

Research paper

No evidence for sex-specific effects of the maternal social environment on offspring development in Japanese quail (*Coturnix japonica*)Esther M.A. Langen^{a,b,*}, Nikolaus von Engelhardt^{a,1}, Vivian C. Goerlich-Jansson^{a,b,2}^a Department of Animal Behaviour, Bielefeld University, Morgenbreede 45, 33615 Bielefeld, Germany^b Department of Animals in Science and Society, Utrecht University, Yalelaan 2, 3508 TD Utrecht, The Netherlands

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ABSTRACT

The social environment of reproducing females can cause physiological changes, with consequences for reproductive investment and offspring development. These prenatal maternal effects are often found to be sex-specific and may have evolved as adaptations, maximizing fitness of male and female offspring for their future environment. Female hormone levels during reproduction are considered a potential mechanism regulating sex allocation in vertebrates: high maternal androgens have repeatedly been linked to increased investment in sons, whereas high glucocorticoid levels are usually related to increased investment in daughters. However, results are not consistent across studies and therefore still inconclusive. In Japanese quail (*Coturnix japonica*), we previously found that pair-housed females had higher plasma androgen levels and tended to have higher plasma corticosterone levels than group-housed females. In the current study we investigate whether these differences in maternal social environment and physiology affect offspring sex allocation and physiology. Counter to our expectations, we find no effects of the maternal social environment on offspring sex ratio, sex-specific mortality, growth, circulating androgen or corticosterone levels. Also, maternal corticosterone or androgen levels do not correlate with offspring sex ratio or mortality. The social environment during reproduction therefore does not necessarily modify sex allocation and offspring physiology, even if it causes differences in maternal physiology. We propose that maternal effects of the social environment strongly depend upon the type of social stimuli and the timing of changes in the social environment and hormones with respect to the reproductive cycle and meiosis.

1. Introduction

Variation in the social environment affects female behaviour and physiology with potential consequences for reproductive investment. Changes in reproductive investment, in turn, modify the prenatal environment of the developing offspring and can thereby profoundly shape offspring's future phenotype (Groothuis et al., 2005; Guibert et al., 2010; Kaiser and Sachser, 2009, 2005). Pre- and postnatal maternal effects of the social environment can bias offspring sex ratios (Clutton-Brock and Iason, 1986; Michler et al., 2013; Minias et al., 2014) and affect offspring development and behaviour, often in a sex-specific way (Kaiser and Sachser, 2009, 2005). Sex-specific maternal effects are thought to have evolved as adaptations, maximizing fitness of male and female offspring for their anticipated environment. Maternal steroid hormones provide important candidate signals, transmitting effects of the social environment across generations. Hormones,

and other compounds, are transferred to the ovum and embryo and can profoundly affect offspring behavioural and physiological development (Groothuis et al., 2005; Groothuis and Schwabl, 2008; Kaiser and Sachser, 2005; Meylan et al., 2012; Radder, 2007; von Engelhardt and Groothuis, 2011).

Oviparous species, such as reptiles and birds, are especially suitable to explore prenatal effects because the maternal and offspring environment can be independently manipulated. Previous studies on avian species have shown effects of the social environment on sex allocation (Michler et al., 2013; Minias et al., 2014). In the great cormorant (*Phalacrocorax carbo sinensis*), social density positively correlates with the proportion of male offspring within broods (Minias et al., 2014). In contrast, female great tits (*Parus major*) bred in areas with experimentally increased nesting densities or who experienced areas with high nesting density as juveniles produce female-biased broods in the following year, whereas females breeding in areas with decreased

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nesting densities or reared in areas of naturally lower nesting density produce male-biased broods (Michler et al., 2013). In many vertebrate species, changes in both primary and secondary offspring sex ratio have been linked to variation in maternal plasma steroids around conception (reviewed by Alonso-Alvarez (2006), James (2008), Krackow (1995), Navara (2013a), Pike and Petrie (2003)). In avian species, increased levels of maternal androgens usually lead to male-biased offspring sex ratios (Goerlich-Jansson et al., 2013; Goerlich et al., 2009; Pike and Petrie, 2005; Rutkowska and Cichoń, 2006; Veiga et al., 2004, but see Correa et al., 2011), whereas increased levels of maternal glucocorticoids often result in female-biased offspring sex ratios (Bonier et al., 2007; Goerlich-Jansson et al., 2013; Love et al., 2005; Pike and Petrie, 2006, 2005, but see Gam et al., 2011; Henriksen et al., 2013). In Japanese quail (*Coturnix japonica*), naturally increased maternal faecal corticosterone (CORT) metabolite concentrations and experimentally elevated maternal plasma CORT concentrations are associated with a female-biased primary sex ratio (Pike and Petrie, 2006). In contrast to the findings in other species, maternal plasma testosterone levels of Japanese quail have been related to both an unbiased (Pike and Petrie, 2006) as well as a female-biased offspring sex ratio (Correa et al., 2011). This indicates that results from experimental and correlational studies are still inconclusive.

In addition to affecting offspring sex ratio, the maternal social environment can affect offspring growth and survival, which may be mediated by changes in maternal circulating levels of androgens and CORT. For example, in American red squirrels (*Tamiasciurus hudsonicus*), increased offspring growth rates at higher social densities have been attributed to the effects of increased maternal CORT (Dantzer et al., 2013). In Japanese quail, social instability resulted in an increase in agonistic interactions and reduced offspring body mass at the age of 1–3 weeks, compared to stable social groups (Guibert et al., 2010). Such effects on growth may be due to increased maternal CORT because artificially increasing maternal circulating CORT reduced offspring growth in Japanese quail (Hayward and Wingfield, 2004). The maternal social environment and maternal hormones can also have sex-specific effects on offspring growth and survival. In guinea pigs (*Cavia aperea*), housing females individually during pregnancy decreased growth of daughters compared to daughters of group-housed females, whereas growth of sons was non-significantly increased (von Engelhardt et al., 2015). Artificially increasing maternal circulating testosterone in zebra finches (*Taeniopygia guttata*) reduced the hatching success of sons and increased the post-hatching survival of daughters (Rutkowska and Cichoń, 2006). Experimental elevation of maternal CORT in European starlings (*Sturnus vulgaris*) increased the mortality of male embryos, led to a female-biased sex ratio at hatching, and reduced early growth in males (Love et al., 2005).

