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Effect of contact incubation on stress, behavior and body composition in the precocial Red jungle fowl

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ABSTRACT

Birds use contact incubation to warm their eggs above ambient temperature required for embryonic development. In contrast, birds in the industry as well as many birds in breeding programs and scientific studies are incubated in conventional incubators that warm eggs via circulating warm air. This means that contact incubated eggs have different thermal properties than eggs incubated in a conventional incubator. In light of previous studies showing that small differences in incubation temperature can affect chicks post-hatching phenotype, we investigated the consequences of incubating Red jungle fowl eggs at the same temperature (37 °C) either via contact incubation or warm air incubation. We found that contact incubated chicks had a more robust body composition, were more explorative and had a higher temperature preference early in life, as well as a sex dependent difference in plasma Corticosterone levels pre-hatch (measured in down-feathers) and post-hatch (measured in plasma) compared to chicks incubated in a conventional warm air incubator. While previous studies have demonstrated that embryonic development and post-hatch phenotype is sensitive to small variations in temperature, our study demonstrates for the first time that the way heat is distributed to the egg has a similar magnitude of effect on post-hatch phenotype and highlights the sensitivity of the incubation period in shaping birds post-hatch phenotype.

1. Introduction

Variation in the pre-hatch environment can have profound effects on birds' post-hatch phenotype and thereby overall fitness (for review see Henriksen et al., 2011 and DuRant et al., 2013). The pre-hatch environment can be subdivided into two distinct components. Firstly, the composition of the egg determines the amount and quality of nutrition available during pre-hatch growth (Williams, 1994) and secondly, the condition under which the egg is incubated determines if and how fast pre-hatch development will proceed (Deeming and Ferguson, 1991). The majority of research on long-term effects of the pre-hatch environment have focussed on effects of alterations in the composition of the eggs (Willems et al., 2016, for review see Henriksen et al., 2011, Groothuis et al., 2005 and Dixon et al., 2016) and only more recently has it become evident that the incubation conditions under which the embryo develops are not only important for hatching success but also influences the birds post-hatch phenotype (DuRant et al., 2013). This line of research is however still scarce.

To develop properly, bird embryos must maintain a high body

temperature during pre-hatch growth. They do not generate sufficient heat themselves to manage this and must rely on heat from one of the parents delivered through a specialized patch of skin on the parents' breast known as the brood patch (Deeming, 2002), which the incubating parent bird presses up against the egg. In the poultry industry and other breeding programs birds do not incubate eggs themselves. Instead, eggs are placed in forced draft (FD) incubators that maintain high (for chickens, 37 °C) ambient air temperature throughout incubation. Eggs placed in conventional FD incubators have a uniform temperature, while eggs warmed by a brood patch have a substantial temperature gradient within, from the warm patch through the egg (Turner, 1994a, 1994b). This means that an egg has very different thermal properties depending on whether it's being warmed in a conventional incubator or is being warmed by a brood patch.

The common notion that the embryo is a mere passive recipient of heat from the parent (or incubator) and at most contributes heat as it grows is too simplified. The embryo has striking physiological capabilities for managing the flow of heat into its egg, most notably through the developing embryonic circulation of blood (Turner, 1997) and during

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the first week of incubation the chicken embryo is able to perform thermo-regulative behavior by moving within the egg to more optimal temperature locations (Li et al., 2014). This means that an embryo, to some degree, is able to redistribute its blood flow or itself to optimize its temperature exposure during incubation. However, these capabilities emerge only when there is a thermal gradient within the egg, which occurs during contact incubation but not when an egg is incubated in an FD (Turner, 1997; Li et al., 2014). During incubation heat production from the embryo increases daily, thereby increasing the temperature of the egg. In FD incubator where the surrounding temperature is high, excess heat is not always lost into the environment and embryos are therefore at risk of overheating during development, which can lead to reduced post-hatch conditions (Wineland et al., 2000a, 2000b).

To what extend conventional FD incubators influence embryonic development and post-hatch phenotype in a way that is different than under natural Contact-incubation has yet to be investigated. Research on incubation temperature in both precocial and altricial birds have demonstrated that differences in incubation temperature of only 1–2 °C can influence embryonic development leading to alterations in early post-hatching body composition, stress sensitivity and mobility (Hepp et al., 2006; Olsen et al., 2008; DuRant et al., 2010; Nord and Nilsson, 2011). Given the very different temperature profile of an egg warmed uniformly by surrounding warm air in a FD incubator and a contact incubated egg, it is likely that similar differences in embryonic growth and post-hatching phenotypic traits will be evident between these two types of incubation condition.

