Transmission of maternal antibodies in Japanese quail (Coturnix japonica) eggs

Author: Anouk van Weert
Solis-id: 3915794
Master-thesis, June 2018
Institute: Utrecht University, Faculty of Veterinary Medicine
Dept. of Animals in Science and Society
Supervisor: V.C. (Vivian) Goerlich-Jansson

Abstract

Antibodies transmitted from the mother to the offspring, contribute to the humoral immune system of the offspring early in life, though the protection contributed by the maternal antibodies differs among species. The diversity and concentration of the transmitted antibodies could be of influence on the disease resistance and the survival chances of offspring. Antibodies are also called immunoglobulins and immunoglobulin Y (IgY) is the avian and reptilian homologue of the mammalian IgG. Social animals living together establish dominance hierarchies. These dominance hierarchies could control e.g. sexual relations and access to resources within groups and dominance determines a part of this social order. The goal of this research is to determine whether there is a relation between female social dominance and yolk IgY antibody concentration in Japanese quails. We expected that female Japanese quails living in social groups become more stable in their dominance rank and that this may be reflected in the IgY concentrations of their eggs. In a previous experiment, eggs were collected and prepared for IgY analysis, during which also the dominance ranking of the mother was determined. For this study a protocol has been refined to measure IgY concentrations of egg yolk from Japanese quail via Enzyme-Linked Immuno Sorbent Assay (ELISA). The results showed no overall correlation between mean body weight and mean IgY concentration of the females. On the other hand, an overall significant correlation was found between yolk mass and IgY concentration. Future research could look into the possible interactions between egg volume, social dominance, yolk steroid concentrations (testosterone, progesterone) and IgY concentrations.

Table of contents

Introduction

Transfer of maternal antibodies and the immune response

Mothers of both mammals and avian species transmit antibodies to their offspring. While the transmission of antibodies to offspring is widely examined in mammals, this process has not been comprehensively explored in avian species (Grindstaff, 2010; Grindstaff et al., 2003).

Maternal antibody transmission by a female immunocompetent adult, can be described as the transfer of antibodies, to an immunologically naive neonate. Transmission of maternal antibodies can take place prenatally through the placenta or postnatally through colostrum and/or, milk. In oviparous species such as birds, fish and reptiles, the transmission of maternal antibodies occurs through the deposition of antibodies to their eggs (Grindstaff et al., 2003).

Transmitted maternal antibodies are catabolized from circulation after birth or hatch (Brambell, 1970; Grindstaff et al., 2003). The duration of antibodies in offspring circulation depends on the species and the initial levels of maternal antibodies (Smith et al., 1994). Progeny with higher initial concentrations of maternal antibodies contain maternal antibodies in their circulation for a longer period of time. These offspring will benefit from a longer immune protection period. Contrary to progeny with lower initials concentration of maternal antibodies (Cáceres et al., 2000; Goddard et al., 1994; Grindstaff et al., 2005). Not only maternal antibodies are provided by maternal transfer, but also antimicrobial proteins and antioxidants. These are also important determinants of posthatching immune functions (Shawkey et al., 2008). After a period of time, the offspring starts to develop its own immune system. In the period between the maternal protection and their own protection, the offspring is susceptible to various diseases (Hasselquist and Nilsson, 2009). Furthermore, it is also presumed that maternal antibodies continue to effect phenotype, growth and development in offspring even after catabolization (Boulinier and Staszewski, 2008; Grindstaff et al., 2003; Hasselquist and Nilsson, 2009).

The transmitted antibodies contribute to the humoral immune system of the offspring early in life, though the protection contributed by the maternal antibodies differs among species. The variety and concentration of antibodies are correlated between mothers and their offspring (Bollen and Hau, 1999; Gasparini et al., 2002; Graczyk et al., 1994; Grindstaff, 2008). The diversity and concentration of the transmitted antibodies could be of influence on the disease resistance and the survival chances of offspring (Al-Natour et al., 2004; Grindstaff, 2008; Heller et al., 1990; Keller and Van Noordwijk, 1993; Leitner et al., 1990; Sahin et al., 2003; Smith et al., 1994; Starck and Ricklefs, 1998).

Antibodies can also be called immunoglobulins. Immunoglobulins are molecules secreted by plasma cells in response to antigen exposure and are molecules composed of two heavy (H) chains and two light (L) chains. The structure of the chains classifies the immunoglobins into isotypes of immunoglobulins such al IgG, IgM and IgA. Immunoglobulin Y (IgY) is also composed of two heavy chains and two light chains and is the avian and reptilian homologue of the mammalian IgG. IgY is an important antibody responsible for the development of the avian offspring immune system, while IgM is the predominant isotype responsible for the avian primary humoral response (Munhoz et al., 2014; Wakabayashi, 2010).

Maternal antibodies levels in progeny differs between the individuals (Bumstead et al., 1993; Graczyk et al., 1994; Pardue et al., 1990). This could be explained by variation in antibody transmission to individual eggs or be due to variation in capacity of offspring to absorb IgG (Bumstead et al., 1993; Pardue et al., 1990). The capacity of offspring to absorb maternal antibodies depends on the length and metabolic rate of gestation period, receptor density on epithelial cells and receptor binding affinity for IgG (Linder et al., 2000; Muggli et al., 1984). The transport of IgY from serum to egg yolk is proportional to IgY concentration in the serum of the female (Munhoz et al., 2014). Thus, interaction between the maternal and offspring genome could be determine the levels of maternally derived

antibodies absorbed by offspring. The interaction between transmission of maternal antibodies and development of active immunity still needs more research for a better understanding (Grindstaff, 2010).

It was also discovered that differences in living circumstances of the females prior to reproduction influences the diversity and quantity of transmitted antibodies. Mainly the exposure to various pathogens is presumed to be a determining factor (Grindstaff, 2010, 2008; Grindstaff et al., 2003). But also, the social environment, food accessibility and disease expose can affect the immune status of a female and therefore the amount and types of antibodies transmitted (Chandra, 1975; Gasparini et al., 2001; Grindstaff et al., 2005, 2003; Lemke and Lange, 1999; Lundin et al., 1999; Michalek et al., 1975; Roulin and Heeb, 1999; Saino et al., 2002).

Social dominance

In most avian species, it has been observed that complex social structures exists within groups. Social animals living together establish dominance hierarchies. Dominance hierarchies could control e.g. sexual relations and access to resources within groups and dominance determines a part of this social order. Dominance hierarchies can have a profound effect on the survival of many social birds (Ficken et al., 1990; Noble, 1939; Sheppard et al., 2013). In previous experiments, dominance hierarchies in Japanese quails have been studied. According to several researches, dominance hierarchies in Japanese quail are of the peck order type (Boag and Alway, 1981; Masure and Allee, 1934; Mills et al., 1997; Nol et al., 1996; Edward, 2018). The priority of Japanese quail is gaining access to resources. According to Boag et al 1981, dominance hierarchies in Japanese quail have a genetic basis. Parents of high dominance rank were more likely to produce high dominance offspring than parents of low dominance rank (Boag and Alway, 1981). Thus, both genotype and phenotype has an effect on the dominance status of an individual (Nol et al., 1996).

Aim of the study

The goal of this research is to determine whether there is a relation between female social dominance and yolk IgY antibody concentration in Japanese quails. We expect that female Japanese quails living in social groups become more stable in their dominance rank and that this may be reflected in the IgY concentrations of their eggs. During this research we also hope to get a better insight in the relation between female body weight - IgY concentrations and the relation between yolk mass - IgY concentrations. Further, we investigated the variation in IgY concentration within and between the females. Finally, we investigated if there would be a change in IgY concentration over the period of several months.

