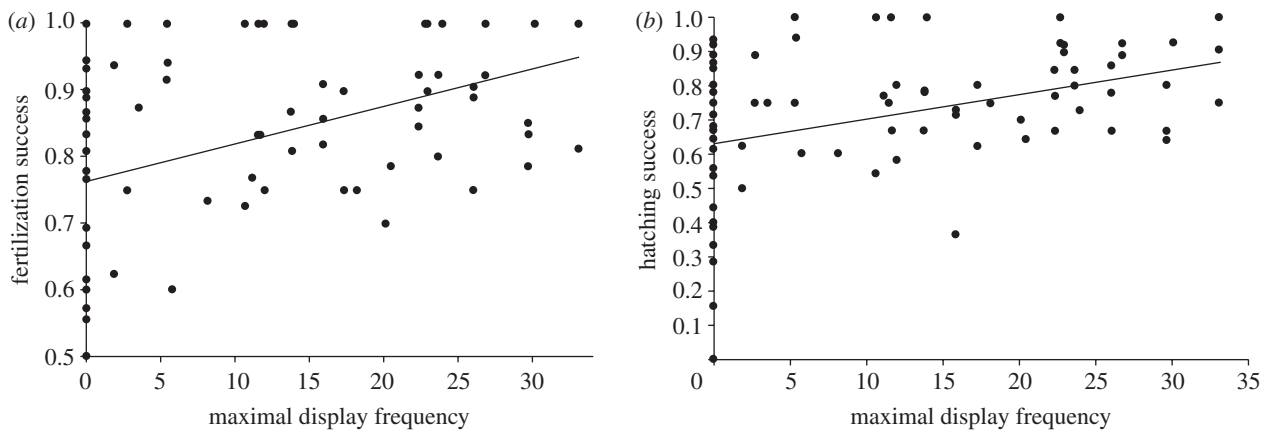


Figure 1. Relationship between the maximal display frequency observed by the female and (a) fertilization success and (b) hatching success.

Table 1. Best generalized linear mixed models for the number of eggs laid per female, fertilization success, hatching success and yolk testosterone. The bold values indicate the  $p$  is significant at the 0.05 level. Best models were selected based on their AICc. The four best models are presented in electronic supplementary material, table S1.

dependent variable	independent variables	d.f.	$F$	$p$	AICc	$w$
number of eggs per female	mother's condition	1,87	6.89	<b>0.0103</b>	-2426	0.964
fertilization success	maximal display frequency observed	1,77.2	5.64	<b>0.0201</b>		
	mother's age	1,83.2	4.90	<b>0.0296</b>		
	number of eggs per female <i>model</i>	1,77.08 3	8.92 10.53	<b>0.0038</b> <b>&lt;0.0001</b>	-10 309	0.988
hatching success	maximal display frequency observed	1,72.7	4.09	<b>0.0467</b>		
	number of eggs per female	1,88.69	8.30	<b>0.0050</b>		
	egg rank <i>model</i>	1,1054 3	6.38 9.52	<b>0.0117</b> <b>&lt;0.0001</b>	-9591	0.940
yolk testosterone	experimental treatment	1,27	5.25	<b>0.0300</b>		
	number of eggs per female <i>model</i>	1,27 2	10.49 8.06	<b>0.0032</b> <b>0.0018</b>	-1045	1

not affect the probability of hatching (GLMM, treatment:  $F_{1,87.12} = 0.88$ ,  $p = 0.3508$ ; GLMM, maximal display frequency observed:  $F_{1,87.75} = 1.04$ ,  $p = 0.3099$ ).

### (b) Testosterone in egg yolk

Stimulation of the mother increased yolk testosterone levels (GLMM, treatment:  $F_{1,27} = 5.25$ ,  $p = 0.0219$ ; GLMM, maximal display frequency:  $F_{1,27} = 0.91$ ,  $p = 0.3493$ ; table 1), as eggs laid by  $D^+$  females contained similar testosterone levels to eggs from wild populations (figure 2a). In contrast, watching poorly displaying males did not increase testosterone levels compared with the control group (figure 2a). When including only fertilized eggs in the models, yolk testosterone level of the sacrificed eggs did not explain the hatching success of the fertilized non-sacrificed eggs (GLMM, testosterone:  $F_{1,32.41} = 0.34$ ,  $p = 0.5664$ ).

### (c) Chick testosterone levels and growth

Circulating testosterone levels in chicks at the age of 15 days was best explained by yolk testosterone of the corresponding sacrificed eggs (GLMM, yolk testosterone of the first laid egg:  $F_{1,20.95} = 17.53$ ,  $p = 0.0002$ ; table 2), assuming a relationship in the testosterone levels between the first and subsequent eggs. Therefore, maternal

testosterone received by the embryo during its development probably enhanced the production of endogenous testosterone in chicks. When yolk testosterone was discarded from the analysis (increasing sample size), the chick testosterone level was best explained by the experimental stimulation of the mother (GLMM, treatment:  $F_{1,72.6} = 6.11$ ,  $p = 0.0025$ ; GLMM, maximal display frequency:  $F_{1,70.57} = 1.29$ ,  $p = 0.2003$ ; figure 2c), with chicks from  $D^+$  females having higher circulating testosterone level than chicks from both C and  $D^-$  females. Our experiment had no effect on the chick sex ratio (GLMM, treatment:  $F_{1,629} = 0.15$ ,  $p = 0.6974$ ; GLMM, maximal display frequency:  $F_{1,629} = 0.47$ ,  $p = 0.4931$ ; yolk testosterone of the sacrificed eggs:  $F_{1,631} = 2.46$ ,  $p = 0.1169$ ; table 2; electronic supplementary material, figure S3). The treatment positively influenced chick growth rate (table 2 and figure 3; electronic supplementary material, figure S4), with the chicks from  $D^+$  females growing faster than the chicks from C and  $D^-$  females.