In this study, we investigate if contact incubation affect the pre-hatch development and thereby post-hatch phenotype of precocial birds differently than conventional FD incubation using the Red jungle fowl as a model species. The Red jungle fowl (RJF) is the wild progenitor of the domesticated chicken and in the wild they incubate their eggs in a nest on the ground (Collias and Collias, 1967). A female will lay an egg every day until she has a clutch of around 6–10 egg and once the last egg is laid she will start to incubate continuously until the chicks hatch. During the incubation period, the female only leaves the nest for 0.5–1.0 h every 1 or 2 days (Sherry et al., 1980). The eggs hatch (asynchronously) after 19–20 days of incubation over a period of 7–33 h (Meijer and Siemers, 1993). The Red jungle fowl have similar requirements during incubation to the domesticated chicken, regarding temperature and humidity, but hasn't been incubated in FD incubators for as many generations as domestic chickens, thereby limiting potential adaptation to this type of incubation. In turkeys and chickens, incubation temperature has been reported to influence thermoregulation, post-hatching growth and hatchling morphology (Hulet et al., 2007; Nichelmann and Tzschentke, 2002), while in wild birds, incubation temperature has been reported to influence HPA-axis sensitivity and thermoregulation (DuRant et al., 2013). We therefore choose to focus on these traits, since they all affect young birds' ability to survive and cope with their environment. Additionally, we also measured the birds' fearfulness, cognitive ability as well as their general behavior in an undisturbed environment, to get insight into variation in coping style. Finally, to assess any difference in pre-hatch stress-levels between the two incubation environments, we measure corticosterone (CORT) in the down feather of the newly hatched chicks. CORT have previously been measured in bird feathers and used as an indicator of stressful conditions in the post-hatch environment (Harms et al., 2010), but to our knowledge this is the first study to measure hormones in down-feathers.

2. Methods

2.1. Ethical note

This study was approved by Linköping Council for Ethical Licensing of Animal Experiments, ethical permit no 122–10.

2.2. Animals and housing

We used one-year-old Red jungle fowl females (n = 11) from a captive, pedigree-bred population kept at Linköping University, Sweden. This population was kept in the facility for the purpose of a breeding program as part of ongoing research in behavior genetics and has not been bred or used for commercial purposes and their behavior is therefore similar to that observed in wild red jungle fowl. Full details of animal housing and husbandry systems are given elsewhere (Campler et al., 2009).

2.3. Incubators and design

Using a newly developed incubator (Brinsea Contaq Z6, https://www.incubators.org/brinsea-contaq-z6-incubator.html) specifically designed to mimic contact incubation by pressing artificial skin inflated by warm air down on top of the egg (mimicking a bird's brood patch, see Supplementary Fig. 1) we investigated the consequences of incubating Red jungle fowl eggs in warm air (FD incubators, Masalles Mod.25-l HLC, http://www.masalles.com) versus via contact incubation (Brinsea Contaq Z6, see Supplementary Fig. 2). Using a split-brood design we allocated sibling-eggs collected within the same week from the same RJF hens (n = 11) to either a FD incubator or the Contact incubator.

2.4. Egg measurements during incubation

Eggs were stored at 13 °C for up to 1 week until they were all placed in one of the preheated incubators. The FD incubator's temperature was 37 °C and the humidity was held at 58%. The contact incubators contact zone was 37 °C and humidity was 45%. The eggs were turned automatically every 6 h in both incubators. After 18 days of incubation, all eggs were placed into a new FD hatcher with cameras and separated into separate glass-containers to record exact hatching time for all chicks and humidity was raised to 80% in the FD incubator. All eggs were weighed before (day 0) and during incubation (day 7 and day 14). Exact hatching time of all chicks was recorded using video-cameras installed in the incubators.

2.5. Hatchlings handling and down feather sampling

The hatchlings were removed from the incubator and wing-tagged as soon as their feathers were completely dry, approximately 18 h of hatching. Between 8 and 15 mg of down feather were sampled from each chick as soon as they were removed from the incubator. This was done by cutting of a small section of down feather from the back of their neck. The cut was made ½ cm above the skin, leaving behind the lower part of each down feather. The down feathers were stored in plastic bags for up to 6 months in a − 80 °C freezer. The offspring were raised in pens measuring 70 × 77 m, in groups of 11–12. All pens were equipped with fresh water and food ad libitum. Ambient temperature in the room was kept at 21 °C and during the first 2 weeks of life the chicks had access to heating lamps.

2.6. Growth

Chicks were weighed to the nearest 0.01 g on day 0 (hatching day), day 5, day 11, day 19 and at 4, 5 and 6 weeks of age. Their tarsus length, from the hock of the bent leg to the joint of the back toe was measured with a slide caliper, to acquire information about the birds' structural size when the birds were 0 and 5 days old and at 6 weeks of age. In order to explore if there was an overall difference in body composition between the two groups, we estimated body condition from the mass and tarsus length data for each individual using Peig and Green's (2009) scaled mass index (SMI). This index accounts for the covariation between body size and body mass components by standardizing body mass

at a fixed value of a linear body measurement (tarsus length) based on the scaling relationship between mass and length. This index is calculated using the equation SMI = Mi (Lo / Li)ˆbsma where Mi and Li are the body mass and the linear body measurement of individual i respectively, bSMA is the scaling exponent estimated by the SMA regression of M on L, L0 is the arithmetic mean value for the study population.

2.7. Behavioral measurements

2.7.1. Temperature preference

Early in life chickens have not yet developed the mechanisms necessary to maintain a constant body temperature and their main source of heat is the mother. Their main thermoregulatory mechanism is to seek heat (the mother) when they experience a drop in temperature. The chicks' temperature preference was measured at 3 days of age by placing them in an 80 cm × 20 cm long arena with a temperature gradient that gradually increased from 25 °C at one end of the arena to 40 °C in the other end (see Fig. 3 in Supplementary material). The birds were placed individually at one end of the arena at 25 °C, after which their placement was noted every 15 s for 6 min, to determine if there was any difference in temperature preference between FD and contact incubated chicks.

