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Transfer of humoural immunity over two generations in urban pigeons

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Maternal antibodies (MatAb) are known to provide passive protection early in life for young vertebrates but their effects on the development of offspring immune response across generations are still unknown. Here, we investigated the effects of antigen exposure (keyhole limpet haemocyanin, KLH) experienced by urban pigeon (*Columba livia*) females on the amount of antigen-specific antibodies (Abs) transferred into the egg yolk of their daughters and on the humoural immune response towards this same antigen in their grandchildren. We found that chicks from KLH-injected maternal grandmothers had a higher humoural response than chicks from sham-injected grandmothers. However, we did not detect a significant effect of female KLH exposure on the ability of their daughters to transmit anti-KLH Abs into their eggs. These results suggest that antigen exposure at one generation may shape the immune profile of offspring over two next generations, although the underlying mechanisms remain to be investigated.

1. Introduction

The transfer of maternal antibodies (MatAb) in vertebrates is a well-documented example of maternal effect [1,2] by which mothers can confer protection against parasites to their offspring [3] and may educate their developing immune system [4,5]. Some recent empirical studies suggest that the effect of MatAb could span over two generations [6]. Indeed, in the developing immune system of the neonate, MatAb could prime the mother's antigen repertoire to which the grandmother has been exposed [6] and enhance the immune system of the mother, which could in turn influence the immune system of their grandchildren through MatAb. For instance, F_0 grandparents exposed to parasites would produce Abs towards the parasite. Such specific Abs will be then transferred to the F_1 generation and would enhance the immune response towards the same parasite in the F_1 generation through educational effects. This F_1 MatAb would be transferred to the F_2 generation and could trigger a better immune response towards the same antigen in the F_2 generation (transgenerational educational effects). We define epigenetic as the inheritable modifications of gene expression without changes in the underlying DNA sequences [7,8]. In the context of immune maternal effects, the phenotypic level of parasite resistance induced by the transfer and the educational properties of MatAb would be a non-genetically inherited phenotype from mothers and therefore could constitute a good example of an epigenetic effect.

Recently, the evolutionary importance of epigenetic effects in inclusive heritability and fitness [8] has been the focus of fascinating debates [9] but most empirical work mainly focused on cultural heritability [10] and on DNA switch-off [11]. Here, we hypothesized that specific MatAb would be a non-genetic indirect messenger of the phenotypic level of parasite resistance over two

successive generations. This hypothesis therefore predicted that the humoural immune response of juveniles towards a specific antigen would be positively linked to the level of antigen exposure towards this same antigen of their mothers and grandmothers. Although previous studies on pigeons did not bring clear evidence of educational effects of MatAb on short timescales [12], offspring long-term immune repertoires could be modified by MatAb [13] and may enhance the transfer of MatAb to the next generation. If this hypothesis is true, we predict that daughters of antigen-exposed mothers would transfer more antibodies (Abs) into their eggs than non-exposed mothers. We tested these predictions in urban pigeons (*Columba livia*) by investigating the effects of antigen exposure experienced by F_0 grandmothers on the amount of Abs transferred into the egg yolk of their F_1 daughters and on the humoural immune response towards this same antigen in their F_2 grandchildren.

2. Material and methods

The experiment was conducted between 2010 and 2012; 120 adult feral pigeons (60 females and 60 males) from three suburban locations near Paris were captured by the SACPA company (France) under the authorization of local authorities and kept in 10 outdoor aviaries located at the CEREEP field station (CEREEP-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, St-Pierre-les-Nemours). Each aviary contained six males and six females in similar conditions (F_0 generation).

The F_0 generation was injected in 2010. Sixty birds (three breeding pairs chosen randomly in each aviary) received a subcutaneous injection of a 100-µl solution containing 0.5 mg ml^{-1} of keyhole limpet haemocyanin (KLH). We chose to use KLH, a novel antigen for pigeons, and not a natural antigen, to ensure that females had no pre-existing specific Abs that could have masked the effects of the experimental treatment. The 60 remaining birds were injected with phosphate-buffered saline (PBS). A second injection was performed two weeks later to ensure that blood anti-KLH Ab levels differed between KLH and sham-injected treatment groups. In a previous study, we found that these injections induced a higher transmission of maternal anti-KLH Ab from the F_0 mother to the F_1 generation [12].