Among the 750 chicks produced, 55 chicks were produced by experimental fathers (6 fathers from the  $D^-$  group and 10 fathers from the  $D^+$  group). The growth rate of these chicks was explained neither by the group of the father ( $F_{1,25.74} = 0.75$ ,  $p = 0.3949$ ) nor by the display rate of the father ( $F_{1,21.62} = 1.80$ ,

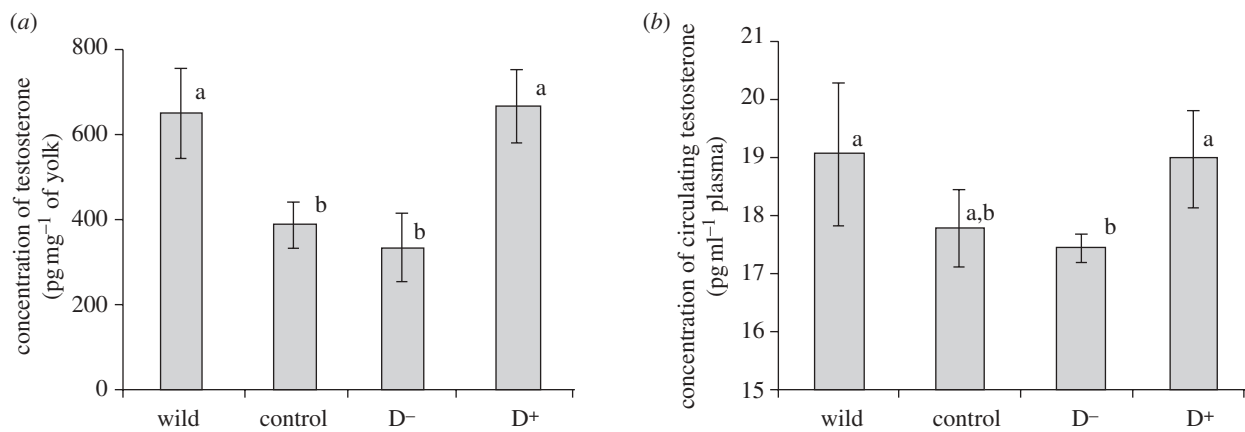


Figure 2. Relationship between treatment and (a) egg yolk testosterone and (b) concentration of circulating testosterone of 15-day-old chicks. Wild eggs were eggs collected in the wild and artificially incubated, control eggs were from females housed in front of females, D<sup>-</sup> eggs were from females housed in front of poorly displaying males and D<sup>+</sup> eggs were from females housed in front of highly displaying males. Represented values are means  $\pm$  s.e. (error bars), calculated from row data. Different letters indicate statistical difference significant at the 0.05 level (Tukey post hoc tests).

Table 2. Best generalized linear mixed models for chick circulating testosterone, chick sex ratio and chick growth. The bold values indicate the  $p$  is significant at the 0.05 level. Best models were selected based on their AICc. The four best models are presented in electronic supplementary material, table S1.

dependent variable	independent variables	d.f.	$F$	$p$	AIC	$w$
chick circulating testosterone (reduced sample)	yolk testosterone of the sacrificed eggs	121.8	19.26	<b>0.0002</b>	-948	0.752
	mother's inbreeding rate	120.69	0.51	<b>0.4844</b>		
chick circulating testosterone (full sample)	standardized hatching date	1153	2.67	<b>0.0401</b>	437	0.900
	experimental treatment	172.6	6.11	<b>0.0025</b>		
	model	2	10.91	<b>&lt;0.001</b>		
chick sex ratio	yolk testosterone of the sacrificed eggs	1633	0.26	<b>0.6121</b>	99	1
	standardized hatching date	1633	3.75	<b>0.0527</b>		
	model	2	2.86	<b>0.0636</b>		
chick growth	chick age	15 304	18 615.5	<b>&lt;0.0001</b>		
	chick squared age	15 285	1659.32	<b>&lt;0.0001</b>		
	chick sex	1192.5	65.54	<b>&lt;0.0001</b>		
	mother's inbreeding rate	1215.8	0.31	<b>0.5758</b>		
	experimental treatment	1201.5	4.44	<b>0.0129</b>		
	father identity	111 253.3	1.39	<b>0.0174</b>		
	model 1	6	72 224.2	<b>&lt;0.0001</b>		
chick growth	chick age	15 306	18 614.8	<b>&lt;0.0001</b>		
	chick squared age	15 286	1658.85	<b>&lt;0.0001</b>		
	chick sex	1189.9	65.94	<b>&lt;0.0001</b>		
	mother's inbreeding rate	1214.2	0.39	<b>0.5348</b>		
	father identity	111 249.4	1.61	<b>0.0012</b>		
	maximal display frequency observed	1187.2	12.65	<b>0.0005</b>		
	model 2	6	72 225.7	<b>&lt;0.0001</b>		

$p = 0.1942$ ), suggesting that father quality did not strongly affect chick growth in this experiment.