The study therefore asks the following questions:

- Is there a relation between IgY concentrations and the dominance rank of the females? And would there be a change in IgY concentrations over a period of time?
- Is there be variation in IgY concentrations within and between the females?
- Is there a correlation between IgY concentrations and body weight of females?
- Is there a correlation between IgY concentrations and yolk mass?

Materials and methods:

Japanese quail (Coturnix japonica)

The Japanese quail, a terrestrial bird, belong to the order Galliformes and to the family of Phasianidae and is indigenous to China, Japan, Korea and Indochina (Ainsworth et al., 2010; Chang et al., 2009; Hubrecht and Kirkwood, 2010; Mills et al., 1997). Japanese quails originally bred as domestic songbirds, became popular for egg and meat production. Later around 1960, the Japanese quail became a common laboratory species, especially for hormonal, neurological and behavioural studies (Ainsworth et al., 2010; Hubrecht and Kirkwood, 2010; Mills et al., 1997; Shanaway, 1994). They are small in size, mature rapidly and are highly adaptive to a wide range of husbandry conditions, in comparison with other domesticated poultry. (Ainsworth et al., 2010; Mills et al., 1997). The species is sexually dimorphic, female Japanese quails are larger than male Japanese quails and they differ in plumage pattern. The plumage pattern is predominantly brown in both sexes, but differ in shades of brown and in some of the markings on the throat and breast (Cheng and Kimura, 1990; Mills et al., 1997; Shanaway, 1994).

Ethical statement

No animals were used for this experiment. Eggs were already collected in previous studies. The eggs were collected over several weeks and dominance rank of female Japanese quails was determined in a behavioural test. For this experiment eggs were collected and prepared for the analyses of IGG antibodies via Enzyme-Linked Immuno Sorbent Assay (ELISA). Non-invasive antibody and hormone assessments were performed to minimalize harm to the animals.

Dominance experiment

Japanese quail were housed at the experimental location (Central Animal Facility, Faculty of Veterinary Medicine at Utrecht University). After being part of a socialization experiment for one month, new social groups were formed and kept for two months. Each group consisted of one wildtype male, two wildtype females and one cream coloured female. Prior to the start of the dominance experiment, new groups were formed again. Directly afterwards, the first and second week of dominance testing started. To stabilize the groups, no experimental treatment had been carried out for three months. After the three months of stabilization, the experiment ended with the final two weeks of dominance testing (see figure 1). A set-up based on food competition was used to test for dominance between two females (Nol et al., 1996). One session took 10 minutes per group. At the end of one week all females were tested against each other. The dominance experiment was performed four times. The first two weeks were used to test for dominance in an unstable social environment and the last two weeks were used to test for dominance in a stable environment. Each female Japanese quail has been assigned to a dominance rank score based on the food competition test. The dominance ranks consist out of three categories: (1) most dominant, (2) intermediate and (3) subordinate (de Groot; van der Borgh; Smits, 2017).

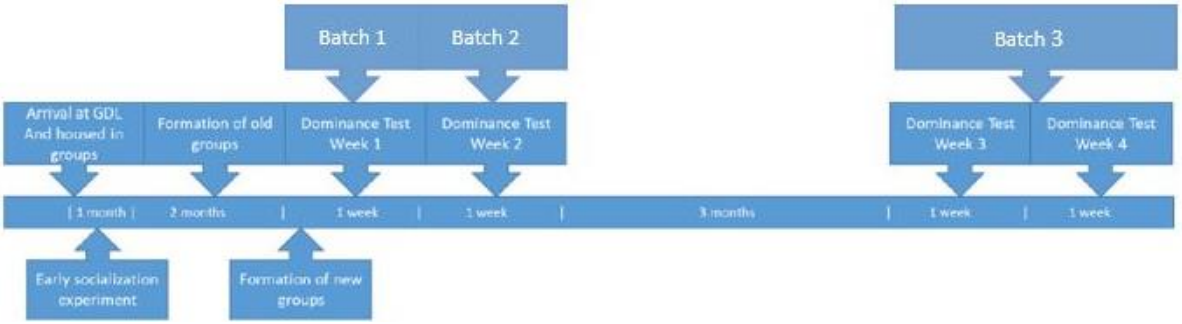

Figure 1: timeline of the dominance experiment. The dominance experiment was performed four times; week one, two, three and four. The eggs collected in the first week of the trial was named batch 1, the eggs collected in the second week of the trial was named batch 2 and the eggs collected in week three and four was named batch 3 (de Groot, 2017).

Japanese quail eggs

In the wild, Japanese quails reproduce during spring and summer. In laboratory circumstances, Japanese quail can be maintained in reproduction through the year if there is a sufficient long daylength (Ainsworth et al., 2010; Chang et al., 2009; Hubrecht and Kirkwood, 2010; Mills et al., 1997). Quail eggs vary in shell colour, mottling pattern, shape and size between females, but are consistent for an individual female (Mills et al., 1997; Sezer and Tekelioglu, 2009). The colour varies from white to blue and pale to dark brown. Japanese quail eggs weight about 10 gram and are 30mm in length. Compared to chicken eggs, Japanese quail eggs are small (Ainsworth et al., 2010; Hubrecht and Kirkwood, 2010; Mills et al., 1997; Shanaway, 1994). The three components that formed the egg are the albumen, yolk and shell. With an average weight of 10 gram; the quail egg consist of 57% albumen, 33% yolk and 10% shell (Panda and Singh, 1990). In addition, the age of the Japanese quail has influence on these physical characteristics of the egg (Yannakopoulos and Tserveni-Gousi, 1986).

Egg collecting and cross-linking to mother

In the first groups, eggs were collected for two weeks, to establish which egg belong to which female. Each group would have multiple eggs with 3 specific egg profiles. During the dominance experiment period, eggs were collected again for each week after each session of the dominance experiment. It was possible to define mother specific egg profiles, through cross-linking between the egg profiles from the old and new groups. Egg characteristic such as pattern, (yolk)weight, length, width and volume were written down. The eggs were categorized in three batches. The eggs collected in the first week of the trial was named batch 1, the eggs collected in the second week of the trial was named batch 2 and the eggs collected in week three and four was named batch 3 (see figure 1). The eggs and yolk mix were temporarily frozen (-20°C) for later analysis (de Groot; van der Borgh; Smits, 2017).

Protocol optimalisation

The collected eggs were prepared for the analyses of IGY antibodies via ELISA. The ELISA protocol has been preliminary established based on a published protocol (Okuliarova et al., 2014). For the complete protocol see attachment 1. The protocol has been refined to measure IgY concentrations of egg yolk from Japanese quail. The protocol contains components for both preparation of the yolk mix from frozen egg samples as well as the ELISA itself. ELISA protocols in various studies were compared. The main differences were the antibodies used to coat the 96 wells plate, the sample dilution, addition of the conjugate and the addition of reaction stopper. Blocking, washing and reading of the plate is about the same in each of the ELISA protocols compared (Grindstaff, 2013, 2010, Grindstaff et al., 2006, 2005; Okuliarova et al., 2014; Wakabayashi, 2010).

First, practice ELISA's were performed to test the protocol, to get experiences with pipetting, to produce a standard curve and to investigate intra- and inter assay variation. A standard curve is drawn, from graded results of various standard concentrations by using standard preparation and by plotting the standard concentration on the X-axis and the absorbance on the Y-axis. The absorbance of the sample dilutions of unknown concentration should fall within the range of the standard curve. Assay value is obtained by comparison of the sample dilution results with the standard curve.