2.7.2. Undisturbed explorative behavior

Explorative behavior consists of a range of behavioral acts, but combined they are all concerned with gathering information about the environment. At 8–9 days of age the chicks' explorative behavior was measured in a novel undisturbed arena (see Fig. 4 in Supplementary material). The chicks were placed in pairs (from same treatment group) in an 80 × 80 cm arena with access to food, water, shade and different levels of elevation. The chicks were tested in pairs to minimize the level of anxiety in the novel environment and the observer were blind to the chicks' treatment and not visible to the chicks during the testing. During the testing period, it was noted how long (seconds) it took for the birds to leave the 'start zone', were they were placed at the start of the testing period. Additionally the location (zones, see Fig. 4 in Supplementary material) and whether they were active (moving or standing) or lying down was scanned every 15 s.

2.7.3. Fear test

The birds' fear level was assessed at 4 weeks of age using an emergence test (Jones et al., 1991). Emergence from a dark box into a lighted compartment or arena has been successfully used to measure fear in domestic chicks (Jones, 1979) under the assumption that more fearful or timid birds will show longer emergence latencies. The birds were tested individually by placing them in a dark box, measuring 30 × 20 cm with a sliding door. The box was placed in a lighted room, and the box-door was closed and a 2 min acclimatization period was allowed before the sliding door was raised. The latencies from raising the door until the chick a) put its head through the hole and b) moved its entire body out of the box, were recorded.

2.7.4. Cognition

To test if there was any difference in cognitive ability between the 2 treatment groups we performed a simple visual associative learning task. In the literature, visual discrimination is broadly defined as learning to pick one kind of visual stimulus over another. Chicken have good colour vision (Osorio et al., 1999) and we therefore based the test on the birds' ability to discriminate between the colour blue and yellow. The whole testing period took place when the birds were between 12 and 24 days old and had 3 components to it: 1) 1earning 2) memory and 3) reversal learning, with reversal learning referring to the adaption of behavior according to changes in the stimulus-reward contingency (see Fig. 5 in Supplementary material).

All birds were hand-feed mealworms on several occasions from the age of 2 days and all birds were very eager to eat mealworms. When the birds were 12 days old (**day 1** of the test) they were presented with 2 bowls (one blue and one yellow) individually. Four times a mealworm was placed in one of the bowl in front of them. For half of the birds, the mealworm was always placed in the blue bowl, for the other half it was placed in the yellow bowl. On **day 2** the birds learning ability was tested, by placing them in a 20 × 30 cm arena, were both the blue and yellow bowl was attached to one of the walls, at a height low enough for the birds to peak into if they stretched their necks but too high for the birds to see if the bowl contained a mealworm. Each bird was tested twice (with 2 mealworm) and the position of the bowls were switched between tests. When the birds picked (pecked at) the right bowl a mealworm was placed in that bowl. Duration until the bird made the right choice and number of failed attempts were recorded.

On **day 6** the birds were tested again following the same procedure as above to test their ability to remember (memory) the right bowl colour.

On **day 7** the 2 bowls were placed in front of each bird and they were given 4 mealworm each, similarly to day 1. However, this time the mealworm was placed in the opposite bowl (opposite colour) as on day 1. This was done to test the birds' reversal learning. The following 4 days (from **day 8** to **11**) the birds were tested in the same way and in the same arena as on day 2 and 6. However, this time the birds were rewarded when they picked the bowl in which the mealworms had been placed on day 7. The reward colour (bowl colour) was balanced over treatment.

2.8. HPA-axis sensitivity

To test the birds HPA-axis sensitivity post-hatch all birds underwent a stress test to assess the reactivity of their HPA-axis at 7 weeks of age. This was done by quantifying the CORT response to a standard stressor (the bag protocol or capture stress protocol, Wingfield et al., 1992). Birds were blood sampled from the wing veins and baseline samples were obtained within 3 min after the person entered the room. After a blood sample was collected, each bird was placed in a cloth bag that allowed light to penetrate, in order to avoid a calming effect of darkness. The birds were blood sampled again 10 min and 30 min after being placed into the bag and returned to their pen after the last sampling. Blood was collected in EDTA-coated tubes, kept on ice and centrifuged (800g for 5 min.) within 2 h of sampling and then stored at −20 °C until further analysis (see below). CORT secretion was calculated as area under the total response curve (see Fig. 3) using the trapezoid formulas AUCg and AUCi (Area Under the Curve, g = ground, i = increase) according to Pruessner et al. (2003). With AUCg representing the total amount of hormone produced over time with respect to a starting value of zero, thus not accounting for baseline levels of circulating hormone, and AUCi characterizing the sensitivity of the HPA axis by evaluating the amount of hormone produced above the starting baseline level.