Including both circulating testosterone level and treatment effect in the same model (which reduced sample size) revealed that the variation in chick growth was mediated by the level of circulating testosterone of the chicks (GLMM, treatment model: testosterone:  $F_{1,154.3} = 10.57$ ,  $p = 0.0023$ ; treatment effect:  $F_{1,159.6} = 0.18$ ,  $p = 0.6723$ ; GLMM, maximal display frequency model: testosterone:  $F_{1,154.3} = 10.57$ ,  $p = 0.0014$ ; maximal frequency:  $F_{1,159.8} = 2.39$ ,  $p = 0.1238$ ).

Wild chicks grew faster than captive-bred chicks, as evidenced by their gain of body mass over time, and at

the age of 15 days they had higher circulating testosterone levels than captive-bred chicks (figure 3).

#### 4. DISCUSSION

Our results demonstrate that females exposed to male courtship behaviours modulate maternal allocation according to the observed display rate. Allowing females to observe males with high display frequencies resulted in higher hatching success, fertilization success, yolk testosterone levels, chick growth rate and increased circulating testosterone levels in chicks. However, the experimental treatment did not affect the number of

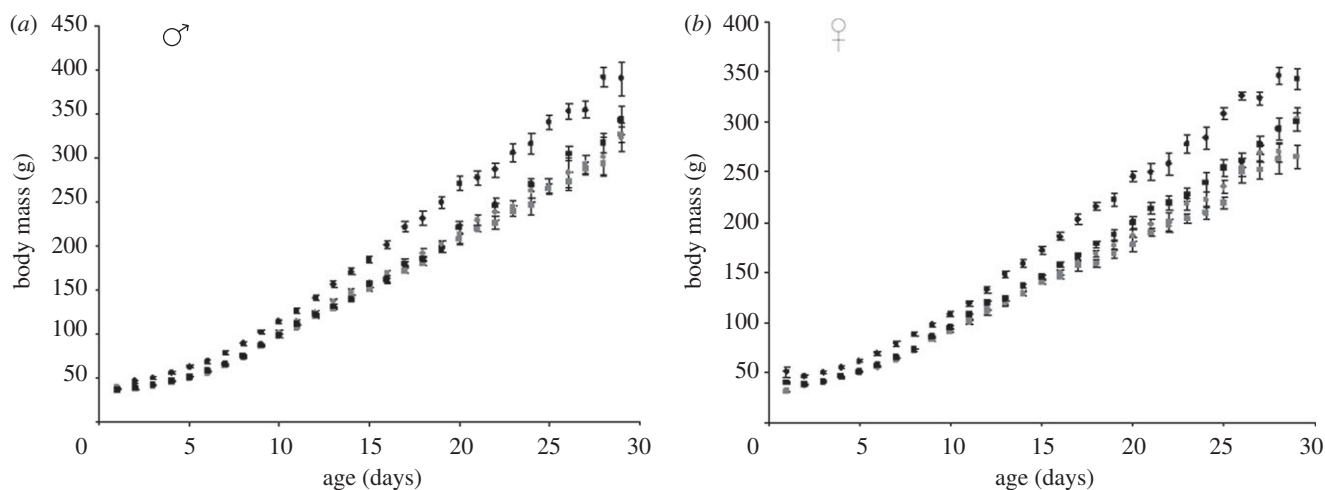


Figure 3. Relationship between treatment and early growth of (a) male and (b) female chicks. Wild chicks were chicks hatched from eggs collected in the wild and artificially incubated; data are represented by black dots. Control chicks were from females in front of females; data are represented by grey dots.  $D^-$  eggs were from females housed in front of poorly displaying males, data are represented by grey squares.  $D^+$  eggs were from females housed in front of highly displaying males; data are represented by black squares. Represented values are means  $\pm$  s.e. (error bars), calculated from row data. Different letters indicate statistical difference significant at the 0.05 level (Tukey post hoc tests).

eggs produced per female nor offspring sex ratio. The differential allocation hypothesis states that females adjust their investment to male attractiveness to trade off their own future fitness with current offspring fitness (Burley 1988). In the Houbara bustard, display activity is an honest signal of male quality (Chargé *et al.* in press). Females mated with high-quality males are expected to obtain increased benefits in terms of offspring fitness, which is in line with the differential allocation hypothesis.

Gil *et al.* (2004) and Tanvez *et al.* (2004) exposed female canaries to recorded songs of varying attractiveness and collected the infertile eggs laid. They found a gradual effect of the stimulation on yolk testosterone, but were unable to further investigate the consequences of maternal allocation on the offspring. Our study design allowed the opportunity to evaluate how signal-modulating maternal allocation is perceived and processed, because we used artificial insemination. This technique allowed us to decouple the paternal effects and the degree to which the mothers were exposed to the signal on offspring fitness. In our experiment, the fitness consequences observed were exclusively attributable to the female's perception of her environment (i.e. male display). Our results thus demonstrate that a purely sensory pathway, via female visual stimulation, is able to trigger maternal allocation.