Maternal effects on offspring growth and survival may be attributed to (sex-specific) modulation of offspring endocrine physiology (Groothuis et al., 2005; Groothuis and Schwabl, 2008; Kaiser and Sachser, 2005) since both growth and survival can relate to circulating hormone levels (e.g., Braasch et al., 2011; Brown et al., 2005; Goodship and Buchanan, 2006; Goutte et al., 2010; Groothuis and Ros, 2005; Hull et al., 2007; Müller et al., 2009; Ros, 1999; Wada and Breuner, 2008). Studies on transgenerational effects of the maternal social environment on offspring physiology are scarce, especially in birds. However, in Japanese quail, maternal social instability increases the offspring's emotional reactivity scored in different behavioural tests, suggesting possible effects on the hypothalamic–pituitary–adrenal axis (HPA-axis) regulating the release of CORT (Guibert et al., 2010). This assumption is corroborated by studies on guinea pigs, which even find sex-specific effects of the maternal social environment on the HPA-axis in the offspring (Kaiser and Sachser, 2001; von Engelhardt et al., 2015).

In our previous study on Japanese quail, we have shown that the social environment during breeding affects female physiology (Langen et al., 2017). Females housed in pairs (one male, one female) had higher plasma androgen concentrations and tended to have higher plasma

CORT concentrations than females housed in groups (one male, three females; see Langen et al. (2017) for more details). Here, we examined the offspring of those females to investigate whether the maternal social environment affects offspring sex ratio and has sex-specific effects on mortality, growth and endocrine physiology. We expected overall positive effects on daughters of pair-housed females, i.e., a bias towards female offspring because higher maternal androgen (Correa et al., 2011) and CORT levels (Pike and Petrie, 2006) have been linked to a female-biased offspring sex ratio in Japanese quail. Furthermore, we expected increased growth and decreased mortality in daughters of pair-housed mothers because elevated maternal plasma androgen or CORT levels had positive effects on daughters and negative effects on sons in other avian species (Love et al., 2005; Rutkowska and Cichoń, 2006). In contrast to female-biased reproductive investment of pair-housed mothers, we expected a potentially male-biased offspring sex ratio, increased growth and decreased mortality in sons of group-housed mothers. We also investigated whether offspring from pair-housed and group-housed mothers differ in their circulating androgen levels and the sensitivity of the HPA-axis.

2. Materials and methods

2.1. Ethics statement

All experimental procedures and humane endpoints for minimizing suffering were approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Recklinghausen, Germany (licence number 84-02.04.2013-A127). Animal facilities were approved for keeping and breeding Japanese quail for research purposes by the local government authority responsible for health, veterinary and food monitoring (Gesundheits-, Veterinär- und Lebensmittelüberwachungsamt Bielefeld, Germany).

2.2. Parental generation

The parental generation originated from eggs generously provided by the INRA in Nouzilly, France (Experimental unit 1295 (UE PEAT) and UMR 85, Physiologie de la Reproduction et des Comportements, INRA-CNRS-IFCE-Université de Tours, Val de Loire Center, Nouzilly, France). These eggs were produced by females from a non-selected control line, bred next to quail lines selected for low or high social reinstatement (Mills and Faure, 1991). They were incubated and reared at Bielefeld University (Germany) and placed into their social treatments at 29 days of age. The social treatment was either pair-housing or group-housing, with groups ($n = 12$) consisting of three females and one male, pairs ($n = 24$) of one female and one male. Siblings or half siblings were never housed in the same cage. The birds were kept indoors, in two adjacent rooms with artificial lighting and no natural daylight. The light–dark cycle was 14:10 h, and the temperature was set to 20 °C. Pair cages measured 75 × 80 × 40 cm, group cages 150 × 80 × 40 cm. The distribution of the cages across and within rooms was balanced across treatments. The birds were kept on wood shavings, and all cages contained a sand bath and one shelter hut per female. Food (GoldDott Hennenmehl, Derby Spezialfutter GmbH, Münster, Germany) and water was provided ad libitum. On a weekly basis, the standard diet was supplemented with mealworms and shell grit. After collecting eggs for breeding the next generation, the parental females were tested for their hormonal response to a stressor (at 66–67 days of age; see Fig. 1) and to an injection with gonadotropin releasing hormone (at 72 days of age; see Fig. 1), and we measured growth, reproductive output, and egg yolk testosterone concentrations (Langen et al., 2017).

