Short communication: Effects of in-ovo injection of endocrine disruptors and methyltransferase inhibitor on quail growth and egg-laying performances

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Animal 16 (2022) 100464

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

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ARTICLE INFO

Article history:
Received 27 July 2021
Revised 11 January 2022
Accepted 13 January 2022
Available online 15 February 2022

Keywords:
5-Aza-2′-deoxycytidine
Bisphenol A
Coturnix Japonica
Epigenetics
Genistein

ABSTRACT

Early experiences, including prenatal environment, are known to influence a wide variety of mechanisms involved in the phenotype elaboration. We investigated the effect of the addition of endocrine disruptors or of a methyltransferase inhibitor during the embryonic development of quails from different genetic backgrounds (four different quail lines) on their growth and egg-laying performances. Fifty-four pairs of parents per line were used and fertilised eggs from each pair were randomly divided into five groups: a control group without any injection, an injected control group treated by injection into the egg of sesame oil, and three groups treated by injection of Genistein, Bisphenol A or 5-Aza-2′-deoxycytidine. All quails were individually weighed at 8, 21, 36 and 78 days. The age at first egg laid and the number of eggs laid were recorded. These analyses revealed a significant impact of the treatment on growth but no influence on the egg-laying traits. All three molecules significantly affected at least one of the analysed growth traits. In conclusion, we showed that the injection of endocrine disruptors or DNA methyltransferase inhibitor into the egg had significant effects on quail development; these effects were specific to each treatment, but no interaction between line and treatment was observed.

Implications

Early experiences, including exposure to biologically active substances during embryonic development, can affect a wide variety of mechanisms involved in the construction of the individual. Inappropriate early-life rearing conditions may lead to the development of unfavourable consequences on animal health and welfare. This study demonstrates the significant effects on adult phenotypes of endocrine disruptors and methyltransferase inhibitors during embryonic development in quail. It paves the way to study the extent to which the individual early environment can influence future generations, notably in the case of contamination by endocrine disruptors into the maternal diet and accumulation into the egg.

Introduction

The prenatal environment is known to influence the adult phenotype in several species, in part through epigenetic mechanisms. Epigenetic phenomena are "mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Riggs et al., 1996). These mechanisms take part in the regulation of gene expression and can induce modifications of phenotype. There are different types of epigenetic phenomena including histone modifications, changes in chromatin structure, effects of non-coding RNAs, and DNA methylation. Modifications in the embryo environment, such as the presence of chemical contaminants, can induce epigenetic changes during the development of somatic cells and eventually of germ cells affecting the offspring of the next generations (Skinner, 2011). Here, we propose to analyse in quail the impact of *in-ovo* exposure to endocrine disruptors, Genistein and Bisphenol A (**BPA**), and of a DNA methyltransferase

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https://doi.org/10.1016/j.animal.2022.100464
1751-7311/

inhibitor, 5-Aza-2′-deoxycytidine (**5Aza** or Decitabine) on transmissible traits, in different genetic backgrounds.

Several molecules have been shown to induce epigenetic modifications in many vertebrate species such as humans, mice, rats and zebrafish (Silva et al., 2019; Qin et al., 2021). Genistein is a phytoestrogen notably found in soy, known to have protective effects against metabolic diseases or cancers that has been shown to induce epigenetic changes (Silva et al., 2019). Bisphenol A is a widespread environmental contaminant that has notably been demonstrated to promote epigenetic modifications (Qin et al., 2021). The DNA methyltransferase inhibitor 5Aza is a drug that has long been known to be beneficial in the treatment of leukaemia (Momparler et al., 1997). For this study, four different quail lines were used to analyse the putative interaction between the injected treatment and the genetic background. This study hypothesised that changing the embryo environment by injecting molecules into the egg may trigger phenotypic alterations later in life, putatively depending on the molecule or the genetic background.