In the previous experiment of de Groot and van der Borgh, 2017, were the collected eggs prepared until storage of yolkmix according to the ELISA protocol. To test if the yolkmix samples were not degraded, practice eggs and yolkmix of practice eggs have been prepared according to the yolkmix sample preparation and IgY isolation steps in the ELISA protocol. After it was established that the yolkmix samples were suitable for analysis, the yolk mix samples were used in several practice ELISA's. Various aspects of the protocol have been tested. First, different sample dilutions have been used in the practice ELISA's. Sample dilutions 1:50 and 1:500 were not suitable for use, because the ELISA values did not give good standard curves. Second, sodium hydroxide is added to stop the colour reaction and according to the protocol the reaction should be stopped at 19 min. However, the ELISA plate seemed already saturated at 19 min. At the next ELISAs, the colour reaction was stopped at 10 min. and 15 min. At 10 min. the ELISA values did not give a good standard curve, but at 15 min. the ELISA values did give a good standard curve. Third, different serially diluted standards were tested for the calibration line, but the dilutions according to the protocol gave the best outcome. Subsequently, ELISA's were performed with different amount of coating (30µl instead of 15µl) and with a refreezed and rethawed chicken IgY standard aliquots (TA.x). In both cases, no remarkable results were observed. Thus, the two major adjustments made in protocol are the adjustment of the sample dilution of 1:50 to 1:30, in which the 1:30 dilution proved to be most within the limits of the linear part of the standard curve and the adjustment of the stop of the colour reaction of 19 min. to 15 min.

Assay quality control

The 96 wells plates were placed into the ELISA reader and measured at 405nm and 650nm with the programme SoftMax pro 5.4. The optical densities (ODs) of the 96 wells plates were analysed by calculating averages, standard deviations and coefficients of variation (CVs). The CV is used to define the level of variability within a data set and to express the precision of the immunoassay results. The CV is divided in the intra-assay CV and inter-assay CV. The variance between sample replicates within the same plate, represents the intra-assay CV. The intra-assay variation was calculated by the standard deviation (STD) of the ODs multiplied by 100 and divided by the means of the ODs. Wells with an intra-CV value >10% were excluded from the analysis. The variance between sample replicates on different plates, represents the inter-assay CV and that can be used to estimate plate-to-plate consistency. The inter-assay variation was calculated by dividing the STD of the ODs means of each plate by the mean ODs of the plates and multiplying by 100. Wells should have inter-CV values <15% (de Groot, 2017; Conrad, 2018).

GraphPad Prism was used to constitute standard curves and get measured concentrations of each plate. The standard curves of the ELISAs were made by plotting the standard concentration on X-axis and the absorbance on Y-axis. Before analysis with GraphPad Prism concentrations of the standard dilutions were transformed to logarithmic values (de Groot; Conrad, 2018).

Protocol IGY ELISA

For the ELISA experiment 280 yolkmix samples had to be prepared. The yolkmix samples named egg IDs 1-162 and egg IDs N001-N123 were prepared according to the IgY isolation step in the ELISA protocol. After IgY isolation, the supernatants of the egg IDs were frozen (-20°C) in duplicate.

A distribution set-up was made for 10 ELISA's with the 280 yolkmix samples. For the complete set-up see attachment 2. Each ELISA plate had to meet the following requirements. Each individual ELISA plate

had to include all the eggs of one female Japanese quail, the three categories of the dominance ranks and the three categories of batches. And the amount of each of the three categories of dominance ranks, needed to be equally divided among the ELISA plates. Furthermore, each ELISA plates had to include blanks (blnk) to control for background, zero standards (B0) for negative control, total activities (TA) for positive control and IgY samples (quail ID 90) with known concentration for control between plates. The set-up of the distribution table is shown in table 1.

Ten 96-wells plates were coated with 100 µl anti-chicken IgY (Sigma-Aldrich, Cat. No. C6409) added to each well of the plates. 150µl undiluted anti-chicken IgY dissolved in 120 ml carbonate buffer (pH=9.6) was needed for 10 plates and to dilute 1:800. The plates were incubated overnight at 4 °C. Next day, incubated plates were washed three times with PBS / 0,05% Tween-20 (PBS-T) using a squirt bottle and 100 µl PBS / 2,5% milk-powder (PBS-M (2,5%)) blocking solution was added to each well on the plates. Then the plates were incubated for 2 h at 37 °C. The yolkmix supernatants were thawed during incubation and diluted 1:25.000 in PBS / 0,2% milk-powder (PBS-M (0,2%)). These dilutions were then diluted again in two steps. Step one: 2 µl yolkmix supernatant was transferred to 998µl PBS-M (0,2%) to dilute 1:500. Step two: 10 µl yolkmix supernatant was transferred to 290 µl PBS-M (0,2%) to dilute 1:30. For the IgY samples with known concentration 50µl yolkmix supernatant was transferred to 1450 µl PBS-M (0,2%) %) to dilute 1:30. For the standard dilution preparation, chicken IgY standard (Promega, Cat. No. G1161) was serially diluted in 8 steps from 400 pg/ml to 3.125 pg/ml. 60 µl chicken IgY standard (TA.x) diluted in 5940 µl PBS was needed for 10 plates. Prepared dilutions were stored at 4°C. Incubated plates were washed again one time with PBS-T using a squirt bottle and 100 µl of the standards dilution and sample dilutions was added to the each well on the plates. To the blank and B0 wells 100 µl PBS was added, to control for background and to control for effect of the buffer. To the TA wells 100 µl 40 µg/ml chicken IgY standard was added to control for 100% total activity. The TA wells should produce the highest possible colour reaction, because of the oversaturating of the anti IgY coating with IgY and the following maximum possible binding of the conjugate. The plates were incubated again at 37 °C for 1h. After incubation, the plates were washed again one time with PBS-T using a squirt bottle and 100 µl of the alkaline phosphatase conjugated anti-chicken IgY (Sigma-Aldrich, Cat. No. A9171) was added to each well of the plates except for the blank wells. To the blank wells 100 µl PBS was added. 120µl conjugated antibody dissolved in 120 ml TBS / 0,2% milk-powder (TBS-M (0,2%)) was needed for 10 plates and to dilute 1:1.000. The plates were incubated again at 37 °C for 1h. The incubated plates were washed for three times with TBS / 0,05% Tween-20 (TBS-T) using a squirt bottle and 200 µl p-nitrophenyl in substrate buffer (p-NPP) was added to each well on the plates. 5 ml 50x p-Nitrophenyl phosphate (Calbiochem, Cat. No. 487663) dissolved in 50 ml p-Nitrophenyl phosphate substrate buffer (Calbiochem, Cat. No. 487664) and 195 ml miliQ was needed for 10 plates. The plates were incubated in the dark for 15 min. at room temperature, because of the sensitivity of the colour reaction to light. To stop the reaction, 100 µl sodium hydroxide (1 M) was added to each well on the plates and absorbance (ODs) was measured by the ELISA reader at 405 nm and 650 nm. 405 nm was used to measure the OD and 650nm to control for background absorbance.