The F_1 generation contained 88 chicks born in 2010 (42 females and 46 males). All individuals of this F_1 generation were injected with KLH (0.5 mg ml^{-1}) at 21 and 35 days of age. Reproduction of the F_1 generation was then monitored during 1 year after these early injections. From April to September 2011, we collected 142 eggs from 25 females of the F_1 generation. Ninety-two eggs were laid by 14 F_1 daughters from 10 F_0 sham-injected females and 50 eggs were laid by 11 F_1 daughters from eight F_0 KLH-injected females. There was no significant effect of F_0 treatment on the number of eggs laid by F_1 daughters ($F_{1,16} = 0.90$, $p = 0.36$). Egg yolks were isolated and diluted 1 : 1 in PBS, homogenized for 1 min with a homogenizer (ULTRA-TURRAX, T10 Basic Disperser, Science Lab, Houston, USA). Chloroform was then added 1 : 1 and homogenized for 1 min with a vortex. After centrifugation (6 min at 13 r.p.m.), the supernatant was used for Ab assays.

In 2012, eggs laid by F_1 birds were left in the nests to hatch. Thirty-three chicks were born and constituted the F_2 generation. We injected all F_2 chicks at 21 and 35 days subcutaneously with 100 µl of a solution containing 0.5 mg ml^{-1} of KLH. To monitor the dynamics of the humoural immune response towards KLH across time, we collected blood samples from chicks from the brachial vein at 3, 7, 14, 21, 28, 35, 42, 49 and 56 days. Fourteen chicks had KLH-injected maternal grandmothers ($N = 6$ grandmothers) and 19 chicks had sham-injected maternal grandmothers ($N = 7$ grandmothers). Plasma were then extracted and stored at −20°C.

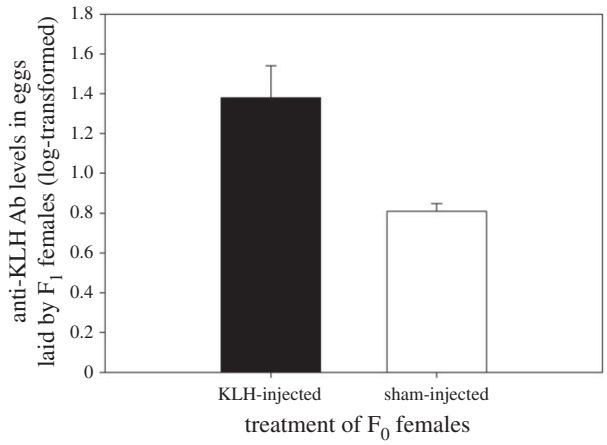

Figure 1. Means ± s.e. of anti-KLH Ab level in egg yolk laid by F_1 daughters of KLH-injected (black bar, $N = 50$) or sham-injected (open bar, $N = 92$) F_0 females. The apparent difference between the two bars is not significant when taking into account the pseudo-replication ($F_{1,117} = 0.76$, $p = 0.38$).

The ELISA technique was used to quantify the amount of anti-KLH Abs in the plasma as described by Jacquin *et al*. [13].

All statistical analyses were performed using SAS (v. 9.4). We first used a linear mixed model with the anti-KLH Ab concentration in eggs of the F_1 generation as the dependent variable and the immune treatment of the F_0 females as an explanatory variable. We added the F_0 and F_1 female identities as random effects to take into account for the non-independence of eggs laid by the same mothers. Second, we used a generalized linear mixed model with the anti-KLH Ab concentration of the F_2 chicks as the dependent variable and the immune treatment of the F_0 grandmothers and F_2 chick age as a fixed effect. Chick, F_0 and F_1 female identities were added as random effects for similar reasons as previously described. Anti-KLH Ab concentrations were log transformed to achieve normality.

3. Results

The concentration of anti-KLH Abs in eggs laid by F_1 daughters was not significantly affected by the immune treatment of the F_0 grandmothers (figure 1, $F_{1,117} = 0.76$, $p = 0.38$). The dynamics of the humoural immune response towards KLH injections of the F_2 chicks was affected by the immune treatment of their F_0 maternal grandmother over time as shown by the significant interaction between chick age and immune treatment of the F_0 maternal grandmother (table 1 and figure 2). At 49 days, anti-KLH Ab concentrations was higher in F_2 chicks from KLH-injected maternal grandmothers compared to chicks from sham-injected maternal grandmothers ($F_{1,20} = 5.09$, $p = 0.04$). No significant differences were detected at the other ages (all p-values > 0.08).

4. Discussion

As predicted, F_2 chicks from KLH-injected F_0 maternal grandmothers had a higher humoural immune response against KLH, than F_2 chicks from F_0 sham-injected grandmothers. Our study therefore shows that immune factors transferred from mothers to offspring can influence the immune response of the next generations.