2.9. Corticosterone measurements in blood and feathers

All down feather samples were weighed to the nearest 0.1 mg and extracted twice. For the first extraction (based on a protocol by Bortolotti et al., 2008), 1 ml methanol was added to each sample and then the samples were placed in a sonicating water bath for 30 min at room temperature before being incubated at 50 °C overnight in a shaker. The next morning the samples were centrifuged for 10 min. and the methanol extraction (~0.8 ml) was transferred to new tubes. The new tubes were placed in a SpeedVac vacuum concentrator until all the methanol had evaporated. Once the methanol had evaporated the remaining pellet in each tube was dissolved in 250 assay buffer (from CORT ELISA kit, see below). For the second extraction, a metal bead was placed in the tubes with the already extracted down feather, after which the tube was dropped in liquid nitrogen for 2 min. Immediately thereafter the tubes were placed in a Tissuelyser (Qiagen TissueLyser II) at 23 Hz for 2 min and then dropped in liquid nitrogen again to repeat the procedure. Then 1 ml methanol was added to each sample and left overnight at room

temperature on a shaker. The next morning all samples were centrifuged and the methanol extraction (~0.8 ml) was transferred to new tubes. The new tubes were placed in a SpeedVac until all the methanol had evaporated. Once the methanol had evaporated, the remaining pellet in each tube was dissolved in 250 assay buffer.

The concentrations of CORT in the feather samples and the plasma samples (from the stress test) were determined using a commercial CORT enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, NY, USA). All samples were tested in duplicate following a standard protocol (see online manual http://www.enzolifesciences.com/ADI-900-097/corticosterone-eia-kit/). Inter-assay and intra-assay coefficients of variation were 7.2% and 9.2% respectively for the plasma analyses, and 9.4% and 6.4% for the feather analyses.

2.10. Statistics

Statistical analyses were performed in SPSS version 22. One-way ANOVA was used to determine between-incubation type differences in egg mass variation during incubation, differences in incubation duration until hatching and for the exploration test where chicks were tested in same treatment pairs. Effects of incubation type on hatchling size and post-hatch growth was analysed using a factorial ANOVA with sex and treatment in the model. The same factorial ANOVA was also used to test for effects of incubation treatment on fearfulness, down-feather CORT concentration and time to solve the cognitive tests. Temperature preference at 3 days of age were analysed using a mixed repeated-measure ANOVA with treatment as between-subject factors and time as within-subject factor. A normal distribution could not be achieved for the cognitive tests score (number of wrong choices). Comparison of differences in cognitive score between incubation type was therefore made via Mann-Whitney U test. The statistical significance level was set at $P < 0.05$.

3. Results

3.1. Egg mass and number

The 11 hens laid 62 eggs in total, and 31 eggs were placed in each incubator. After 3 days of incubation all eggs were candled to determine if they were fertile, 28 eggs in the FD incubator were fertile and 27 eggs in the contact incubator were fertile. In total 24 eggs hatched (10 males and 14 females) in the FD incubator and 22 hatched (11 males and 11 females) in the contact incubator. There was no overall difference in egg mass between the eggs placed in the FD incubator and the contact incubator (Day 0, see Table 1: F = 0.034, df = 1, P = 0.854). After a week in the incubator the eggs in the two treatments did not differ in mass (Day 7, see Table 1: F = 0.907, df = 1, P = 0.478). Two weeks after being placed in the incubators the contact incubated eggs had lost more mass than FD incubated eggs (Day 14, see Table 1: F = 4.439, df = 1, P = 0.041).

3.2. Hatching time and hatchling body composition

Contact incubated chicks hatched significantly later (F = 31.534, df = 1, P = 0.001) than FD incubated chicks (~half a day later, see Fig. 1).

Table 1
Egg mass (gram, mean ± SE) before incubation (day 0) after 1 week in incubator (Day 7) and after 2 weeks in incubator (Day 14). P-values below 0.05 indicated with an *.

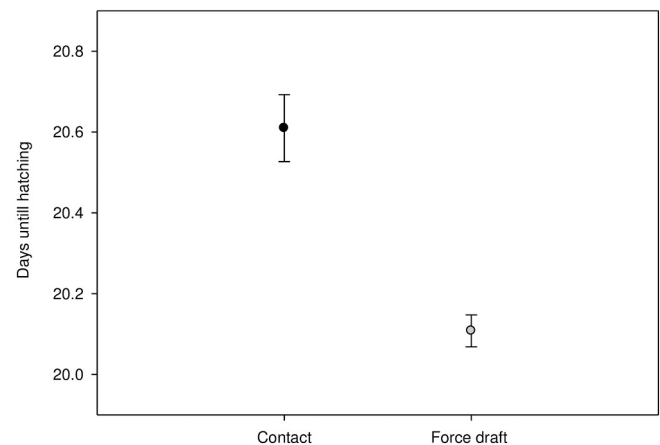| Time of incubation	Egg mass	
	Force draft	Contact
Day 0	40.96 ± 0.46	41.09 ± 0.47
Day 7	39.66 ± 0.40	39.02 ± 0.47
Day 14	38.41 ± 0.46	36.98 ± 0.48*

Fig. 1. Incubation duration (days) until hatching for contact incubated Red jungle fowls (black) and force draft incubated Red jungle fowls (grey).