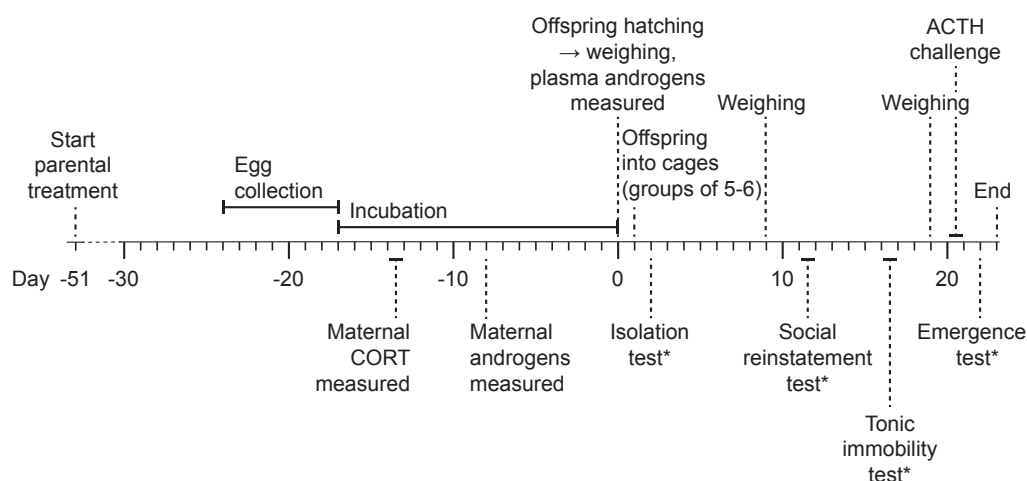


Fig. 1. Timeline of experimental procedures. Procedures marked with * are behavioural tests which are not reported here.

2.3. Egg incubation and hatching

After the parental generation had been housed in their treatment groups for 27 days (at 8 weeks of age), eggs (124 eggs from pair-housed females and 155 eggs from group-housed females) were collected over the course of one week, weighed to the nearest 0.1 g, and incubated to produce the offspring generation. Eggs were incubated in a HEKA-Euro-Lux II incubator (HEKA-Brutgeräte, Rietberg, Germany) in complete darkness to avoid the effects of light on development and because it more likely reflects the situation during natural incubation (Archer and Mench, 2014). Until incubation day 14, the temperature was set at 37.8 °C, humidity to 55%, and the eggs were turned every 2 h. After 9 days of incubation, the eggs were candled to identify embryonic development and non-fertilized eggs were removed (remaining eggs: 107 eggs from pair-housed females and 121 eggs from group-housed females, Table 1). On day 15 of incubation, eggs were moved to hatching trays, the incubation temperature was set to 37.5 °C, the humidity to

75%, and the eggs were no longer turned. The hatching trays were divided into separate compartments (5.5 × 5.5 × 5 cm) for each individual egg so that we could identify which chick hatched from which egg. The compartment walls were made of transparent Plexiglas and the bottom of each hatching tray was made of mesh wire, allowing air flow and olfactory and acoustic communication between the chicks. The offspring hatched after 17 ± 1 days of incubation. Some hatchlings were excluded from the experiment because they had birth defects (two male offspring from two pair-housed mothers, one female offspring from one group-housed mother). In addition, some offspring originated from cages in which birds had to be separated before or during egg collection due to aggression (two male and one female offspring from two mothers housed in the same group and six male and six female offspring from two of five separated pairs; see Langen et al. (2017) for more information). These offspring from separated parental cages were not included as subjects in the present study but used as cage mates. A total of 35 male and 29 female offspring from pair-housed mothers, and

Table 1
Samples sizes for each measurement.

Measure	Offspring from pair-housed mothers				Offspring from group-housed mothers			
	Total	Sons	Daughters	Mothers	Total	Sons	Daughters	Mothers (groups)
Sex ratio								
Primary	107 ¹	49	47	18	121 ¹	53	56	– ² (8)
At hatching	64	35	29	17	68 ¹	34	33	20 (8)
Correlation with maternal androgens	59	33	26	15	49	27	22	15 (6)
Correlation with maternal CORT	47	23	24	12	49 ¹	25	23	16 (7)
At day 23	54	27	27	16	56	28	28	17 (7)
Mortality								
Overall	64	35	29	17	68 ¹	34	33	20 (8)
Correlation with maternal androgens	59	33	26	15	49	27	22	15 (6)
Correlation with maternal CORT	47	23	24	12	49 ¹	25	23	16 (7)
Egg mass	64	35	29	17	68 ¹	34	33	20 (8)
Mass at hatching	4	35	29	17	68 ¹	34	33	20 (8)
Mass at day 9	56	28	28	17	57	28	29	19 (8)
Mass at day 19	54	27	27	16	56	28	28	17 (7)
Hatchling androgens								
Individual samples	17	13	4	16	12	5	7	13 (7)
Pools	7	4 (8) ³	3 (7) ³	16	4	2 (4) ³	2 (5) ³	13 (7)
ACTH challenge								
Baseline	38 ⁴	22 ⁴	16 ⁴	15 ⁴	48 ⁴	26 ⁴	22 ⁴	16 (8) ⁴
Response	37 ⁴	22 ⁴	15 ⁴	15 ⁴	47 ⁴	26 ⁴	21 ⁴	16 (8) ⁴

¹ Sexing was unsuccessful in 23 embryos and 1 hatchling.

² In groups, mothers of embryos were not identified.

³ Number of individuals included in the plasma pool.

⁴ Reduced sample sizes due to insufficient plasma for the CORT analysis.

34 male and 33 female offspring, and one hatchling of unidentified sex from group-housed mothers remained (Table 1).

Birds were removed from the incubator once their feathers had dried (ca. 2 h after hatching), weighed to the nearest 0.1 g and their tarsus was measured to the nearest mm using a digital caliper. To measure circulating androgen levels at hatching and to assign genetic sex and parentage, a small blood sample (max. 50 μ l, or about 0.7% of body weight which does not appear to have long-term effects on adult or developing birds; Sheldon et al. (2008)) was taken by piercing the jugular vein with a sterile 27 gauge needle and collecting the blood in heparinized capillaries (BRAND GMBH + CO KG, Wertheim, Germany). As we were unable to retrieve blood from 25 out of 132 chicks, a piece of egg shell membrane (ca. 2 \times 2 mm) containing blood vessels was collected for genetic sex and parentage assignment. From the remaining 107 chicks, we were able to retrieve at least a small amount of blood for DNA extraction, and 53 of these samples were further used for androgen measurements (Table 1).