Previous studies found a linear effect of male attractiveness on yolk testosterone (Gil *et al.* 2004; Tanvez *et al.* 2004). Different communication channels (canary: acoustic; Houbara: mainly visual) may promote different effects. In addition, female exposure to visual stimulation is expected to vary according to her mating system. Shortly before egg-laying, socially monogamous females mainly interact with their social mate. They are therefore expected to receive and process one signal, and invest in reproduction proportionally to this signal. In contrast, females of lekking species visit leks, an aggregation of displaying males. They consequently receive several signals that are of varying quality, but would usually mate only

with the most attractive male. They should thus invest testosterone according to the best signal received.

Despite accumulating evidence on maternal effects owing to yolk hormone variations, how these effects influence offspring development and survival remains unclear (Groothuis & Schwabl 2008). Hormone levels in the yolk significantly decrease during embryo development. It is generally acknowledged that maternal testosterone is gradually replaced by endogenous testosterone during embryonic development (Carere & Balthazart 2007; Groothuis & Schwabl 2008). Interestingly, our results suggest that maternal testosterone received by the embryo during its development may enhance the genetically determined production of endogenous testosterone in chicks, which in turn may favour chick growth. Therefore, our results are in line with the idea that differential allocation could act as an amplifier of good genes, as suggested by Sheldon (2000).

Our experimental stimulation of the Houbara females had no effect on the chick sex ratio. However, differential maternal allocation can lead to an offspring sex ratio bias in some systems, which could hamper the management of conserved species (Robertson *et al.* 2006). Indeed, supplementary feeding of the critically endangered kakapo *Strigops habroptilus* to enhance breeding frequency and breeding success led to an unfortunate offspring sex ratio bias towards males. Robertson *et al.* (2006) optimized the feeding regime of the kakapo females and successfully removed the bias in sex ratio. Doing so, they documented the first example of a successful manipulation of maternal allocation through female diet for species conservation. To our knowledge, our study is the first example of a successful manipulation of maternal allocation through sensory stimulation for species conservation.

It has been argued that maternal life-history decisions, such as mate preferences and maternal allocation, are adaptive, and that they could favour population growth and viability since they favour offspring fitness (Wedekind 2002). Such preferences are usually not considered in

conservation breeding programmes, where reproduction occurs in captivity and the resulting progeny released to support small wild populations, because these programmes are mainly structured around genetic considerations. Our results show that it is possible to favour maternal allocation independently of the genetic quality of the mate, by stimulating females with attractive males.

In captive breeding programmes, inducing individuals to breed, and especially to breed with an individual selected by the managers to maintain a high genetic diversity in the captive pool, can be problematic. Hence, using assisted reproductive techniques such as artificial insemination has become a common practice (Saint Jalme *et al.* 2003). In this context, our findings have important implications for captive breeding programmes. Our data suggest that using artificial insemination in such programmes can reduce maternal allocation, thereby decreasing both the number of offspring produced and the future survival of the offspring in the absence of stimulation of females by displaying males. Without female stimulation, artificial insemination is expected to have negative effects on population dynamics, and therefore on population viability (Benton *et al.* 2005), compared with natural reproduction. However, as suggested by our findings, high levels of maternal allocation can be maintained if artificial insemination is coupled to an appropriate stimulation of the breeding females.

In short, we demonstrated that maternal effects can be mediated by behavioural signals. Unravelling the proximate mechanisms of maternal differential allocation showed that they are based on sensory stimulation. Male display courtship constitutes an effective signal shaping maternal allocation and provides conservationists with an easy and cheap tool to manipulate and maximize maternal allocation.

We thank His Highness Sheikh Mohamed Bin Zayed Al Nahyan, Founder and Sponsor. We greatly thank His Excellency Mohammed Al Bowardi, General Manager of the Office of Mohamed Bin Zayed Al Nahyan, and Jacques Renaud, Manager of the ECWP, for their support. We thank G. Sorci, M. Saint Jalme, R. Chargé and Y. Hingrat for discussing the project; G. Lévêque for artificial inseminations; F. El Marhoune and A. Rguigui for help in collecting blood; G. Wichmann and I. Lehman for providing lab facilities; D. S. Schmeller, A. S. Chaine, C. Kapfer, H. Kokko, D. B. Miles, A. Roulin and two anonymous referees, who provided valuable comments and discussion on the manuscript; and C. Dormann, R. Strüby and J. Clobert for their statistical advice.