Table 1: standard distribution table for the ELISA with explanation of abbreviations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	1 IgY	1 IgY	9 IgY	9 IgY	17 IgY	17 IgY	25 IgY	25 IgY	B0	S1
B	Blnk	S2	2 IgY	2 IgY	10 IgY	10 IgY	18 IgY	18 IgY	26 IgY	26 IgY	B0	S2
C	B0	S3	3 IgY	3 IgY	11 IgY	11 IgY	19 IgY	19 IgY	27 IgY	27 IgY	B0	S3
D	B0	S4	4 IgY	4 IgY	12 IgY	12 IgY	20 IgY	20 IgY	28 IgY	28 IgY	B0	S4
E	TA	S5	5 IgY	5 IgY	13 IgY	13 IgY	21 IgY	21 IgY	29 IgY	29 IgY	IgY	S5
F	TA	S6	6 IgY	6 IgY	14 IgY	14 IgY	22 IgY	22 IgY	30 IgY	30 IgY	IgY	S6
G	Blnk	S7	7 IgY	7 IgY	15 IgY	15 IgY	23 IgY	23 IgY	31 IgY	31 IgY	IgY	S7
H	Blnk	S8	8 IgY	8 IgY	16 IgY	16 IgY	24 IgY	24 IgY	IgY	IgY	IgY	S8

Abbreviation	Definition	Description	Reason
Blnk	Blank	100 µl PBS (1x)	Control for background
A	Total activity	Add 100 µl 40 µg/ml TA solution instead of sample for maximum activity	Positive control
B0	Zero standard	Add 100 µl PBS (1x) instead of sample to control for no supposed activity	Negative control
S1-S8	Standard dilutions	Add 100 µl 1=400pg/ml, 2=200, 3=100, 4=50, 5=25, 6=12.5, 7=6.25, 8=3.125	Standard curve
Egg ID IgY	IgY sample	100 µl sample	Sample of interest
IgY	IgY sample	100 µl sample	Sample with known concentration → control between plates

Statistical analysis

For statistical analysis IBM SPSS statistics 24 was used. Before any statistical test has been carried out, the data was visually tested for normal distribution by histograms and normal probability plots. The data was also tested for normal distribution by tests of normality (Kolmogorov-Smirnov). For some of the results no statistical analysis have been performed.

To test if there is a relation between IgY concentration in yolk and the different dominance ranks, a variance analysis was performed. Parametric ANOVA test could be applied because the data was normally distributed and the group variances of the population were similar to each other (Test of Homogeneity of Variances). Before the ANOVA was performed, an average dominance rank for each female quail was calculated based on the individual dominance ranks, scored during the dominance experiment. Also, an average IgY concentrations were calculated based on the individual IgY concentration of the eggs of each female. The dependent variable of the One-Way ANOVA was the mean IgY concentration and the mean dominance rank was the independent variable.

To include the different batches, the One-Way ANOVA has been carried out three times, once for each batch. Before the ANOVA was preformed, an average IgY concentration per batch was calculated for each female based on the IgY concentrations corresponding to the three categories of batches. Data was selected on the corresponding batch. The dependent variable of the One-Way ANOVA was the mean IgY concentration per female per batch and the mean dominance rank was the independent variable.

Both mean quail weight and yolk mass have been analysed. First, correlation analysis has been performed for mean quail weight against mean IgY concentration and yolk mass against IgY concentration. Before the correlation was performed, an average body weight for each female was calculated based on the individual body weights of each female, recorded during the dominance experiment. The variables of the first correlation were the mean quail weight and the mean IgY concentration. The variables of the second correlation were the yolk mass and IgY concentration. Depending on whether the data was normally distributed, a parametric Pearson or a nonparametric Spearman correlation was performed.

After the correlations were carried out, another set of correlations were performed, to explore if there is a relation between IgY concentration and quail weight, in combination with the different batches and the different dominance ranks. So, three kinds of data were used; 1) the mean overall batches and ranks, 2) the mean per rank, but overall batches and 3) the mean per rank within batches. Correlations were performed, as described below, one for each batch. Before the correlations were performed, an average IgY concentration per batch was calculated for each female based on the IgY concentrations corresponding to the three categories of batches. And the body weight was also calculated for each female based on the body weight corresponding to the three categories of batches. Data was selected on the corresponding batch of dominance rank. The variables of the set of correlations were the mean quail weight per female per batch and the mean IgY concentration per female per batch. The same set of correlations have been carried out as described above, one for each dominance rank.

The last set of correlations were performed, to explore if there is a relation between IgY concentration and yolk mass, in combination with the different batches and the different dominance ranks. Correlations were performed, as described below, one for each batch. Data was selected on the corresponding batch of dominance rank. The variables of the set of correlations was the yolk mass and the IgY concentration. The same set of correlations have been carried out as described above, one for each dominance rank.

Results

Social dominance

To test if there is a relation between IgY concentration in yolk and the different dominance ranks, a variance analysis was performed, averaging IgY concentration per female over all eggs from all batches. There was no statistically significant difference between groups as determined by one-way ANOVA. Thus, there was no statistically significant difference between the mean IgY concentrations and the different dominance ranks of the females ($F_{2,29}$=1.22, P=0.31).

Females in the intermediate rank showed the largest spread in mean IgY concentrations. Female in the subordinate rank also showed a large spread in mean IgY concentrations. For both the intermediate and subordinate rank, the data is negatively skewed. The mean IgY concentrations of the dominant females are normally distributed. This is shown in figure 2.

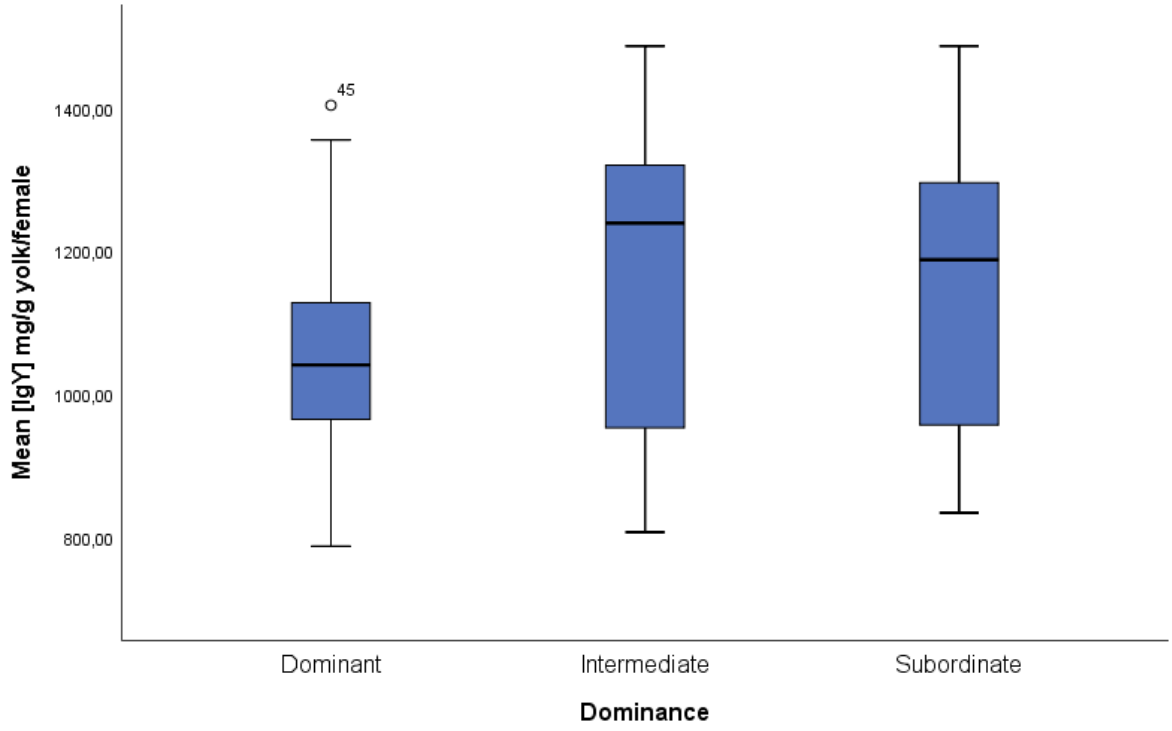

Figure 2: Boxplot of the three categories of the dominance rank against the mean IgY concentration mg/g per female per batch.