After 19 days of incubation, all eggs that had not hatched were removed from the incubator and a tissue sample was taken from dead embryos for genetic sex determination.

2.4. Offspring husbandry

After weighing and measuring, the offspring were all kept together for the first night in a 100 \times 80 \times 80 cm cage on wavy cardboard and with two heating lamps and food (ground pellets: GoldDott Enten-Gänsestarter - no coccidiostat, Derby Spezialfutter GmbH, Münster, Germany) and water provided ad libitum. Lights remained on for the first night. The next day, the birds were placed into smaller groups of five to six unrelated individuals (all from the same parental treatment, n = 14 cages of pair offspring, n = 12 cages of group offspring). At that time, the offsprings' sexes were still unknown, therefore the chicks were randomly allocated across groups. Offspring cages measured 75 \times 80 \times 40 cm, contained heating pads partially covered by a small hut (15 \times 13 \times 13 cm), and ad libitum water and food. The main lights were set to a 14:10 h light-dark cycle (lights on at 5 am), but small night lights were placed approximately 1 m in front of the cages to make sure the birds were able to find food and water during the night. Birds were kept on wavy cardboard until 8 days post-hatching, after which they were kept on wood shavings.

All cages were checked daily, and we recorded whether any of the birds had died to be able to measure differences in mortality between offspring from the two maternal treatments. To analyse offspring growth, all birds were weighed to the nearest 0.1 g on the day of hatching, and on post-hatching days 9 and 19. Between the day of hatching and day 23, the offspring underwent several behavioural tests, the results of which will be described elsewhere (Langen et al. in prep). On post-hatch day 20–21, we assessed the birds' CORT response to an injection with adrenocorticotrophic hormone. For a timeline of all experimental procedures, see Fig. 1. Sample sizes per measure vary (Table 1) since some of the offspring died in the first few weeks or because we were unable to get enough plasma for the physiological measurements.

2.5. ACTH challenge

In order to test the offspring's HPA-axis sensitivity, we measured the plasma CORT increase following an injection of adrenocorticotrophic hormone (ACTH, which stimulates glucocorticoid production in the adrenal glands and is normally released by the pituitary in response to corticotrophin-releasing hormone from the hypothalamus) on post-hatch day 20–21. All birds were tested between 09:00 am and 1:00 pm, and plasma CORT levels did not change significantly during that period ($\chi^2_{(1)}$: 1.55, p = 0.21).

For the ACTH challenge, all birds from one cage were caught and transported to the experimental room in a transport box

(40 \times 30 \times 40 cm). A blood sample was taken to determine baseline plasma CORT concentrations by puncturing the ulnar vein with a sterile 27 gauge needle and collecting 200–300 μ l blood in heparinized capillaries (BRAND GMBH + CO KG, Wertheim, Germany). We recorded the time between opening the cages and taking the baseline blood sample (range: 71–287 s, mean \pm SEM: 155 \pm 6 s).

After the baseline blood sample was taken, the birds were injected in the pectoral muscle with 0.8 μ g ACTH (H-1150.0001, Bachem, Bubendorf, Switzerland) dissolved in 50 μ l PBS (average dosage ca. 10 μ g/kg) and placed back in the transport box. 10 min post injection, the birds were caught again, and a second blood sample was taken to determine the CORT response to ACTH.

2.6. Hormone analysis

After blood samples were taken to determine androgen (at the day of hatching) and CORT levels (in the ACTH challenge), samples were kept on ice for a maximum of two hours and then centrifuged for 10 min at 2000 \times g. The plasma was then collected and frozen at -20 $^{\circ}$ C for future use.

We used a commercial testosterone ELISA Kit (Demeditec Diagnostics GmbH, Kiel, Germany, cat. no. DES6622) to determine plasma androgen concentrations. Cross reactivity of the kit antibody, as reported by the manufacturer, was 23.3% for 5 α -Dihydrotestosterone, 1.6% for Androstenedione, and less than 0.1% for other steroids. Samples were distributed over two assays, balanced for maternal treatment. The inter-assay coefficient of variation (CV) was 1.64% (based on two quail plasma pools measured in each assay). Since we were unable to get sufficient plasma from 24 out of 53 chicks, the 24 samples were pooled by combining samples from two to three hatchlings in each pool, resulting in 11 plasma pools. We pooled plasma samples from hatchlings within the same sex and maternal treatment and, where possible, pools consisted of samples from full siblings. In total, 40 samples were measured in the androgen assay (29 single plasma samples and 11 plasma pools; Table 1). In four out of the 40 samples (two from sons of pair-housed mothers, two from daughters of group-housed mothers), androgen concentrations were below the range that could be estimated using the standard curve and were therefore assigned the lowest measured value (28.2 pg/ml), as a conservative estimate.

Plasma CORT concentrations in the ACTH challenge were determined using a commercial Corticosterone RIA Kit (MP Biomedicals, Orangeburg, USA, cat. no. 07-102102). Cross reactivity of the kit antibody, as reported by the manufacturer, was 0.34% for Desoxycorticosterone, 0.1% for Testosterone, and less than 0.1% for other steroids. Samples were measured together with quail plasma samples from other experiments and distributed over 11 assays, balanced for treatment. The intra-assay CV was 4.51%, the inter-assay CV was 13.86% (based on a chicken plasma pool and two kit controls measured in each assay). 170 samples were measured in the CORT assay (86 baseline, 84 post-ACTH; Table 1), and in 15 cases the CORT values were above the highest assay standard (all post-ACTH samples, from eight sons of pair-housed mothers and seven sons of group-housed mothers). As we were unable to repeat measurements at a higher dilution these samples were assigned a value of 35 ng/ml (based on the value of the highest assay standard) as a conservative estimate.