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Running title: Stimulation improves maternal allocation

2

**Watching sexy displays improves hatching success and offspring  
4 growth through maternal allocation: an application to  
conservation biology**

6

**Adeline Loyau<sup>1,2,3\*</sup> and Frédéric Lacroix<sup>1</sup>**

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## Electronic supplementary material 1

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### Appendix S1 *Supplementary methods*

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**Hormone Assays.** We assessed testosterone and androstenedione levels in the yolk. To assess egg steroid levels, we took the eggs out of the freezer and left them at 4°C during 3 hours. Albumen thaws faster than yolk, allowing for easy separation and treatment. After thawing, we recorded the yolk wet mass (to the nearest 0.01g). We mixed the yolk with a pestle and then with a magnetic stirrer in an equal amount (w/v) of distilled water for 15 min. We transferred aliquots of this 2x dilution into 1.5 ml Eppendorf tubes and froze them at -20°C until extraction. We extracted steroids from a sample of approximately the same volume of yolk/water emulsion that we weighted to the nearest 0.1 mg. We added 1 ml of ethyl acetate to the sample, vortexed for 30 sec and centrifuged for 15 min at 11000 r.p.m. at 4°C. We pipeted the supernatant into a new vial and let it evaporate over night at room temperature. The residue was dissolved in 100 µl of extraction buffer provided by the manufacturer (Neogen, Lexington, USA). For testosterone assays, the sample was diluted 20-fold. We did not dilute the samples before androstenedione assay. We extracted steroids from 100 µl of chick plasma and dissolved the residue in 70 µl of extraction buffer to concentrate the steroids before assay.

Steroid assays were performed in duplicate using a commercially available Enzyme Immunoassay Kit (Neogen, Lexington, USA) according to the manufacturer's protocol. Briefly, we deposited 50 µl of sample in a well of an antibody coated microplate, added 50 µl of enzyme conjugate, then shook and incubated the plate at room temperature in a wet chamber for 1 h. We washed the plate four times with 300 µl of wash buffer and added 150 µl of substrate. After 30 min of incubation, we stopped the enzyme reaction with 150 µl of 1M H<sub>2</sub>SO<sub>4</sub> and immediately read the plate at 450 nm with a spectrophotometer (SPECTRA IMAGE, Tecan, Crailsheim, Germany). The measured values were analyzed with EasyWIN kinetics V6.0a and a standard curve was used to quantify steroid levels. When the concentration did not fit within the range of the standard curve, the samples were further diluted and re-assessed. Dose response of serially diluted extracts yielded a parallel slope to the standard curves, suggesting no sample matrix effects. The intra-assay coefficients of variation of testosterone, androstenedione and corticosterone were below 5 % and inter-assay coefficients were below 9%.

52 Cross reactivity for the testosterone antibody reported by the manufacturer was:  
testosterone (100 %), dihydrotestosterone (100 %), androstenedione (0.86 %), bolandiol (0.86  
54 %), testosterone enanthate (0.13 %), estriol (0.10 %), testosterone benzoate (0.10 %), and  
other compounds had negligible cross reactivity (<0.06 %). Cross reactivity for the  
56 androstenedione antibody as reported by the manufacturer was: androstenedione (100%),  
estrone (1.50%), pregnenolone (0.20%), deoxycorticosterone (0.16%), estrone-3-sulfate  
58 (0.16%), and other compounds had negligible cross reactivity (<0.08 %).

Running title: Stimulation improves maternal allocation

2

# Watching sexy displays improves hatching success and offspring 4 growth through maternal allocation

6 **Adeline Loyau<sup>1,2,3\*</sup>** and **Frédéric Lacroix<sup>1</sup>**

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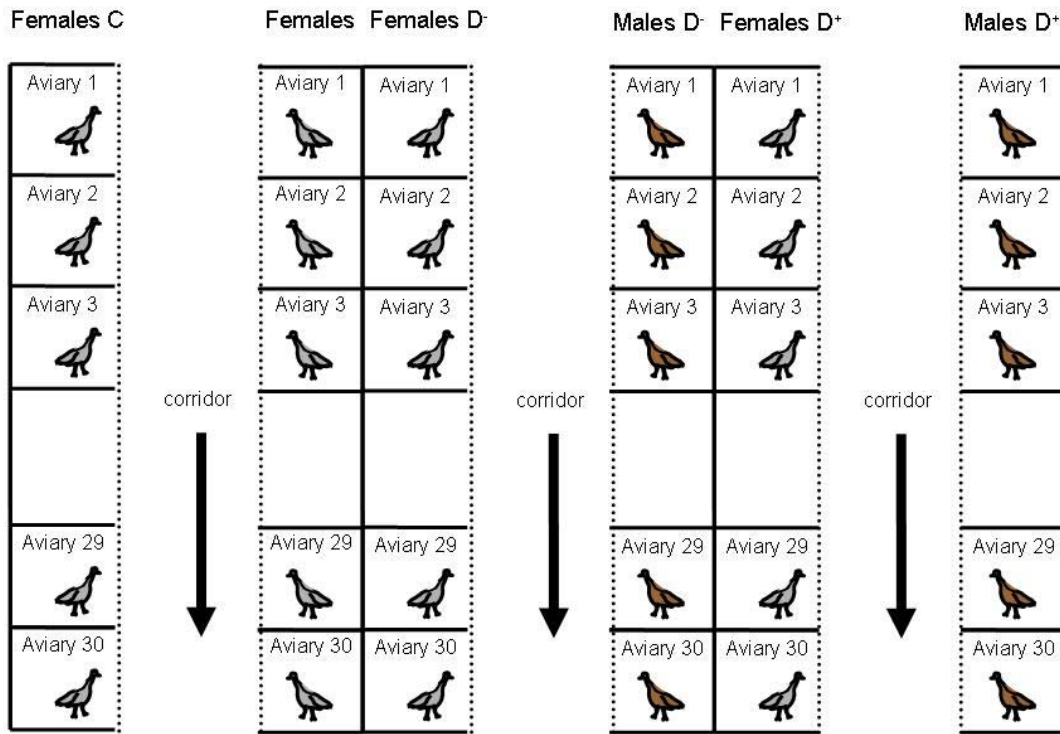
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Number of tables: 2