There was also no statistically significant difference between the mean IgY concentrations and the different dominance ranks of the females with the separate ANOVA analyses for the different batches. $ANOVA_{batch1}$; ($F_{2,26}$=0.57, P=0.57), $ANOVA_{batch2}$; ($F_{2,27}$=0.63, P=0.54) and $ANOVA_{batch3}$; ($F_{2,26}$=1.87, P=0.18).

Changes in IgY concentrations over time

Females in batch 1, 2 and 3 showed spread in mean IgY concentration. In comparison to batch 1 and 2, batch 3 showed the largest spread in mean IgY concentration. The females in batch 2 presented mean IgY concentrations by values for the mean that are higher than the median, thus the data in batch 2 is positively skewed. The mean IgY concentrations in batch 1 and 3 are approximately normally distributed. This is shown in figure 3.

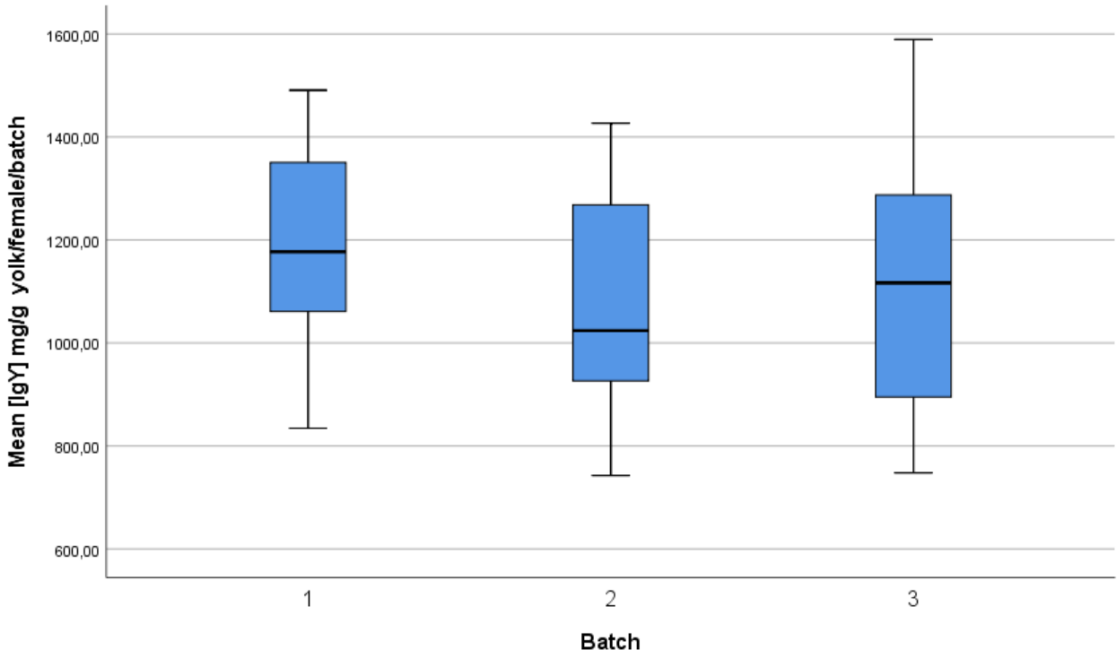

Figure 3: Boxplot of the three different batches against the mean IgY concentration mg/g per female per batch.

The line chart in figure 4A shows the changes in mean IgY concentration of the female Japanese quails across the three batches, thus a period of four months. In each dominant rank the individual females show changes in IgY concentrations over time. Some quail seem to decrease in IgY concentrations over a period of time, while other seem to increase in IgY concentrations over a period of time. And figure 4B shows that there is individual variation in IgY concentration in the eggs of the females and that the variation in IgY concentration between the females is bigger than the variation in IgY within the females.

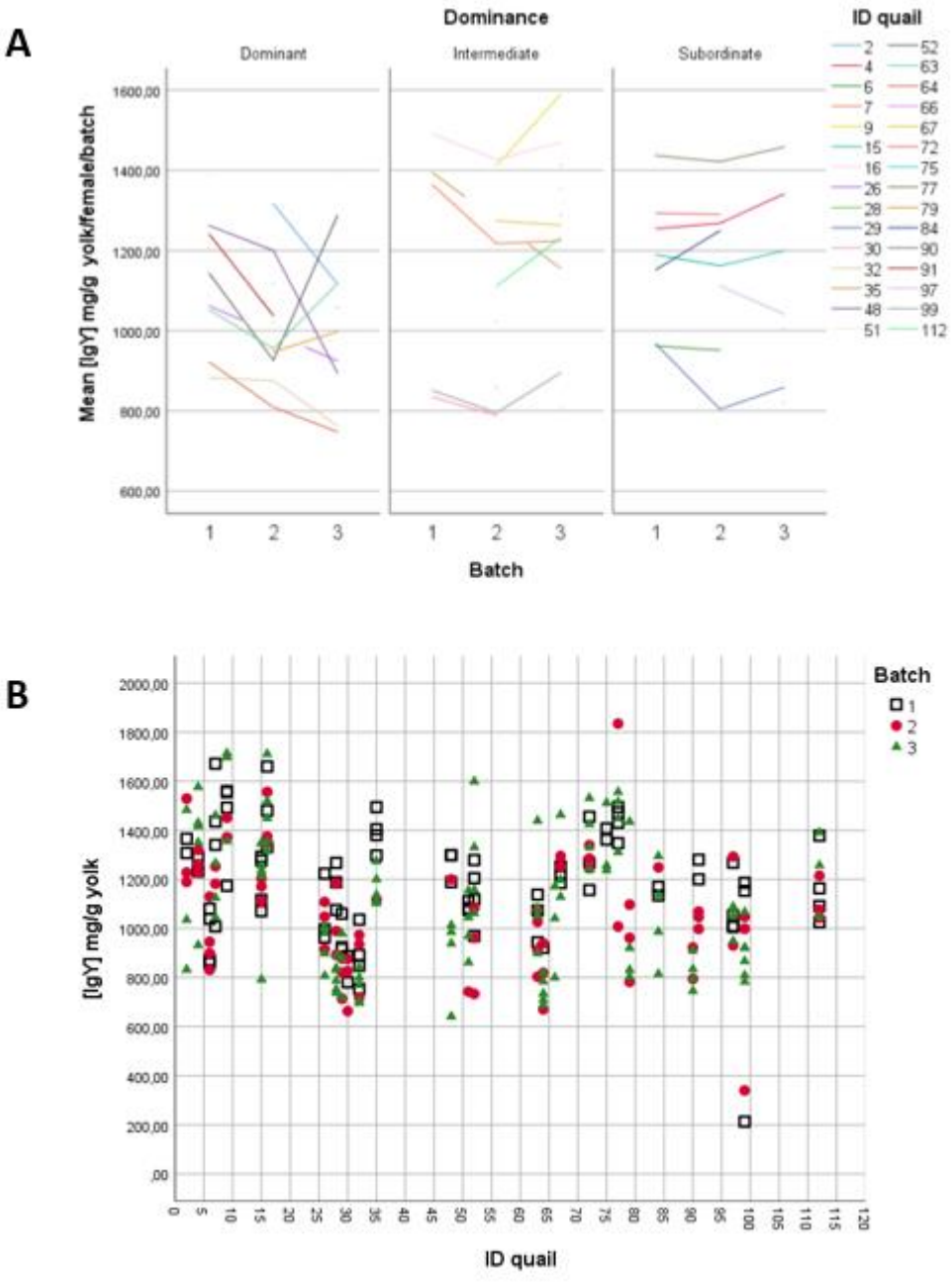

Figure 4: A) Line chart of the three different batches against the mean IgY concentration mg/g per female per batch categorized by quail ID's. Divided into dominance rank. Some of the 'ID quail' lines stop because several female quails change in dominance rank during the dominance experiment. B) IgY concentrations mg/g yolk plotted against ID quail categorized by batch.