2.7. Genetic sex and parentage assignment

We used molecular methods to determine offspring sex and to assign parentage of all hatched offspring to one of the three potential mothers in the group treatment. The concentrated blood cells left over after centrifuging blood for hormone measurements were diluted 1:2 with phosphate buffer saline (10 mM PBS + 6 mM EDTA, pH 7.4) and stored at -20 $^{\circ}$ C. Similarly, tissue samples from non-hatched embryos were frozen at -20 $^{\circ}$ C for future use. Genomic DNA was obtained by a phenol/

chloroform or Chelex extraction (Walsh et al., 1991). Genetic sex determination was then performed using primers 2550f and 2718r (Fridolfsson and Ellegren, 1999).

We genotyped offspring and parents at 22 microsatellite loci using fluorescently labelled primers, as described previously (Langen et al., 2017). Parentage was then manually assigned by identifying which genotype of the three potential mothers in a cage best matched the offspring genotype.

2.8. Statistics

All statistical analyses were done using the lme4 package (Bates et al., 2015) of R 3.2.3 (R Core Team, 2015).

To analyse the effect of the maternal social environment and maternal hormones on offspring sex ratio and mortality, generalized linear mixed models with a binomial distribution and logit link function were fitted. Models included the maternal social environment as a fixed effect. Additionally, models of offspring mortality included a fixed effect of offspring sex and its interaction with the maternal social environment. We tested for a sex-ratio bias in each of the maternal social environments, where a significant effect of the intercept on the logit scale indicates a deviation from parity. Finally, we tested for non-random (extra-binomial) variance of sex-ratios using simulations (see Postma et al., 2011). We generated a distribution of 1000 expected clutch sex ratios based on the observed mean sex ratio and the number of offspring from each mother or each maternal cage (for embryos whose parentage was not assigned) and compared whether the observed variance in sex-ratios fell outside the upper confidence interval (overdispersion) or lower confidence interval (underdispersion) of the simulated data. We then analysed the effects of maternal hormones on offspring sex ratio and mortality using separate models, either with maternal baseline plasma androgen or with baseline plasma CORT levels as fixed effects. Maternal treatment was not included in these models to avoid multicollinearity because maternal hormones differed according to maternal treatment.

General linear mixed models were used to analyse the effect of the maternal social environment and offspring sex on egg mass, offspring mass and offspring circulating hormone levels (androgens at hatching and CORT during the ACTH challenge). Normality of the residuals from all general linear mixed models was assessed visually using histograms and Q-Q plots. To achieve normality and equal variances, we transformed values for offspring plasma CORT levels (square root) and body mass (natural log). Again, fixed effects included the maternal social environment, offspring sex, and their interactions. In addition, models of offspring growth included a categorical fixed effect of age (in days) to model the increase in weight with age. The models also included all two-way and three-way interactions of age with the maternal social environment and sex to test whether the weight increase with age differed between treatments and sexes. Age was treated as a categorical fixed effect because offspring mass was measured at only three time points (day 0, day 9 and day 19). Models analysing effects on plasma CORT during the ACTH challenge included a fixed effect of sample (pre or post-challenge) to test whether CORT increased in response to the challenge. The models also included all two-way and three-way interactions of sample with the maternal social environment and offspring sex to test whether the response to the challenge differed between treatments and sexes. In addition, the models on plasma CORT included as a covariate the time it took to collect the first sample after the initial disturbance of opening the cage.

Maternal cage was included as a random effect in all models, to control for potential non-independence of mothers from the same cage. In addition, models included a random effect of maternal ID nested within maternal cage, except for the models of primary sex ratio (because parentage was only assigned for hatchlings, not for embryos). Models analysing offspring data collected after the day of hatching also included a random effect of offspring cage. When analysing offspring

mortality, models did not converge if both maternal ID and offspring cage were included as random effects, even when increasing iterations to 2×10^9 and trying a number of different optimizers. Offspring cage was therefore removed from these models because it had a smaller effect than maternal ID within maternal cage (see Supplementary Table 8). When a random effect of offspring cage was used in the models instead of the random effect of maternal ID, the main effect of sex was no longer significant, suggesting that controlling for maternal ID increased the sensitivity to detect intrinsic sex-differences in mortality. For the analysis of growth and the ACTH challenge, we included the additional random effects of offspring ID nested within offspring cage and offspring ID nested within maternal ID nested within maternal cage.

We always started with the full model and stepwise excluded all non-significant ($p > 0.05$) interactions and main effects, apart from the main factors of interest: the maternal social environment and offspring sex. Interactions were always excluded before the main effects involved in the interaction. We determined the significance of fixed effects using likelihood ratio tests comparing the models with and without the factor of interest. The results of all models are reported in the Supplementary Data.

3. Results

3.1. Offspring sex ratio and mortality

The maternal social environment did not affect primary sex ratio ($\chi^2_{(1)}$: 0.12, $p = 0.73$; Fig. 2), sex ratio at hatching ($\chi^2_{(1)}$: 0.20, $p = 0.65$; Fig. 2) or sex ratio at day 23 ($\chi^2_{(1)} < 0.01$, $p = 0.99$; Fig. 2). Sex ratios did not differ significantly from parity at any stage in either social environment (all z values < 0.75 , all p values > 0.45 ; Supplementary Table 1), nor was there any evidence of over or underdispersion in sex ratio at any stage (Supplementary Table 2). In addition, maternal baseline plasma hormone levels did not predict offspring sex ratio at hatching (effect of maternal baseline androgens: $\chi^2_{(1)}$: 1.53, $p = 0.22$; effect of maternal baseline CORT: $\chi^2_{(1)}$: 0.36, $p = 0.55$; Supplementary Fig. 1).