Material and methods

Experimental design

For this study, we used two experimental and two commercial quail lines: the high social reinstatement quail line obtained by divergent selection on social motivation (Mills and Faure, 1991), referred to as the "S+" line, the DD line that was selected for early egg production (Minvielle et al., 1999) and the line A and the line B, two parental lines kindly provided by commercial breeders. Fertilised eggs produced from 54 pairs of parents per line were randomly divided into five experimental groups: four received an injection before incubation and one received no injection and served as control. The injection was performed the day the freshly laid eggs were incubated, directly into the egg yolk. Of the four groups that received an injection, one was treated with 50 µl of delivery vehicle (10% ethanol/90% sesame oil, Acros Organics), and three others were treated with an injection of 50 µl of one of the tested compounds dissolved in 100% ethanol, and then diluted ten-fold in sesame oil. The dose used per egg was of 500 µg of genistein (Molekula), or of 200 µg of BPA (Sigma-Aldrich), or of 34 µg (50 µM) of 5Aza (Sigma-Aldrich). Doses were established according to previous studies in quail (Halldin et al., 2001; Leroux et al., 2017), or rodents (Zhang et al., 2013) in order to maximise the effects while keeping the hatching rate as high as possible. Untreated eggs and eggs injected with carrier fluid only were used as controls to analyse the putative impact of the *in-ovo* injection on the phenotypes measured. After hatching, we obtained 1 979 live chicks issued from two distinct batches, representing approximately 50 individuals per sex, line and treatment. All birds were weighted at 8, 21, 36 and 78 days of age. The age at first egg laid and the total number of eggs produced over 30 days of experiment were also recorded (Supplementary Material S1). The record of the egg number is the same period for each line: 30 days from the date of the first egg laying. Growth and egg-laying traits were measured to determine a potential phenotypic effect of the chemical agents.

Statistical analysis

All the analyses were carried out using *R* (v.4.0.2) (Supplementary Material S2). Effect of the different factors on BW at a given age (8, 21, 36 and 78 days) and on laying traits of quails was estimated using linear mixed models. In addition, impact of the different factors on the trajectory of BW over time was evaluated using a random regression (**RR**) model applied to longitudinal BW measurements. For all models, we included the pedigree information (family) as a random effect to take into account the kinship between individuals. According to the non-linearity of the weight evolution relative to the age (Fig. 1), the fixed part of the RR model considered a second-order polynomial (age^2) and the random part consisted in first-order polynomials for the family and animal random effects. Each model included fixed effects such as line, batch, injected treatment, sex and age when appropriate, and their interaction. The *lme* function from the *nlme* R package (v.3.1-149) was used to apply the mixed models (Supplementary Table S1).

The variance was analysed for each trait using the ANOVA function from R *car* package (v.3.0-10) according to the given linear model. As the injection of sesame oil alone may have an effect compared to the non-injected control, the ANOVA comparison between each group was performed using the injected control (**IC**) as the reference group (IC vs control and IC vs treatments). The *stepAIC* function from R *MASS* package (v.7.3-54) was used to perform stepwise model selection by Akaike Information Criteria (**AIC**), and the model associated with lower AIC value was selected (Supplementary Table S1). The posthoc Tukey's Honestly Significant Difference statistical test (*emmeans* function from R *emmeans* package v.1.6.1) was used to perform pairwise comparison of the treatment groups and test for significant differences between their means using a significance threshold for adjusted *P*-values of $\alpha = 5\%$.

Results

Line and sex effects

Analyses of growth and egg-laying phenotyping data of quails highlighted a weight difference between lines and sexes of individuals, notably at 78 days of age (Fig. 1, Supplementary Figure S1, Supplementary Table S2). Line had a significant effect on all measured traits (Table 1). The two experimental lines S+ and DD had a lower weight than the two commercial lines A and B at all measured ages (Fig. 1, Supplementary Figure S1.a, Supplementary Table S2). The S+ line laid the first egg significantly later than the other three lines, and its total egg number was significantly lower than the other lines for the same time period (Supplementary Figure S1.a, Supplementary Table S2). Sex had also a significant effect