Correlations of IgY

Irrespective of rank and batch, we found no significant correlation between mean quail weight and IgY concentrations, r= -0.22, N=30, p= 0.91. But we found a negative correlation between yolk mass and IgY concentration, again irrespective of rank and batch, which was statistically significant, r= -0.25, N=271, p< 0.01. Thus, with increasing yolk mass, IgY concentrations decrease slightly.

To learn more about possible correlations between mean quail weight and yolk mass against (mean) IgY concentrations, the different batches have also been included into the analysis. Thus, a second correlation analysis has been performed for mean quail weight per batch against mean IgY concentration per batch, once for each batch. Batch 1 showed a negative correlation between mean quail weight and mean IgY concentration, which was statistically significant, r_s= -0.27, p= 0.01. Thus, with increasing mean quail weight, mean IgY concentrations decrease slightly. There was no significant correlation between mean quail weight and mean IgY concentrations for batch 2 and 3, r_s= -0.13 and 0.10, p= 0.29 and 0.28. Figure 5 summarizes the results.

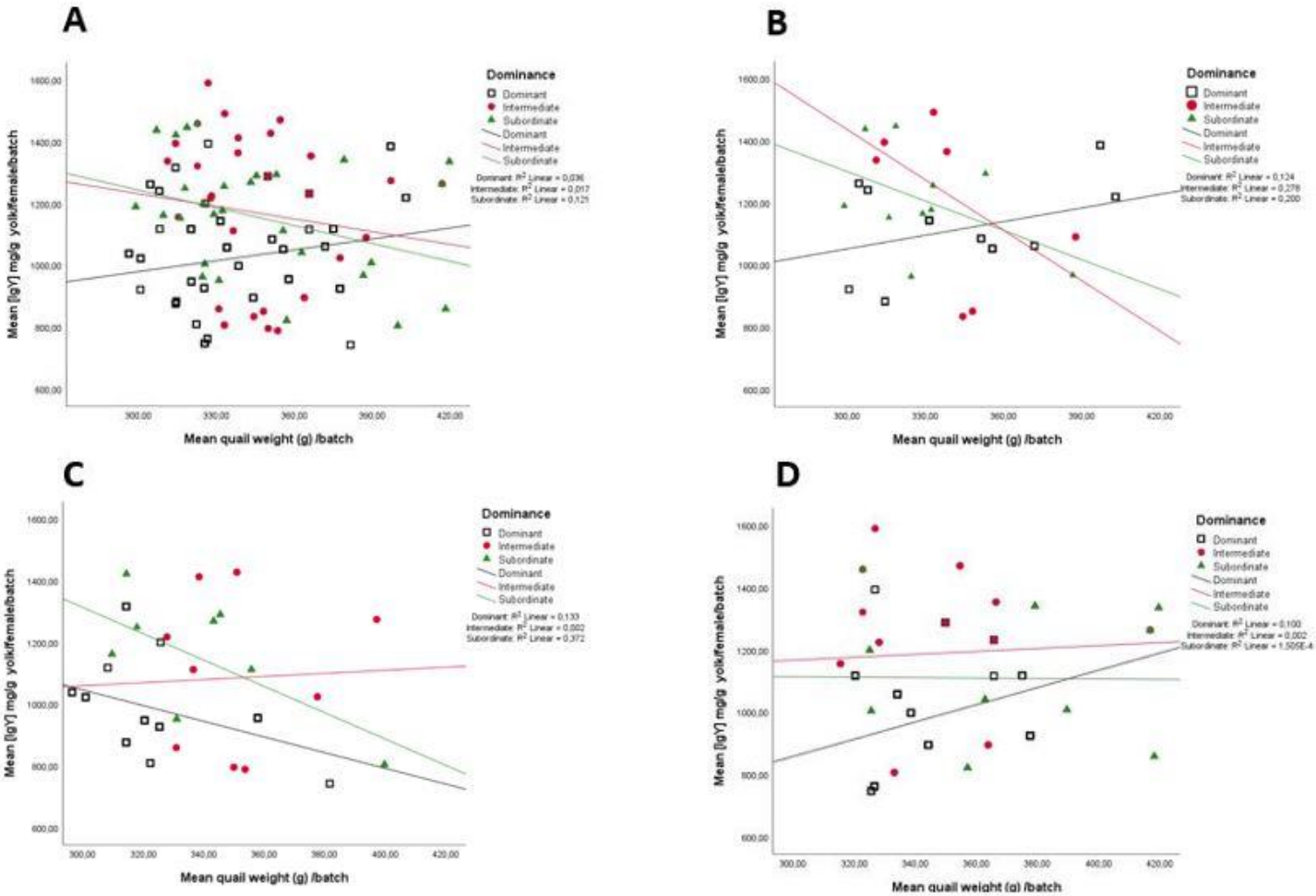

Figure 5: A) Mean quail weight (g) /batch plotted against mean IgY concentration mg/g yolk/female/batch categorized by dominance rank. B) Mean quail weight (g) /batch plotted against mean IgY concentration mg/g yolk/female/batch for batch 1 categorized by dominance rank. C) Mean quail weight (g) /batch plotted against mean IgY concentration mg/g yolk/female/batch for batch 2 categorized by dominance rank. D) Mean quail weight (g) /batch plotted against mean IgY concentration mg/g yolk/female/batch for batch 3 categorized by dominance rank.

Third, correlation analysis has been performed for yolk mass against IgY concentration, once for each batch. There was no significant correlation between yolk mass and IgY concentrations for batch 1 and 2, r= -0.17 and -0.20, N= 84 and 73, p= 0.13 and 0.08. There was a negative correlation between yolk mass and IgY concentration for batch 3, which was statistically significant, r_s= -0.27, p= 0.03. Thus, with increasing yolk mass, IgY concentrations decrease slightly. Figure 6 summarizes the results.