The maternal social environment did not have sex-specific effects on offspring mortality (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 1.80, $p = 0.18$; Fig. 3), nor was there an overall effect of the maternal social environment on mortality ($\chi^2_{(1)}$: 0.20, $p = 0.66$; Fig. 3). However, mortality did differ between the sexes: significantly more male offspring than female offspring died before day 23 ($\chi^2_{(1)}$: 4.48, $p = 0.03$; Fig. 3). This effect disappeared when the random effect of maternal ID was removed and offspring cage was included, which had a weaker effect but could not be estimated together with maternal ID in the same model (see methods), suggesting that cage differences in mortality make it difficult to detect sex-differences when effects of

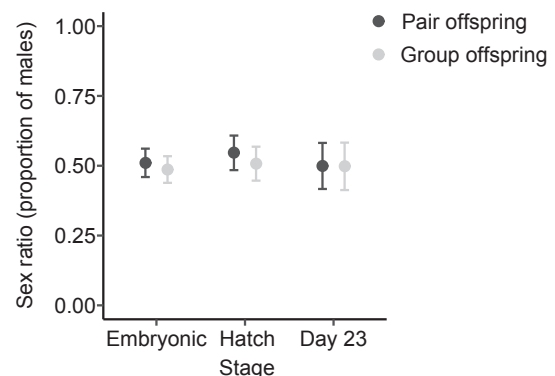


Fig. 2. Offspring sex ratio at the embryonic stage, at hatching and at day 23. Data shown are the estimated means ± 1 SEM (back-transformed from logit).

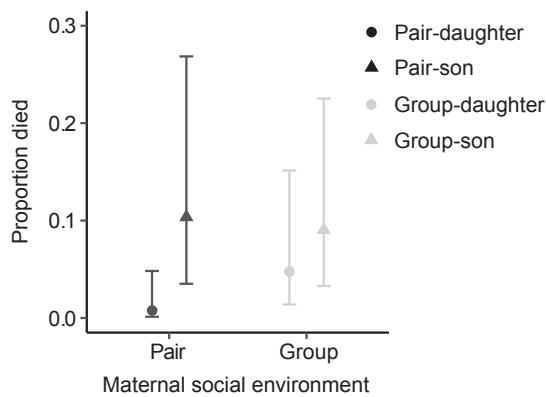


Fig. 3. The proportion of offspring that died before day 23. Data shown are the estimated means \pm 1 SEM (back-transformed from logit).

maternal ID is not accounted for. Maternal baseline plasma hormone levels did not predict offspring mortality (effect of maternal baseline androgens: $\chi^2_{(1)}$: 1.46, $p = 0.23$; effect of maternal baseline CORT: $\chi^2_{(1)}$: 0.65, $p = 0.42$; Supplementary Fig. 1).

3.2. Egg mass and growth

The maternal social environment had no overall ($\chi^2_{(1)}$: 0.27, $p = 0.60$; Fig. 4A) or sex-specific effect on egg weight (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 0.02, $p = 0.89$; Fig. 4A), nor did egg weight differ between the sexes ($\chi^2_{(1)}$: 0.25, $p = 0.62$; Fig. 4A).

All birds increased weight significantly over the course of the experiment (effect of age: $\chi^2_{(2)}$: 1531.30, $p < 0.001$; Fig. 4B). The changes in weight with age did not differ between the maternal social environments (effect of maternal social environment * age: $\chi^2_{(2)}$: 0.49, $p = 0.78$; Fig. 4B) nor between males and females (effect of offspring sex * age: $\chi^2_{(2)}$: 1.43, $p = 0.49$; Fig. 4B) or depending upon the interaction between maternal social environment and offspring sex (effect of maternal social environment * age * offspring sex: $\chi^2_{(2)}$: 4.63, $p = 0.10$; Fig. 4B). There was no difference in average offspring mass according to the maternal social environment, offspring sex, or their interaction (the model included the significant effect of age; all $\chi^2_{(1)} < 1.64$, all p values > 0.20).

3.3. Offspring physiology

The maternal social environment had no sex-specific effects on offspring plasma androgen concentrations at hatching (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 0.02, $p = 0.89$; Fig. 5A). Average androgen concentrations also did not differ between offspring of pair-housed and group-housed mothers ($\chi^2_{(1)}$: 0.45, $p = 0.50$;

Fig. 5A), nor between males and females ($\chi^2_{(1)}$: 1.92, $p = 0.17$; Fig. 5A).

The maternal social environment did not affect the CORT response to an injection with ACTH on post hatch day 20–21 (effect of maternal social environment * sample: $\chi^2_{(1)}$: 0.58, $p = 0.45$; Fig. 5B). Male and female offspring differed in their CORT response (effect of offspring sex * sample: $\chi^2_{(1)}$: 7.11, $p < 0.01$; Fig. 5B) but the sex difference in the CORT response was not affected by the maternal social environment (effect of sample * maternal social environment * offspring sex: $\chi^2_{(1)}$: 2.62, $p = 0.11$). The time between the initial disturbance of opening the cage and the collection of the baseline sample was included as a covariate in all models analysing the effects on ACTH because it significantly affected CORT levels ($\chi^2_{(1)}$: 8.34, $p < 0.01$). This effect did not differ between offspring from the different maternal social environments ($\chi^2_{(1)}$: 0.19, $p = 0.66$). Removing the factor “time until the first sample” from these models did not change the significance or interpretation of the main effects. When analysing CORT baseline and response levels separately, male and female offspring did not differ in baseline CORT concentrations ($\chi^2_{(1)}$: 0.02, $p = 0.89$; Fig. 5B), but males had significantly higher CORT concentrations after the ACTH injection ($\chi^2_{(1)}$: 16.33, $p < 0.001$; Fig. 5B). CORT concentrations increased significantly in both sexes after the ACTH injection (males: $\chi^2_{(1)}$: 146.79, $p < 0.001$; females: $\chi^2_{(1)}$: 86.44, $p < 0.001$; Fig. 5B).