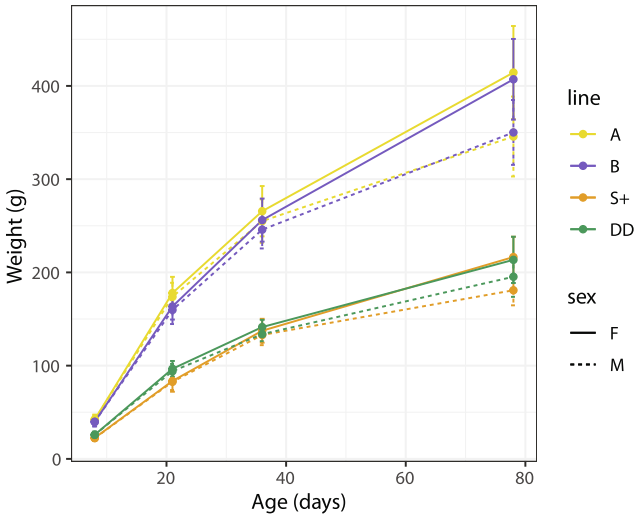

Fig. 1. Plot indicating the weight of quails according to the age, line and sex. The yellow, violet, orange and green straight lines show respectively the quails from A, B, S+, DD lines. The solid lines and dotted lines refer to female (F) and male (M), respectively.

Table 1
Table of ANOVA *P*-values for growth and egg-laying traits of quails (a, top) and comparison of treatments impact on the weight (b, bottom). Results were obtained by combining the following methods: *aov* + *stepAIC*. Only values less than 0.05 are considered as significant.

	Growth and egg-laying traits			
	Weight	Age 1st egg	Egg number	
line	<2.2e−16*	<2e−16*	<2.2e−16*	
batch	1.23e−04*	1.05e−02*	4.78e−10*	
sex	1.06e−01	NA	NA	
age	<2.2e−16*	NA	NA	
age^2	<2.2e−16*	NA	NA	
treat	3.48e−03*	6.39e−02	NR	
age:sex	4.72e−02*	NA	NA	
age:line	<2.2e−16*	NA	NA	
age:batch	1.15e−02*	NA	NA	
treat:age^2	1.64e−02*	NA	NA	
sex:age^2	6.21e−13*	NA	NA	
line:age^2	<2.2e−16*	NA	NA	
batch:age^2	8.36e−04*	NA	NA	
line:batch	NR	5.91e−02	NR	
age:sex:line	1.89e−03*	NA	NA	
treat:sex:age^2	4.74e−02*	NA	NA	
sex:line:age^2	<2.2e−16*	NA	NA	
sex:batch:age^2	4.12e−02*	NA	NA	
	Treatment impact on the weight (vs IC)			
	Con	BPA	Gen	5Aza
treat	6.06e−01	2.02e−01	1.08e−02*	3.34e−02*
treat:age^2	2.58e−01	7.22e−01	3.35e−01	2.19e−02*
treat:age^2:sex	8.76e−01	5.34e−01	7.69e−02	1.13e−02*

Abbreviations: *aov* = R function for analysis of variance; *stepAIC* = R function performing stepwise model selection by Akaike Information Criterion; treat = treatment; NA = untested effect; NR = effect not retained by *stepAIC*; IC = injected control (sesame oil); Con = non-injected control; BPA = Bisphenol A; Gen = Genistein; 5Aza = 5-Aza-2′-deoxycytidine.
* Value significantly different at $P < 0.05$.