22 Number of figures: 3

24 **Electronic supplementary material 2**

26 **Figure S1.** *Disposition of the aviaries during the experimental stimulation of the females*



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### Electronic supplementary material 3

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**Appendix S2** Model selection (k, model rank, AICc and w for the four best models). AGE: chick age, AGE2: squared chick age, EGGRANK: egg rank; EGGW: egg weight on the day of laying, FATHER: father's identity, FMAX: maximal display frequency observed by a female, GPE: treatment effect, HATCHDATE: standardized hatching date (z-score), LAYDATE: standardized laying date (z-score), MAGE: mother's age, MCOND: mother's condition, MINBREE: mother's inbreeding rate, NEGGS: number of eggs laid by a female over the breeding season, YOLKTESTO: yolk testosterone level of sacrificed eggs, SEX: sex of the chick.

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Model	k	rank	AICc	w
<b>Number of eggs per females</b>				
MCOND	1	1	-2426	0.964
MINBREE	1	2	-2277	0.036
MCOND + MINBREE	2	3	-2419	3.03e-07
GPE	1	4	-2242	6.02e-33
<b>Fertilization success</b>				
FMAX+ NEGGS + MAGE	3	1	-10309	0.988
NEGGS + EGGRANK + MAGE	3	2	-10300	0.011
FMAX + NEGGS + LAYDATE	3	3	-10293	3.32e-04
GPE + NEGGS + EGGRANK	3	4	-10285	1.01e-07
<b>Hatching success</b>				
FMAX + NEGGS + EGGRANK	3	1	-9591	0.940
GPE + NEGGS + EGGRANK	3	2	-9586	0.005
FMAX + NEGGS + LAYDATE	3	3	-9581	0.011
FMAX + EGGRANK + LAYDATE	3	4	-9575	4.97e-04
<b>Yolk testosterone</b>				

GPE + NEGGS	2	1	-1045	1
GPE + NEGGS + LAYDATE	3	2	-1015	2.96e-07
NEGGS + LAYDATE	2	3	-997	4.87e-11
NEGGS	1	4	-996	2.43e-11
<b>Chick circulating testosterone</b> (reduced sample)				
YOLKTESTO + MINBREE	2	1	-948	0.752
YOLKTESTO + MINBREE + GPE	3	2	-947	0.125
YOLKTESTO	1	3	-939	0.060
YOLKTESTO + MINBREE + GPE + NEGGS	4	4	-943	0.018
<b>Chick circulating testosterone</b> (full sample)				
GPE + HATCHDATE	2	1	437	0.900
GPE + HATCHDATE + SEX	3	2	447	0.005
GPE + SEX	2	3	450	0.001
SEX + HATCHDATE	2	4	451	0.001
<b>Chick sex ratio</b>				
YOLKTESTO + HATCHDATE	2	1	99	1
HATCHDATE + FATHER	2	2	185	3.16e-19
YOLKTESTO + FATHER	2	3	194	2.93e-21
HATCHDATE + EGGW	2	4	226	2.9e-28
<b>Chick growth</b>				
AGE + AGE2 + SEX + FATHER + MINBREE + GPE	6	1	61787	0.982

AGE + AGE2 + SEX + FATHER + MINBREE + FMAX	6	2	61783	0.0001
AGE + AGE2 + SEX + FATHER + GPE	5	3	61799	0.00385
AGE + AGE2 + SEX + FATHER + MINBREE	5	4	61805	0.000137