There was no difference in hatching mass between FD and contact incubated birds (see Table 2: F = 1.408, df = 1, P = 2.42), but contact incubated hatchlings did have significantly shorter tarsus length (see Table 2: F = 13.484, df = 1, P = 0.001). SMI revealed no significant difference in body condition between the two treatment groups (SMI: FD = 28.34 ± 0.0.421; Contact = 29.54 ± 0.49: F = 2.776, df = 1, P = 0.103).

3.3. Body mass and growth

At 5 days of age, contact incubated chicks weighed significantly less (see Table 2: F = 4.755, df = 1, P = 0.035) and had a significantly shorter tarsus length (see Table 2: F = 10.526, df = 1, P = 0.002) than FD chicks. Contact incubated chicks had a significantly higher SMI than FD incubated chicks (Contact = 38.58 ± 0.6165, FD = 36.11 ± 0.63: F = 6.445, df = 1, P = 0.015), indicating a more robust body composition. Contact incubated chicks continued to have a smaller body mass at age 11 days of age (see Table 2: F = 4.847, df = 1, P = 0.033) and at 19 days of age (see Table 2: F = 6.259, df = 1, P = 0.016). After this age there was no longer any significant difference in body mass or tarsus length between the two treatment groups (P > 0.05). There was no significant interaction between treatment and sex on body mass or tarsus length (P > 0.05), but males were generally heavier than females from 4 weeks of age (P < 0.05).

Table 2
Body mass (gram, mean ± SE) and tarsus length (mm, mean ± SE). P-values below 0.05 indicatec with an *.

Age	Body mass	
	Force draft	Contact
Hatchling	29.27 ± 0.43	28.43 ± 0.45
Day 5	38.49 ± 0.79	35.90 ± 0.83*
Day 11	64.14 ± 1.45	59.60 ± 1.51*
Day 19	107.43 ± 3.66	97.76 ± 3.82*
4 weeks	164.15 ± 4.47	158.22 ± 4.67
5 weeks	238.57 ± 6.73	230.97 ± 7.03
6 weeks	317.49 ± 9.56	312.80 ± 9.99

Age	Tarsus length	
	Force draft	Contact
Hatchling	24.21 ± 0.43	23.60 ± 0.45
Day 5	26.53 ± 0.23	25.42 ± 0.24*
Week 6	59.32 ± 0.89	58.54 ± 0.93

3.4. Down feather corticosterone

The amount of CORT in down feather (1st and 2nd extraction combined, see Fig. 2) were significantly affected by the interaction between treatment and sex (F = 5.407, df = 1, P = 0.025), with FD males having more CORT in their down feathers than contact incubated males (MALES, FD = 155.19 ± 11.29, contact incubated = 119.02 ± 12.37; F = 4.725, df = 1, P = 0.01), there was no significant difference between the females (FEMALES, FD = 112.32 ± 12.37, contact incubated = 120.237 ± 12.37; F = 1.048, df = 1, P = 0.313).

3.5. HPA-axis sensitivity

With endocrinological data, it is often assumed that the use of the AUC_G will result in a measure that is more related to 'total hormonal output', whereas the use of AUC_I is more related to the sensitivity of the system. Total CORT output (AUCg) was significantly affected by the interaction of sex and incubation condition (F = 4.445, df = 1, P = 0.041) with contact incubated females having significantly higher total CORT output compared to FD incubated females and both FD and contact incubated males (P > 0.05). There was, however, no significant effect of incubation condition on the sensitivity of the birds HPA-axis (AUCi, F = 0.456, df = 1, P = 0.503), nor was there any significant difference between the sexes (F = 1.079, df = 1, P = 0.305) or significant interaction between sex and incubation condition (F = 2.819, df = 1, P = 0.101) on AUCi.

3.6. Temperature preference

The two treatment groups both moved towards warmer temperatures during the temperature preference test (treatment x time: F = 4.679, df = 5, P = 0.002). After 3 min contact incubated chicks had moved to a warmer zone than FD incubated chicks (see Fig. 4: F = 8.995, df = 1, P = 0.005) and from 4 min until the end of the testing period at 6 min, there was no effect of time, and neither of the treatment groups moved significantly to warmer areas (P > 0.05).

3.7. Exploration behavior

Contact incubated chicks left the start zone and started exploring the arena significantly sooner than FD incubated chicks (see Table 3, F = 8.888, df = 1, P = 0.003). There was no difference in overall time spend being active (F = 2.231, df = 1, P = 1.441) passive (F = 0.525, df = 1, P
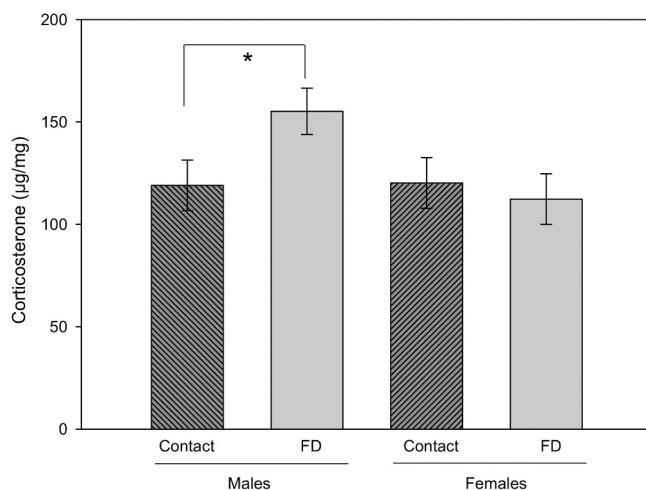