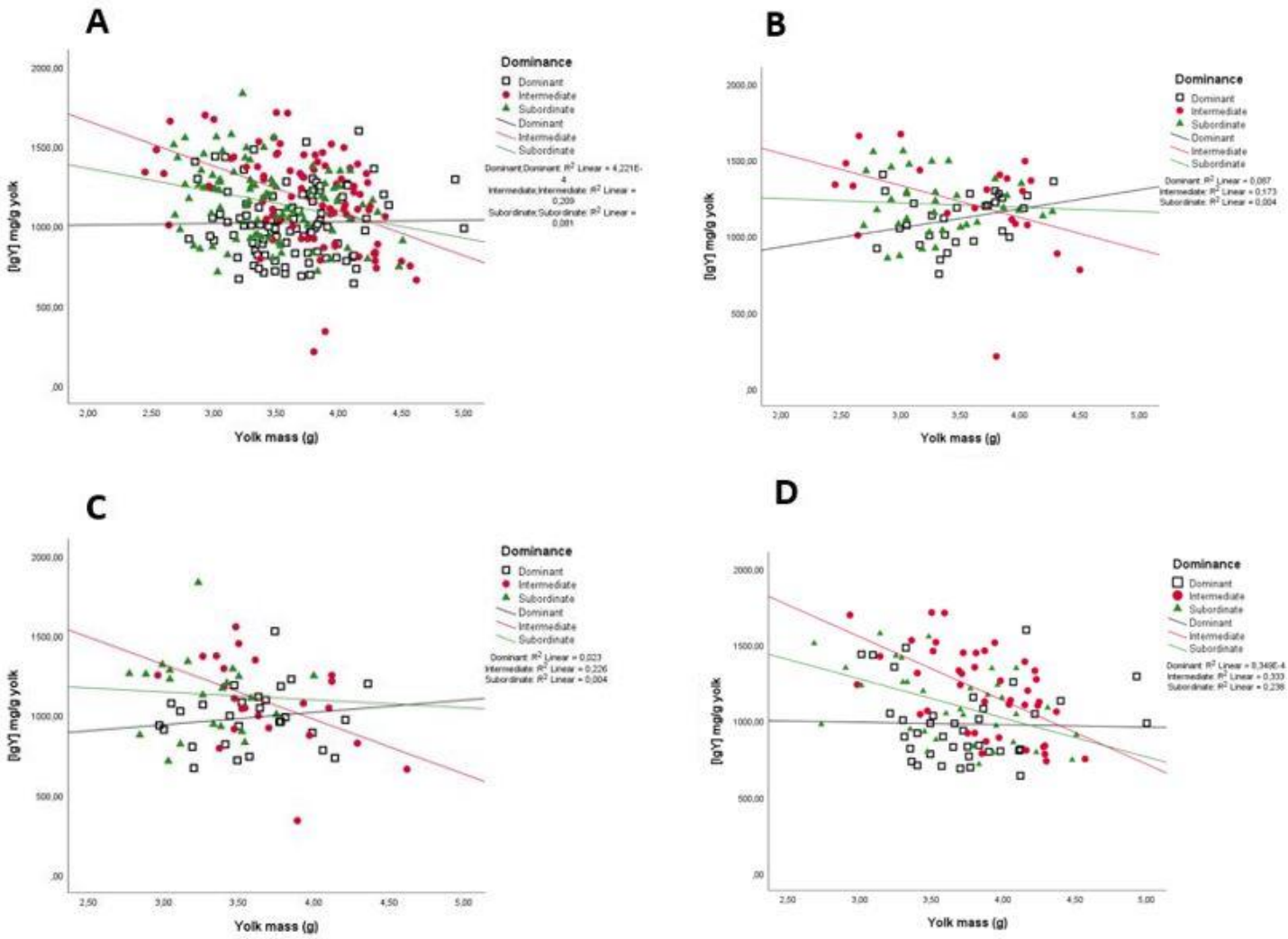

Figure 6: A) Yolk mass (g) plotted against IgY concentrations mg/g yolk categorized by dominance rank. B) Yolk mass (g) plotted against IgY concentrations mg/g yolk for batch 1 categorized by dominance rank. C) Yolk mass (g) plotted against IgY concentrations mg/g yolk for batch 2 categorized by dominance rank. D) Yolk mass (g) plotted against IgY concentrations mg/g yolk for batch 3 categorized by dominance rank.

The different dominance ranks of the female Japanese quail have also been included into the analysis. Correlation analysis has been performed for mean quail weight per batch against mean IgY concentration per batch, once for each dominance rank. There was no significant correlation between mean quail weight and mean IgY concentrations for dominance rank 1 and 2, r_s= 0.14 and -0.13, p= 0.19 and 0.23. There was a negative correlation between mean quail weight and mean IgY concentration for dominance rank 3, which was statistically significant, r_s= -0.30, p= 0.04. Thus, with increasing mean quail weight, mean IgY concentrations decrease slightly.

Last, correlation analysis has been performed for yolk mass against IgY concentration, also once for each dominance rank. There was no significant correlation between yolk mass and IgY concentrations for dominance rank 1, r= 0.21, N= 95, p= 0.84. The female Japanese quails with dominance rank score 1, appear to be relatively stable in IgY concentration in relation to yolk mass. There was a negative correlation between yolk mass and IgY concentration for dominance ranks 2 and 3, which was statistically significant, r= -0.46 and -0.29, N= 86 and 90, p= 0.00 and 0.01. For both dominance rank 2 and 3; with increasing yolk mass, IgY concentrations decrease slightly.

Discussion

IGY ELISA optimalisation

The protocol to quantify yolk IgY by ELISA was tested and refined by performing different test ELISA's. The two major adjustments made in the protocol were adjusting sample dilution of 1:50 to 1:30 and adjusting the stop of the colour reaction of 19 min. to 15 min. The test ELISA's proved that the protocol could be used to measure IgY concentrations in the eggs of the female Japanese quails reliably, as the CV's were below 15%, constituting a low degree of between sample variation.

Regarding the sample preparations, it is important to keep in mind that it takes time to thaw and dilute the yolkmix supernatants. On the day of the ELISA experiment 280 sample dilutions had to be prepared and these preparations took more time than expected. It is possible that this could have an influence on the results because of how long the preparations took and the possible decrease in accuracy over time. It would have been easier if the sample dilutions could be prepared in advance, but other research pointed out that thermal denaturation of standards already occurs when kept overnight at 4 °C instead of -20 °C (de Groot, 2017). This probably also applies to sample dilutions, so it is preferred to prepare the dilutions on the day of the experiment, but this could be tested in future experiments.

One point of concern was that the yolkmix samples were stored over a longer period of time. The yolk mix samples are almost one year old and according to chapter eight of the research of Shanaway (1994), the yolk and albumen of the eggs retain the same quality for only 60 days and remained acceptable for 78 days, if stored at 5 °C. Good oil-coated quail eggs could be retained for 120 days, if stored in a refrigerator. To test if the yolkmix samples can still be used, practice eggs and yolkmix of practice eggs were prepared according to the yolkmix sample preparation and IgY isolation steps in the ELISA protocol. It was established that the yolkmix samples are suitable for analysis. It could still be possible that the storage period of the yolkmix samples has an influence on the results of this study and this factor should be taken into consideration in further research.

Effects of dominance

The main aim of this study was to determine whether there is a relation between female social dominance and yolk IgY antibody concentration in Japanese quails. Second, we were interested if there would be a change in IgY concentration over a period of several months, because we expected that female Japanese quails living in social groups become more stable in their dominance rank and that this may be reflected in the IgY concentrations of their eggs.

The results showed that there was no statistically significant difference between the mean IgY concentrations and the different dominance ranks of the females. The effect of the different batches on the yolk IgY concentrations of the females was also taken into consideration, but also here the results showed that there was no statistically significant difference between the mean IgY concentrations and between the different dominance ranks of the females. These results indicate that there is no relation between female social dominance and yolk IgY concentrations in Japanese quail.

Other results showed that the females of the intermediate rank varied the most in mean IgY concentrations and the dominant rank varied the least. In addition, the females in batch 3 showed the largest spread in mean IgY concentrations and the females in batch 1 and 3 showed normally distributed IgY concentrations. In that case, the most dominant females are the most stable in IgY antibody concentrations in relation to the different batches, compared to the intermediate and subordinate females. Because variation in IgY concentrations is shown by the females in the different dominance ranks, it could be said that there is a possible link between dominance and IgY concentrations.

It is also possible that the results of IgY concentrations in relation to the dominance rank are not completely accurate, because of the changes in dominance rank during the dominance experiment of some female quails and some of the dominance scores given to the female quails were based on (strong) presumption instead of certainty. According to van der Borgh (2017), there could be an indication that Japanese quail do have a continuing stable ranking in dominance and it could be possible that Japanese quail have other dominance hierarchies than the linear one, like a triangular structure, which makes it complicated to determine dominance ranking (Bayly et al., 2006). The abovementioned factors could all have an influence on the interpretation of the results.