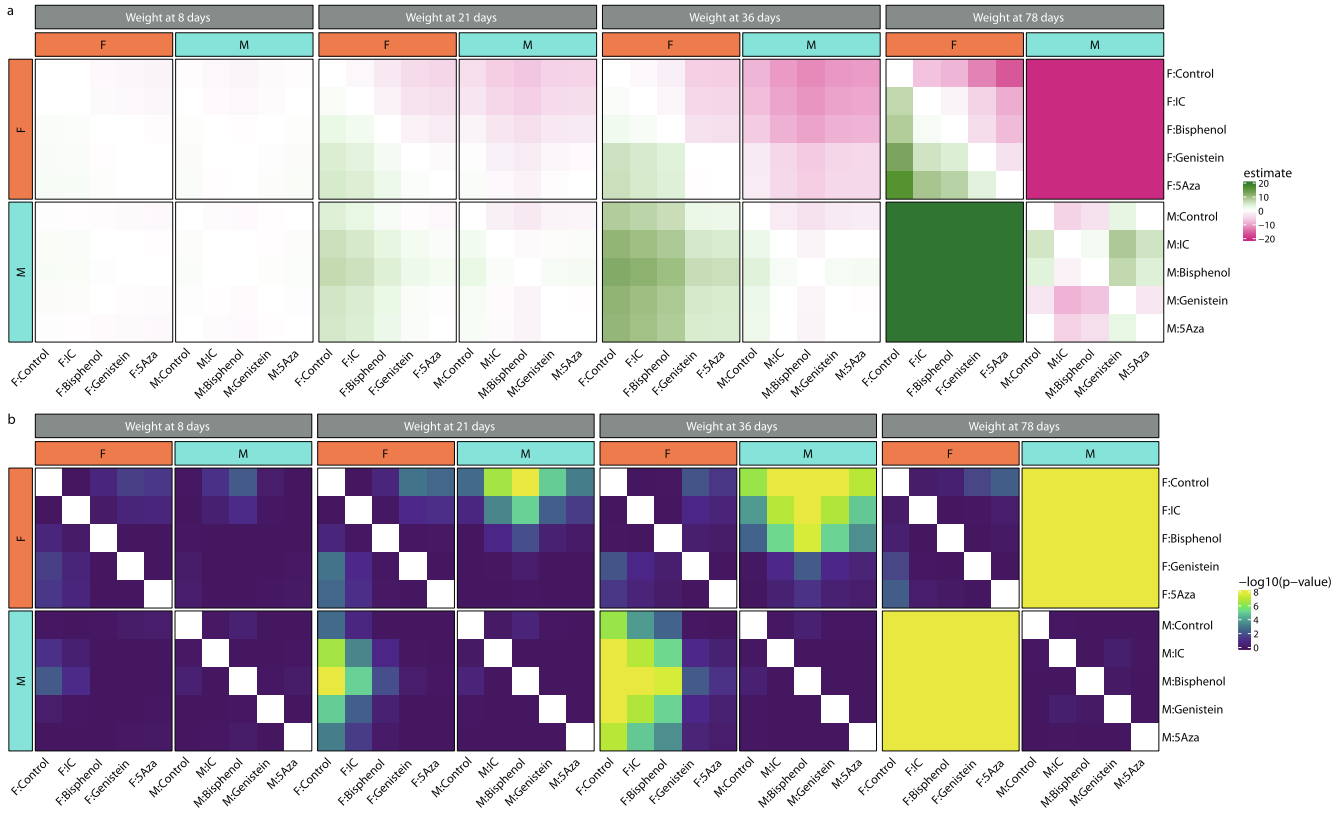

Fig. 2. Heatmaps representing tables of Tukey HSD contrast estimate values (a) and -log10(*P*-values) (b) for each pairwise treatment comparison according to the sex at each measurement age of quails. The estimate value refers to the column condition minus the line condition. The colour gradient (blue to green) is proportional to the significance of Tukey test: blue and green for respectively low and high Tukey test *P*-value. The diverging colour gradient refers to a loss (pink) or a gain (green) of weight. Abbreviations: HSD = honestly significant difference; F = female; M = male; IC = injected control (sesame oil); 5Aza = 5-Aza-2′-deoxycytidine.

on BW at all ages (Supplementary Table S3, Supplementary Figure S1.b), females being generally heavier than males (Fig. 1, Supplementary Figure S1.b). As illustrated by the evolution of the estimate values between males and females in Tukey Honestly Significant Difference (**HSD**) tests (Fig. 2.a), females were heavier than males in all lines, the difference between sexes increasing along life.

Treatment effect

Treatment had a significant effect on BW and a significant interaction between treatment, sex and age^2 effect was observed (Table 1.a), reflecting different weight gain dynamics. The analysis according to the age revealed a significant treatment effect at 8, 21 and 36 days (Supplementary Table S3). We observed a significant interaction between sex and treatment for BW at 8 and 21 days. No treatment effect was detected for egg-laying traits. We did not observe any line × treatment interaction (Table 1.a, Supplementary Table S3).