Fig. 2. Down feather corticosterone concentration (mean ± S.E.M.) in male and female contact incubated hatchlings (Contact, black) and force draft incubated hatchlings (FD, grey).
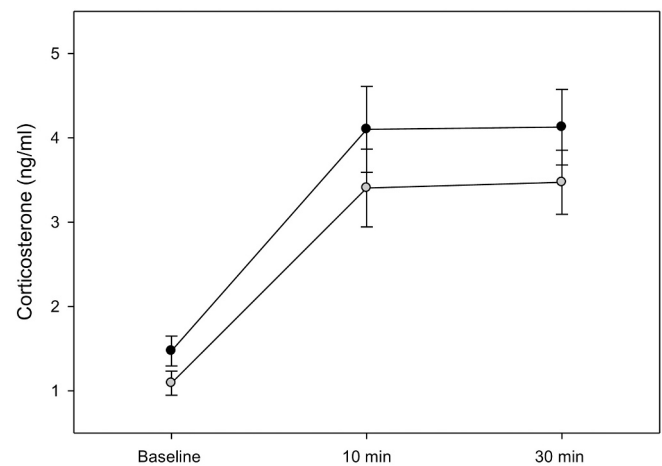

Fig. 3. Plasma corticosterone (mean ± S.E.M.) of contact incubated Red jungle fowls (black) and force draft incubated Red jungle fowls (grey) within 3 min. of catching (baseline) and after 10 and 30 min of physical restrain stress at age 7 weeks.
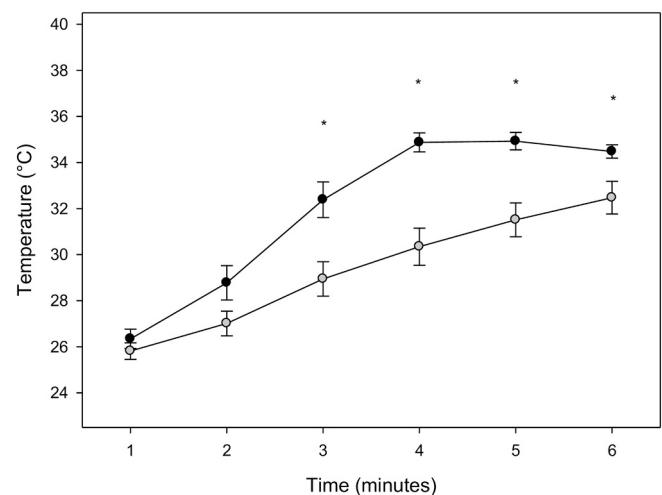

Fig. 4. Temperature preference (mean ± S.E.M.) every minute for 6 min in 3 day old contact incubated (black) and force draft incubated (grey) Red jungle fowls.

Table 3
Undisturbed behavior in novel arena, tested in pairs at age 8–9 days. Time (s) in start zone (mean ± S.E.M.) until chicks started to explore the rest of the arena. Scan counts of activity (mean ± S.E.M.) and location (mean ± S.E.M.) in novel arena time in each zone. P-values below 0.05 indicated with an *.

Trait	Force draft	Contact
Time in start zone	162.67 ± 65.59	46.45 ± 12.67
Activity		
Active	30.17 ± 5.03	35.09 ± 4.80
Passive	12.67 ± 3.56	10.91 ± 3.34
Lying	13.33 ± 2.69	11.36 ± 3.26
Location		
Food - zone	21.17 ± 6.14	19.91 ± 5.94
Shade - zone	2.33 ± 1.74	8.36 ± 5.45
Field - zone	11.50 ± 3.34	12.45 ± 2.86
Elevated - zone	1.08 ± 0.60	1.82 ± 1.53
Start - zone	23.67 ± 6.90	17.27 ± 6.49

= 0.428) or lying (F = 1.441, df = 1, P = 0.699). Nor was there any difference between the treatment groups in time spend in the different zones (P < 0.05, see Table 3).

3.8. Cognition

Although contact incubated chicks were on average faster at solving the cognitive tasks, this difference was not significant neither during the learning task (Contact = 5.53 ± 1.756 s, FD = 10.131 ± 3.547 s: F = 2.825, df = 1, P = 0.108), memory task (Contact = 3.611 ± 0.377 s, FD = 5.397 ± 1.248 s: F = 1.385, df = 1, P = 0.251) or during the reversal task (Contact = 4.43 ± 0.877 s, FD = 6.031 ± 1.533 s: F = 1.074, df = 1, P = 0.311). There was also no significant difference (Mann-Whitney *U* test, P > 0.10) between the two treatment groups in the number of mistakes made during the learning task (Contact = 0.150 ± 0.073 occasions, FD = 0.145 ± 0.056), memory task (Contact = 0.100 ± 0.045, FD = 0.065 ± 0.035) or during the reversal task (Contact = 0.364 ± 0.075, FD = 0.354 ± 0.087).