Running title: Stimulation improves maternal allocation

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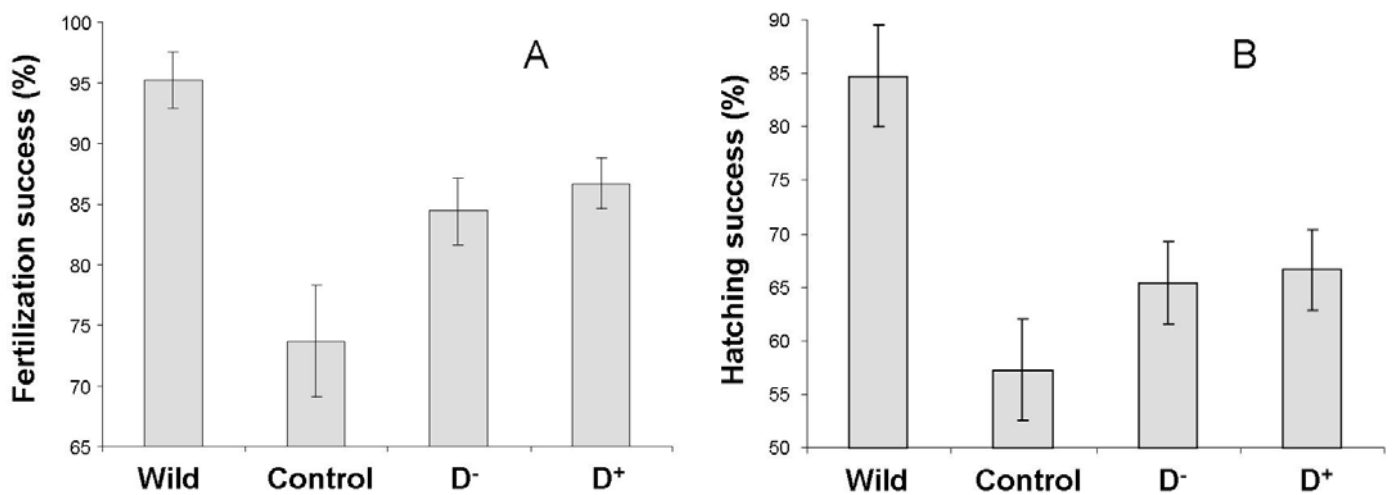
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#### Electronic supplementary material 4

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#### Figure S2. Relationship between treatment and (A) fertilization success and (B) hatching

22 success. Wild eggs were eggs collected in the wild and artificially incubated, Control eggs  
24 were from females housed in front of females, D<sup>-</sup> eggs were from females housed in front of  
26 poorly displaying males, and D<sup>+</sup> eggs were from females housed in front of highly displaying  
males. Represented values are means  $\pm$  SE (error bars), calculated from row data. Different  
letters indicate statistical difference significant at the 0.05 level (Tukey post-hoc tests).



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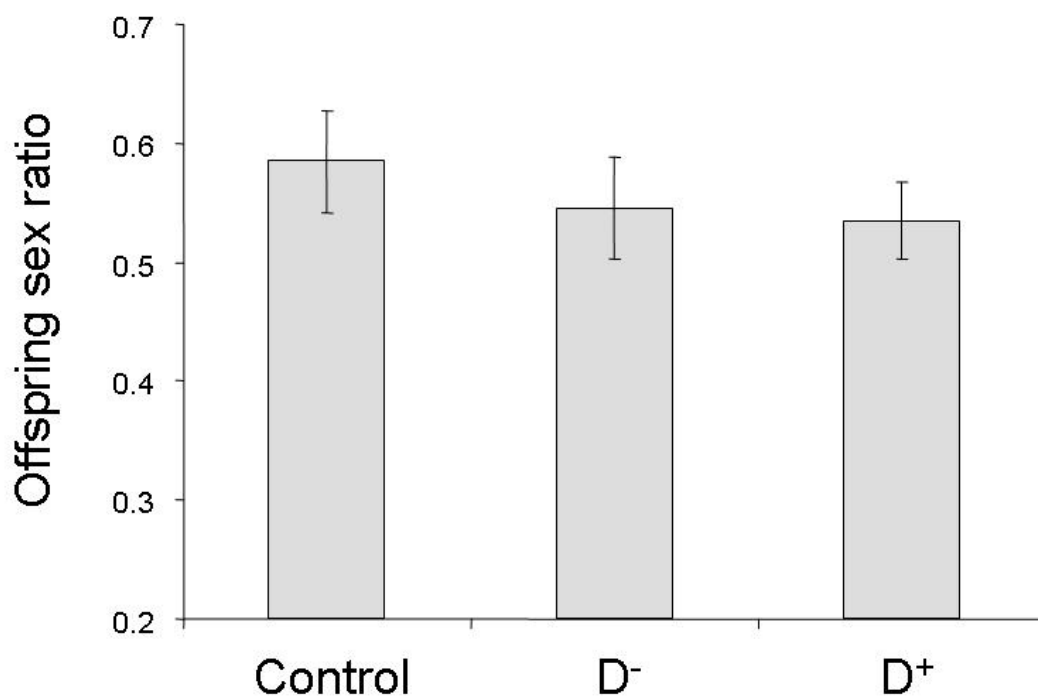
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### Electronic supplementary material 5

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**Figure S3. Relationship between treatment and offspring sex ratio.** Control eggs were from females housed in front of females, D<sup>-</sup> eggs were from females housed in front of poorly displaying males, and D<sup>+</sup> eggs were from females housed in front of highly displaying males. Represented values are means  $\pm$  SE (error bars), calculated from row data.