4. Discussion

The social environment a female is exposed to during reproduction has been reported to induce variation in offspring sex-ratio, mortality, development, and endocrine physiology in a number of vertebrate species (Dantzer et al., 2013; Guibert et al., 2010; Kaiser and Sachser, 2009, 2005; Michler et al., 2013; Minias et al., 2014; von Engelhardt et al., 2015). Maternal hormones are candidate signals involved in such transgenerational effects (Dantzer et al., 2013; Guibert et al., 2010; Hayward and Wingfield, 2004; Henriksen et al., 2013), and they are thought to represent important proximate mechanisms in adaptive sex allocation (Navara, 2013a, 2013b), also by affecting secondary offspring sex ratios (Love et al., 2005; Rutkowska et al., 2007; Rutkowska and Cichon, 2006).

We did not find evidence that the maternal social environment (pair versus group housing) affects offspring sex ratio in Japanese quail, even though pair-housed females had increased circulating androgen levels and a non-significant trend of higher cortisol levels compared to group-housed females, as reported in our previous study (Langen et al., 2017). We had predicted that pair-housed females would produce a female-biased offspring sex-ratio because increased androgen and CORT levels were associated with a female-biased sex-ratio in other studies on Japanese quail (Correa et al., 2011; Pike and Petrie, 2006). Offspring from pair-housed mothers and offspring from group-housed mothers also did not differ in growth, mortality, circulating androgen levels or circulating CORT levels. Moreover, maternal circulating levels of androgens

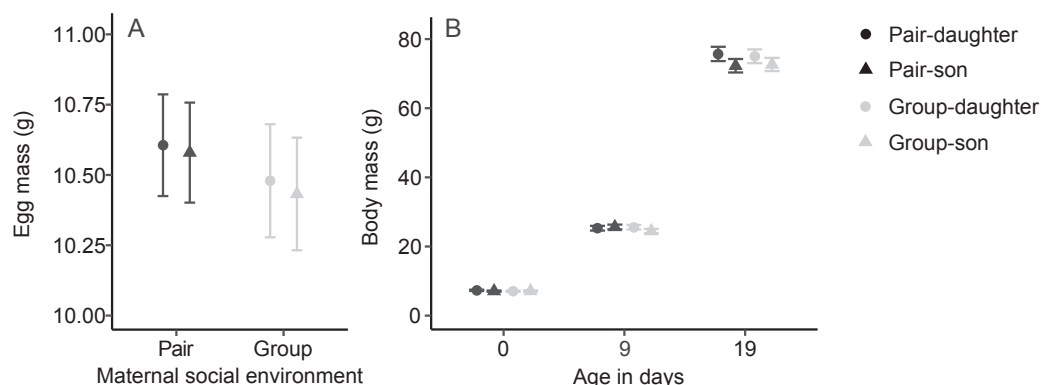


Fig. 4. A: egg mass. B: offspring growth (back-transformed from natural log). Data shown are the estimated means \pm 1 SEM.

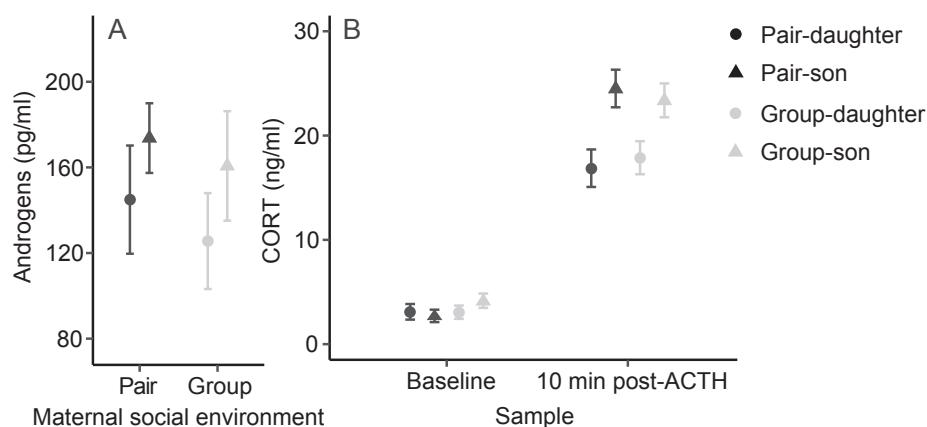


Fig. 5. A: offspring plasma androgen concentrations (pg/ml) at hatching. B: offspring plasma CORT concentrations (ng/ml) before and 10 min after the ACTH injection (back-transformed from square root). Data shown are the estimated means \pm 1 SEM.

and CORT did not correlate with offspring sex ratio and mortality.