3.9. Fearfulness

The two treatment groups did not differ in latency to emerge their head during the emergence test (Contact = 51.5 ± 10.1, FD = 52.6 ± 10.8: F = 0.024, df = 1, P = 0.871) or latency to emerge their whole body (Contact = 60.2 ± 12.03, FD = 59.4 ± 13.04: F = 0.006, df = 1, P = 0.941) during the emergence test.

4. Discussion

This study shows for the first time that contact incubation, mimicking natural parental incubation via a brood patch, leads to chicks with a different post-hatch body composition, altered temperature preference and increased exploration behavior as well as altered plasma CORT levels compared to conventional FD incubated chicks. Phenotypic alterations that all have the potential to affect how these chicks cope with their surrounding environment. To date, studies investigating the implications of incubation temperature on birds' phenotype have relied on FD incubators to test the effect of different incubation temperature within the range of natural nest temperatures. These studies tend to find that the performance of chicks is lowest when temperature differ (either slightly higher or lower) from the intermediate nest temperature, suggesting that small differences in incubation temperature by the parents can have a significant negative effect on the chicks' phenotype and possible fitness. As discussed below our results don't indicate that alteration to the chicks posthatch phenotype merely reflects reduced incubation temperature during contact incubation, but instead demonstrate that the different thermal properties of a contact incubated egg versus a FD-incubated egg (see Supplementary Fig. 2), affect the prenatal development and thereby post hatch phenotype of precocial Red jungle fowls.

One of the most notable effects of incubation condition found in this study was on the chicks' post-hatch growth. Although contact incubated eggs lost more mass during the first 2 weeks of incubation and that chicks from these eggs hatched on average half a day later, contact incubated chicks did not weigh less than FD chick at hatching, nor was there any difference in overall body composition (measured via scaled mass index, SMI). This suggest that pre-hatch growth was slower for contact incubated chicks and that they therefore did not use up their pre-hatch nutrition as quickly as FD incubated chicks. At 5 days of age contact incubated chicks were significantly smaller both regarding body mass and structural size (tarsus length) than FD incubated chicks. However, the SMI was significantly higher for contact incubated chicks than FD incubated chicks. This indicates that although contact incubated chicks were smaller at this age than FD incubated chicks, they had a more robust body composition. Contact incubated chicks continued to have a smaller body mass than FD incubated chicks until 19 days of age, after which there was no difference in body mass or structural size between the 2 treatment groups. Demonstrating that effects of incubation on growth were transient and didn't last more than a few weeks. Previous studies looking at the body-composition of precocial birds

incubated at reduced temperature (1 °C) have found that these birds are structurally larger but with fewer energy reserves (Hepp and Kennamer, 2012, DuRant et al., 2010, 2012), which is opposite to our finding on contact incubated chicks which were structurally smaller than force draft incubated chicks and more robust. This indicates that the effects we see on growth due to incubator conditions are not due to reduced temperature during contact incubation.

For precocial birds one of the most important traits influencing the survival of hatchlings is the early development of thermoregulatory ability (DuRant et al., 2013). In domesticated chickens reduced incubation temperature (1–2 °C) have been reported to reduce the neonate's ability to thermoregulate (Black and Burggren, 2004a, 2004b). We tested whether incubation conditions would affect the chicks' thermoregulatory behavior when the birds were 3 days old. Although all control elements of the thermoregulatory systems are functional at hatching in precocial birds, chickens are not fully homoeothermic until day 10 after hatching (Nichelmann and Tzschentke, 2002) and until then they are dependent on heat from the mother's body or from another heat source. We found that contact incubated chicks preferred a higher temperature at 3 days of age than FD incubated chicks. Although contact incubated chicks were smaller than FD incubated chicks, their SMI index at hatching and at 5 days of age suggest that their body composition was similar to or more robust than FD incubated chicks and it therefore seems unlikely that their body composition made them less cold tolerant. Precocial chicks are able to increase their own heat production immediately after hatching, with this ability increasing with age (Nichelmann and Tzschentke, 2002). Differences in heat production abilities or the development of other thermoregulatory control elements, such as changing cutaneous blood flow or growth of plumage could explain the difference in heat preference between the 2 treatment groups, if these were more developed at 3 days of age in FD incubated chicks, than contact incubated chicks. This could potentially signify reduced survival chance for contact incubated chicks, since they might be more sensitive to a reduction in ambient temperature, and also because they might need to spend more time under the mother's brood patch instead of searching for food. However, it can't be excluded that FD incubated chicks perhaps had the same temperature preference as contact incubated chicks but were just slower at moving to this zone during the testing period (6 min) and would have reached the same preferred temperature as contact incubated chicks, had the testing period been longer. Support for this last claim comes from the explorative behavioral test, where contact incubated chicks left the start zone much faster to explore the rest of the arena than FD incubated chicks. In this test there was no difference in overall level of activity or overall explorative behavior as both groups spend similar amounts of time in the different zones of the arena and showed the same level of activity. In the cognitive test contact incubated chicks were on average also faster at solving the task, although this did not react significance. The faster initiative of contact incubated chicks than FD incubated chicks did not result in them being better at solving the tasks, but it does indicate, together with their behavior in the exploration test, that the contact incubated chicks were less hesitant, potentially indicating a more proactive personality type (Cockrem, 2007).