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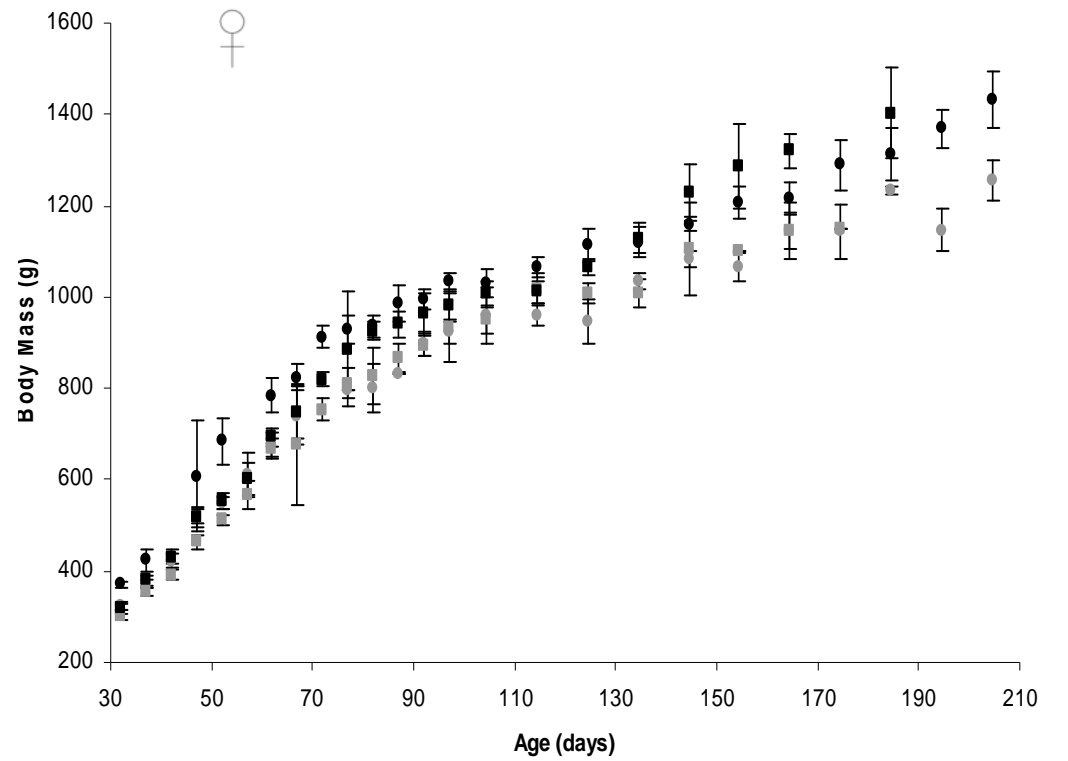
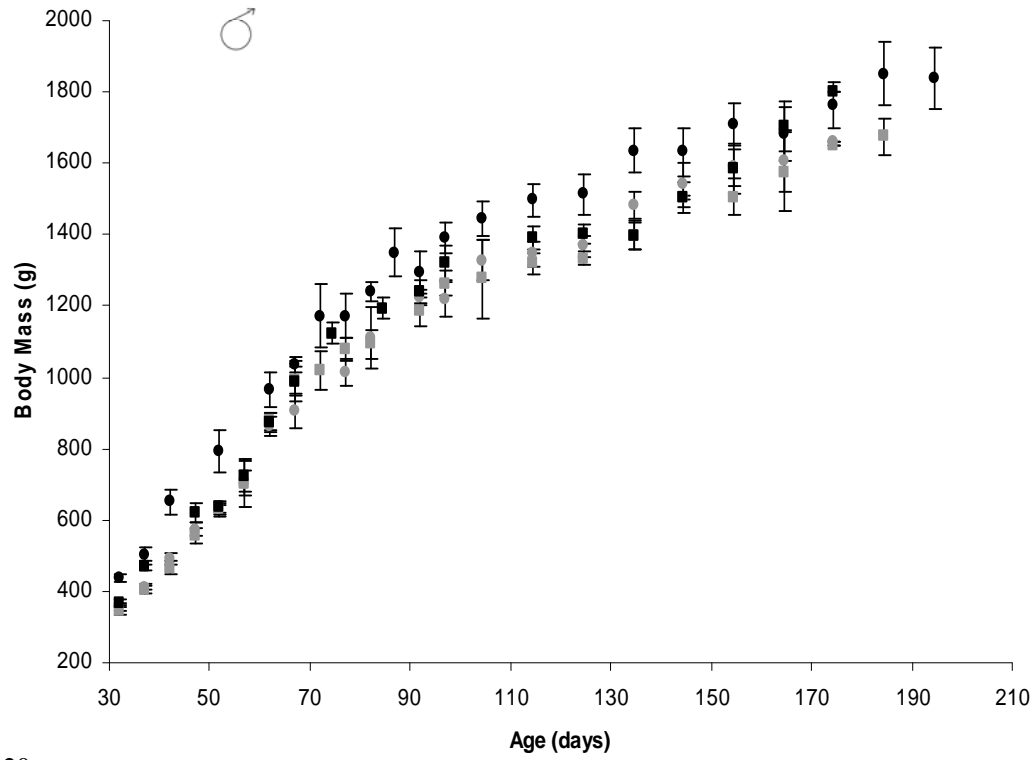
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Electronic supplementary material 6

18 **Figure S4.** Relationship between treatment and chick later growth



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24 **Figure S4. Relationship between treatment and chick later growth.** Wild chicks were chicks from eggs collected in the wild and artificially  
incubated, data are represented by black dots. Control chicks were from females in front of females, data are represented by grey dots. D<sup>-</sup> eggs were  
26 from females housed in from of poorly displaying males, data are represented by grey squares. D<sup>+</sup> eggs were from females housed in front of highly  
displaying males, data are represented by black squares. Represented values are means  $\pm$  SEM (error bars).

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