Individual treatment comparison

The groups' comparison showed that the injection of sesame oil had no significant effect on overall growth (Table 1.b), but sesame oil seemed to decrease the weight at 78 days (Supplementary Table S3, Fig. 2.a). A treatment effect on weights at 8, 21 and 36 days was observed for Genistein and 5Aza when compared to the IC groups, whereas BPA had an effect only at 8 days but no influence was identified on the growth (Table 1.b, Supplementary Table S3).

According to the multiple comparison analyses (Tukey HSD), individuals injected with a chemical agent had a lower weight compared to the non-injected controls (Fig. 2.a, Supplementary Table S4). Genistein and 5Aza groups had a significant lower weight compared to non-injected control group for females at 8, 21, 36, 78 days and a trend is observed compared to the IC group only at 21 days whereas no mean difference was observed for the BPA group (Fig. 2.b, Supplementary Table S5). We observed no significant effect of the treatment on the male growth.

Discussion

In this study, we compared in different quail lines the effects on growth and egg-laying related phenotypes of three compounds expected to modify the embryonic environment after injection into the egg. These lines were of different origins and as expected, the commercial lines (A, B) were heavier than the experimental lines (S+, DD). The observed increase in the weight difference between sexes and between S+/DD and A/B lines over time can explain the line by sex effect observed for BW at 36 and 78 days (Supplementary Table S3). The observed difference between the injected and non-injected controls may demonstrate an influence of an intervention on the egg at an early stage of development.

The effects of treatments were highly significant on BW until 36 days of age, and significant on overall growth. The less significant effect on BW observed after 36 days of age is in accordance with previous studies that used BPA in quail (Halldin et al., 2001) or genistein in chicken, for which the effect on BW gain was significant at 21 days of age but not at 42 days of age (Lv et al., 2018b). More specifically, only 5Aza and Genistein impacted growth compared to the injected control. Previous studies have shown an increased BW gain in 21-day-old broiler chickens after a low-dose genistein supplementation into the diet of their mother, which results in genistein presence into the egg (Lv et al., 2018b), but a decreased embryo weight after high-dose genistein exposure by feed supplementation of the mother (Lv et al., 2018a). No treatment effect on egg-laying traits was observed.

Conclusion

Our study showed significant effects of injection of endocrine disruptors or methylation modifiers into the egg on adult phenotypes. These effects do not seem to depend on the genetic background tested. These compounds may be found in animal diet (genistein) or as chemical contaminants (BPA) and their effect on phenotypes, both from the exposed animals and their progeny, need to be further analysed. Mechanisms of action may involve epigenetic regulations, notably through DNA methylation alterations, and need to be further analysed, including the analysis of putative non-genetic inheritance effects. Improved knowledge of genome-to-phenome relationship, accounting for genetic and non-genetic mechanisms, should ultimately improve livestock production systems.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100464.

Ethics approval

Animals were bred at the INRAE Poultry Experimental Facility (PEAT) in Nouzilly, France (https://doi.org/10.15454/1.5572326250887292E12) in accordance with European Union Guidelines for animal care with an approval by the local ethical committee in animal experimentation (Val de Loire) and the French Ministry of Higher Education and Scientific Research (authorization n°4609-2016032115196879). Animals were maintained under standard breeding conditions and were subjected to minimal disturbance.

Data and model availability statement

All data and scripts used during this study are public. They are available on Github (https://github.com/ccerutti88/Quail_Phenotypes.git).

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Declaration of interest

None.

Acknowledgements

The authors want to acknowledge PEAT staff for their conscientious animal care and phenotyping, and Caillor (https://caillor-genetics.com/home) and Caille Robin (https://www.cailles-robin.fr/) breeders for providing animals. We are grateful to C. Leterrier for providing the S+ experimental line and helpful discussions.

Financial support statement

This work was supported by the EPIQUES grant (INRAE, SelGen Metaprogramme). C. Cerutti is supported by a PhD scholarship from Région Occitanie and INRAE Animal Genetics Division.

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