In a previous study, we showed that F_0 antigen exposure triggered a higher transmission of MatAb to the F_1 generation [12]. These MatAb were found to decrease the F_1 humoural

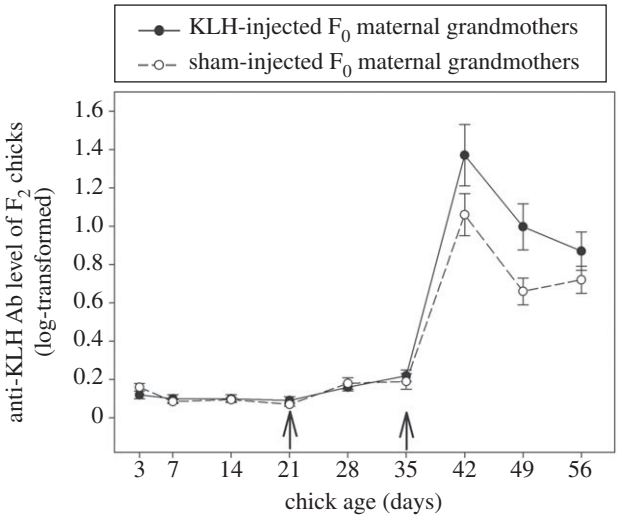

Figure 2. Humoural immune response of F_2 chicks over time following KLH injection at 21 and 35 days old, measured by anti-KLH Ab level in relation to age for F_2 chicks that had KLH-injected maternal grandmothers (black dots, $N = 14$) and chicks that had sham-injected maternal grandmothers (white dots; $N = 19$). Arrows represent KLH injections.

Table 1. Output of the best-fitted generalized mixed models explaining variations in anti-KLH Ab levels over time of F_2 chicks (F_2 chick age) following two injections (at 21 and 35 days old) in interaction with the treatment of their F_0 maternal grandmothers. d.f. represents the degrees of freedom, the *F* and *p* represent, respectively, the *F* statistics and the *p*-values.

F_2 anti-KLH humoural response			
effects	**d.f.**	***F***	***p***
F_2 chick age	8, 241	99.65	<0.0001
F_0 grandmother treatment	1, 241	1.96	0.16
F_2 chick age $\times$ F_0 grandmother treatment	8, 241	2.83	0.005

response of nestlings injected at 21 and 35 days of age, though a classic blocking effect of MatAb [1]. Here, we hypothesized that F_0 MatAb would however increase the long-term immune response of F_1 birds through an educational effect and allow them a higher transmission of anti-KLH Abs into F_1 eggs transferred to the F_2 chicks. However, our results do not support this hypothesis because eggs from F_1 daughters from KLH-injected F_0 females did not contain a significantly higher amount of MatAb than eggs of F_1 daughters from sham-injected F_0 females. Therefore, we cannot explain why KLH-injected grandmothers produced F_2 grandchildren with a better humoural response against KLH. Three hypotheses can however be proposed to explain this intriguing result. First, our statistical power might not be high enough to detect the difference observed even if specific MatAb are the transgenerational messenger. Second, the Ab and their educational properties might not be the transgenerational messenger and another physiological mechanism may play this role. For instance, there is growing evidence that nutrients and hormones packed into the egg could affect several physiological traits such as the immune system [14]. Prenatal immune activation of F_1 generations could positively affect the investment of nutrients and/or hormones into the egg yolk which could affect the development of the immune system over the next generation. Third, the anti-KLH MatAb transferred from F_0 to F_1 could prime the F_1 immune repertoire that could then be transmitted to F_2 chicks and shape their immune memory, without involving the transmission of a higher amount of Abs into F_1 eggs [15]. Our results are in favour of this last explanation, because only the secondary immune response (involving the formation of immune memory) was affected by F_0 injection in F_2 chicks but not the primary response (figure 2). This calls for further studies to test this interesting hypothesis, for instance through the direct manipulation of immune factors in the egg. In addition, here we used an artificial antigen to which pigeons were never exposed (KLH), and further works are now needed to test the fitness consequences of grand-maternal exposure to natural parasites.

In conclusion, this study shows that the grandmother's immunological memory can be transferred to the egg yolk and affect the immune quality of the second generation, therefore impacting the ontogenic trajectory of individuals across at least two generations. This suggests the existence of a transgenerational epigenetic effect of maternal parasite exposure on offspring immune profile over *two* generations which can play a major role in evolutionary ecology. More knowledge is now needed to decipher the physiological messengers of such an epigenetic effect of the antigen repertoire to which the mother has been exposed over her lifetime.

Ethics. All experiments were conducted under the approbation of the French Veterinary Services of the DSV77 (authorization N°77-05).

Data accessibility. Data available from the Dryad Digital Repository: http://datadryad.org/review?doi=doi:10.5061/dryad.nc862.

Authors' contributions. A.I. designed the study, collected field data, carried out the statistical analyses and drafted the manuscript; L.J. participated in the design and conceived the study, helped to collect field data and helped draft the manuscript; C.H. carried out immunological analyses and helped draft the manuscript; S.P. helped to collect field data and helped draft the manuscript; J.G. conceived, designed and coordinated the study, participated in data analysis and helped draft the manuscript. All authors gave final approval for publication. All the authors agree to be held accountable for all aspects of the work.

Competing interests. We have no competing interests.

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