Our results contradict the general pattern in avian species which suggests that higher maternal androgens lead to a male-biased offspring sex ratio (Goerlich et al., 2009; Navara, 2013a, 2013b), but we corroborate earlier findings in Japanese quail showing no such relationship (Pike and Petrie, 2006). However, in Japanese quail, higher maternal androgens have also been linked to female-biased sex ratios (Correa et al., 2011), indicating that the effect of maternal androgens on offspring sex ratio is still unclear. Our results also do not confirm earlier reports that higher maternal CORT levels are linked to a female-biased offspring sex ratio in avian species (Navara, 2013a, 2013b), including Japanese quail (Pike and Petrie, 2006). Studies investigating the relationship between maternal plasma hormone levels and offspring sex ratio differ substantially regarding methods of hormone manipulation or quantification which might explain differing results between studies. For example, Correa et al. (2011) found the temporary peak in circulating androgen levels following mating to be correlated with a female-biased sex ratio. On the other hand, Pike and Petrie (2006), who found no relationship between offspring sex ratio and androgens, analysed faecal androgen metabolite concentrations, which do not reflect short-term fluctuations but an integrated measure of androgen concentrations over several hours. They also found no effect of treating females with androgen implants, which likely affected circulating androgen levels over a longer time period. In addition, multiple steroid hormones are thought to be involved in sex ratio adjustment, and their effects may interact (Navara, 2013a). In the present study, the opposing effects of higher maternal androgens and higher CORT on offspring sex ratio may have cancelled each other out, explaining why the offspring sex ratio of pair-housed mothers did not differ from parity nor from that of group-housed females. Moreover, the elevation in maternal plasma androgen and CORT levels in pair-housed females may not have been large enough to induce a shift in offspring sex ratio. Finally, it has been suggested that effects on primary sex ratios may be largely due to variation in levels of progesterone during meiosis, which is the main follicular steroid produced during this phase (Correa et al. 2005) but was not measured in our study.

The lack of an effect of the maternal social environment on offspring growth, mortality or physiology might partly be explained by the fact that the maternal social environment did not induce differences in yolk androgens, as shown in our previous study (Langen et al., 2017), or in egg mass, as shown here. Yolk hormones are considered a key mechanism in transferring the effects of the maternal social environment to offspring (Gil, 2008; Rutkowska and Cichoń, 2006; von Engelhardt et al., 2006; von Engelhardt and Groothuis, 2011), and differences in the maternal social environment and physiology alone may not suffice to induce changes in the prenatal environment. The fact that we found no effects on egg mass can also explain why offspring growth and

mortality did not differ, egg size being another important mediator of maternal effects (Cunningham & Russell 2000; Hadfield et al. 2013; Krist, 2011; Pick et al. 2016; Williams, 1994). We also found no sex differences in egg mass, confirming previous suggestions that there is little evidence overall for sexual size dimorphism in eggs across avian species (Rutkowska et al., 2014).

We did find a difference in the physiological stress response (increase in CORT) after an ACTH injection between male and female offspring, irrespective of the maternal social environment. While baseline CORT concentrations did not differ between males and females, males showed a higher CORT response, suggesting increased sensitivity of their HPA axis. This is in line with many studies in birds, including Japanese quail, that report a stronger stress response in males compared to females (Astheimer et al., 1994; Goerlich et al., 2012; Hayward et al., 2006; Hazard et al., 2008; Krause et al., 2015; Madison et al., 2008; Romero et al., 2006; Schmeling and Nockels, 1978). Other studies, however, report no differences (Dufty and Belthoff, 1997; Hazard et al., 2008; Satterlee and Johnson, 1988; Sockman and Schwabl, 2001). In addition to having a higher stress response, significantly more male offspring died before day 23 than female offspring. Similar patterns in juvenile mortality are reported in a large number of species (reviewed by Clutton-Brock (1991)), suggesting that males are more vulnerable to environmental challenges. However, it is still unclear what the underlying mechanisms are (Jones et al., 2009). Interestingly the sex-difference in mortality was only detected when controlling for maternal ID, not when controlling for offspring cage. This made it harder to estimate intrinsic sex-differences in mortality because offspring from the same mother were allocated to different offspring cages so that the sex-differences in mortality could be attributed to cage effects. While perhaps not surprising, this also suggests that it is important to consider how the genetic effects, maternal environmental effects and the posthatching environment may interact in affecting sex-specific mortality.

Overall, contradictory findings regarding the effects of maternal physiology and maternal social environment on offspring sex ratio and phenotypes indicate that the mechanisms underlying such maternal effects are still insufficiently understood. An important factor explaining differences between studies, including our own and previous research, might be the timing of manipulations and measurements of the social environment and the endocrine system. Effects of the maternal environment and physiology on developing follicles and offspring may occur only during critical windows (Okuliarova et al., 2017). For example, for sex ratio adjustment, an influence of maternal steroids on the segregation of the sex chromosomes during the first meiotic division has been proposed (Correa et al., 2005; Goerlich-Jansson et al., 2013; Navara, 2013a, 2013b; Pinson et al., 2011; Rutkowska and Badyaev, 2008). Also, circulating hormone levels differ

between life stages and seasons and can change significantly during a single day, even within minutes, in response to the environment, such as social stimuli (Adkins-Regan, 2005; Creel et al., 2013; Hazard et al., 2005; Oliveira, 2004; Ottinger et al., 2001). A single measurement of physiological status does not take such fluctuations into account and might reduce the chance of detecting maternal effects. We may have also missed important effects by not measuring maternal hormones during the time window during which genetic sex determination takes place (meiosis I) and by only measuring maternal androgens and corticosterone, not other steroids such as progesterone (Correa et al., 2005).

Finally, differences between the social stimuli investigated may explain the contradictory results between studies. Social factors such as maternal social instability (Guibert et al., 2010; Kaiser and Sachser, 2009), social density (Dantzer et al., 2013; Minias et al., 2014; von Engelhardt et al., 2015), mate attractiveness (Köllicker et al., 1999; Korsten et al., 2006; Rutstein et al., 2005; Sheldon et al., 1999; Svensson and Nilsson, 1996), pair bonding (Hirschenhauser, 2012; Le Bot et al., 2014; Schweitzer et al., 2014), and social status (Dloniak et al., 2006) are likely to differ in their functional significance and therefore also in their effects on offspring phenotypes and sex ratio. To gain a better understanding of the underlying mechanisms and the function of maternal effects of the social environment, it is therefore necessary to establish which social stimuli are most important for offspring, and at which time maternal effects manifest in relation to the prenatal and postnatal developmental stages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ygcen.2018.04.015>.

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