The less hesitant behavior of contact incubated chicks did not seem to be caused by differences in fear level as no difference was found between the two treatment groups when comparing their behavior in a fearfulness test or measuring their CORT production during a stress test. There was no difference between groups in the sensitivity of the HPA axis evaluated by the amount of hormone produced above the starting baseline level (AUCi), but contact incubated females did have significantly higher overall CORT production (AUCg). Corticosterone and glucocorticoids in general have many functions ranging from regulation of glucose metabolism (McMahon et al., 1988) to being part of the feedback mechanism in the immune system (Coutinho and Chapman, 2011) to its multiple effects on fetal development, such as lung maturation (Lupien et al., 2009). It is therefore almost impossible to

hypothesize about the cause and potential function of the higher CORT production in contact incubated females. Also, although the HPA-axis is fully functional at hatch in Red jungle fowl, it still goes through a maturation process during the initial weeks post-hatch with decreasing CORT levels and response to stressors (Ericsson and Jensen, 2016). The difference in overall CORT production at 6 weeks of age in the birds from this study could therefore also reflect difference in the speed of maturation, and it is therefore not possible to conclude whether this difference was permanent (lasting beyond sexual maturity, at 4–5 months of age) or transient. Again, it seems unlikely that these effects are due to reduced incubation temperature during contact incubation, since reduced incubation temperature has been shown to increase baseline and stress induced HPA-axis activity in precocial birds (DuRant et al., 2010) and reduced mobility (Hopkins et al., 2011).

Force draft incubated males seem to have a higher pre-hatch CORT production, as indicated by the higher CORT concentration in their down feathers. Feather CORT concentration has previously been linked to different environmental condition in both adult birds and nestling (Harms et al., 2010; Koren et al., 2012), however, this is the first study to measure down-feather CORT and link it to the pre-hatch environment. The significantly higher concentration of CORT in the feather of FD incubated males could suggest that FD incubation might have been more stressful or energetically demanding than contact incubation, but only for the males.

Down-feathers buds are visible on the chicken embryo from around embryonic day 10 and soon after this the feathers start to grow and continues until the end of incubation, with the most rapid growth occurring when the embryo is around 2 weeks old (Meyer and Baumgärtne, 1998). Down-feather growth therefore mainly occurs during the second half of incubation when the risk of overheating increases for the embryo (Molenaar et al., 2010). The HPA-axis is functional in chickens around the 14th day of incubation (Jenkins and Porter, 2004), although the presence of CORT in the blood of chick embryos has been confirmed already around the 10th day of incubation (Jenkins and Porter, 2004). The ability of chicken embryos to activate their HPA-axis to cope with environmental factors therefore correlates with down feather growth. Our discovery that CORT can be measured in down feathers could have great importance for the field of pre-hatch stress (see Henriksen et al., 2011) in precocial birds as a non-invasive way of measuring the impact of maternal stress during egg formation or parental stress during incubation.

The humidity set for the force draft incubator in this study was based on supplier instructions (58%), whereas the contact incubator (being more of an open design, like a nest) stabilized at 45%. Previous studies have demonstrated that humidity can affect several traits in the newly hatched chicks (Molenaar et al., 2010) and we can therefore not exclude that the effects of incubation type in our study aren't partly due to differences in humidity. However, the methods used to alter humidity in previous studies, such as inlet of air or water, also indirectly influence the temperature of the egg close to the treatment area. In fact, it has been shown that in chickens, effects of humidity variation on development and post-hatch phenotype disappear when egg temperature are kept constant (Van der Pol et al., 2013). This, together with the fact that nest humidity in Red jungle fowls has been reported to be between 38 and 41% (depending on the study, Rahn et al., 1977, Chattock, 1925, Koch and Steinke, 1944), indicate that variation in humidity might have a larger effect in force draft incubators than during contact incubation, where heat is transferred via contact as opposed to warm circulating air.

The fact that we find FD incubation to have significant effects on birds' phenotype compared to contact incubation even when both types of incubation are fixed at 37 °C, questions the use of FD incubators when testing effect of different nest temperatures, since these incubators might not correctly mimic effects of varying nest temperature and thereby potentially overestimates the effects of incubation temperature. It would be interesting to test different temperatures within the range of naturally occurring nest temperatures using the Contact incubator in

future studies, to see just how much the embryo can buffer potential effects of incubation temperature when contact incubated.

5. Conclusion

While slight temperature differences in incubation temperature have previously been shown to have significant effects on chicks' post-hatch phenotype, the findings from this study demonstrate for the first time that the way heat is distributed to the egg can also significantly affect birds post-hatch phenotype. Our findings add another factor to the growing field of effects of the pre-hatch environment in birds, by demonstrating that contact incubation creates a different pre-hatch environment and chicks with a significantly different phenotype than conventional warm air incubators. Additionally, our finding that CORT can be measured in down-feathers and that differences in CORT concentration between individuals can be related to the pre-hatch environment, provides a potential useful tool for studying pre-hatch CORT production in future studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2020.104892.

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