Effects over time

The results showed that IgY concentration in the eggs of females changes over time and the changes in IgY concentrations of the individual females were present in each dominance rank. Some of the females seem to decrease in IgY concentrations over a period of time, while others seem to increase in IgY concentrations over a period of time.

Previous studies have shown that living in unstable social environments increase the amount of stress in Japanese quail and it is thereby less profitable for animals to reproduce (Guibert et al., 2010). Other studies have shown that females living in unstable social environments lay eggs with higher antibody and testosterone concentrations than females living in stable social environments (Dixon et al., 2016). It was also shown that chicks of unstable females hatch later, developed more slowly and have higher emotional reactivity than chicks of stable females. The influence of females via yolk steroid content of their eggs could be a possible attempt to adapt the offspring to a stressful environment (Dixon et al., 2016; Guibert et al., 2010). This is in contradiction with the results of this study, because the females in batch 1 and 2 who represented the unstable social environment did not evidently show higher antibody concentrations in their eggs in comparison to the females in batch 3 who represented the stable environment. This also could not explain why the females in the stable environment showed the largest spread in antibody concentrations. More research needs to be done to become aware of the effect of time and the possible link to IgY concentrations.

Another aim of this study was to determine the variation in IgY concentration within and between the females. The results showed that there is individual variation in IgY concentration in the eggs of the females and that the variation in IgY concentration between the females is bigger than the variation in IgY within the females. This might be due to individual differences in the reproductive cycle of the females. In other researches yolk antibody concentrations also seemed to show high individual variability and it also differ between stages of the reproductive cycle (Coakley et al., 2014; Meer and Oers, 2015; Okuliarová et al., 2009)

Correlations of IgY

The last aim of this study was to determine whether there is a relation between IgY concentrations and the body weight of female Japanese quails and to determine whether there is a relation between IgY concentrations and the yolk mass. The results showed that there was no statistically significant correlation between mean body weight and IgY concentrations, but there was a statistically significant correlation between yolk mass and IgY concentration.

Although, the results showed no overall correlation between mean body weight and mean IgY concentration of the females, a statistically significant correlation was found for the females in batch 1. This indicates that the mean IgY concentrations of the females slightly decrease, with the increasing of mean body weight. This is in contrast with a previous study in which, positive correlations have been found between maternal body condition and IgY yolk levels, whereby the body condition of the females were established as the residual of a linear regression of body mass on tarsus length (Hargitai et al., 2006). On the other hand, an overall significant correlation was found between yolk mass and IgY concentration and besides that a significant correlation has been found between yolk mass and IgY

concentration for the females of batch 3. This indicates that the IgY concentrations of the females slightly decrease, with the increasing of yolk mass. The results could indicate that there is a stronger relation between IgY concentrations and yolk mass than between female body weight and IgY concentrations.

The different dominance ranks of the female Japanese quail have also been included into the analysis. As been said, the results showed no overall correlation between mean body weight and mean IgY concentration of the females, but a statistically significant correlation has been found for the females of dominance rank 3. In previous studies a correlation has been found between body weight and dominance in mammals and some avian species, and it could possibly apply for Japanese quails as well (Agnvall et al., 2014; Weiß et al., 2011). According to van der Borgh (2017), the size of the female could possibly be of influence on the dominance rank and the development of offspring, because of the possibility that the intermediate and mostly the subordinate females were allowed less access to the food dispenser than the dominant females, and as a result remain smaller. Another explanation of the possible dominance rank effect could be that the dominant females are most able to obtain good nutrition and therefore have the best ability to invest in reproduction and therefore are most stable in IgY antibody concentrations in relation to body weight. The effect of maternal diet quality on both female mass and investment in reproduction e.g. number and size of eggs, has been found, but the proof of that the reduced protein intake has an effect on the maternal antibody transmission to the eggs, has not yet been found (Grindstaff et al., 2005). It could possibly be that only the combination of dominance rank and body weight is of influence on the IgY concentration, and therefore no overall link between mean quail weight and mean IgY has been found.

There was an overall statistically significant correlation between yolk mass and IgY concentration and a statistically significant correlation between yolk mass and IgY concentration for the females of dominance rank 2 and 3. For both the intermediate and subordinate females, it indicates that the IgY concentrations slightly decrease, with increasing yolk mass. It is possible that the intermediate and subordinate females adapt in IgY concentrations in relation to yolk mass. In addition, in the study of de Groot, was found that the intermediate females produce more yolk mass in comparison with the most dominant and subordinate females, who produced about the same amount of yolk mass. In that case, intermediate females could possibly have more resources to devote to egg production in comparison with the subordinate females, because of the possibility of higher stress levels due to being lowest in hierarchy and the need to devote resources to defensive behaviour instead of egg production. The results could again indicate that there is a stronger relation between IgY concentrations and yolk mass than between female body weight and IgY concentrations and that there could be a possible link between dominance, yolk mass and IgY concentrations. Further research could look into this.

Statistical analysis

Variance analysis were performed to test if there is a relation between IgY concentration in yolk and the different dominance ranks and several correlation analysis were performed to test for correlations between mean quail weight and mean IgY concentrations and between yolk mass and IgY concentrations. To include the different batches and different dominance ranks, analysis have been carried out multiple times, once for each batch and once for each dominance rank. For further analyses, linear mixed models could be used to include more factors into the analysis. A disadvantage of this is that the female quails differ in number of eggs laid and not all females have eggs representing the three different batches. This could complicate the execution of the linear mixed models. Next to the other recommendations, several factors should also be analyzed, such as the possible interactions between egg volume, social dominance, yolk steroid concentrations (testosterone, progesterone) and IgY concentrations.

Conclusion

No significant relation was found between female social dominance and yolk IgY antibody concentration in Japanese quail, but it could be said that there is a possible link between dominance and IgY concentrations, because of the variation in IgY concentrations shown by the females in the different dominance ranks. Besides that, the individual females showed changes in IgY concentrations over time in each dominance rank. More research is necessary to gain knowledge about the effect of dominance and the effect of time and group stability and the possible link to IgY concentrations. Furthermore, individual variation in IgY concentration was found in the eggs of the females. There was no correlation found between mean body weight and IgY concentrations, but there was a statistically significant correlation found between yolk mass and IgY concentration. It could be possible that only the combination of dominance rank and body weight is of influence on the IgY concentration and that there is a stronger relation between IgY concentrations and yolk mass than between female body weight and IgY concentrations. Future research could look into the possible interactions between egg volume, social dominance, testosterone, progesterone and IgY concentrations.

Acknowledgements

First, I want to thank my supervisor Vivian Goerlich-Jansson for the opportunity to be a part of the Japanese quail study and for the advice during the analyses and writing. I would like to thank dr. Susanne Kirchhoff and Judith Hendriks for the supervision, lab assistances and many advices. Especially dr. Susanne Kirchhoff who has been a big part of the ELISA experiment. I would also like to thank student Roy van Stralen for helping my tremendously during the ELISA experiment. Finally, I am also grateful for the statistic help and advice from Esther Langen.

Attachments

1. Protocol Quail Yolk IgY ELISA

Yolkmix sample preparation

- Take eggs out of the freezer and let them thaw for ~10 min.
- Record the weight (0,01 g), length and width of the eggs in the meantime
- Label 2x 1,5 ml Eppendorf tubes: one with egg ID and "emb" and one with egg ID and "emb+".
- Store at -20 °C for later possible DNA analysis
- Crack open egg in petri dish using tweezers
- If fertilized, remove the embryo using clean tweezers and place it in an Eppendorf tube labeled "egg ID emb" and prepare "egg ID emb+" by adding a small segment of the embryo to the miliQ
- Wait 10 min. to thaw the egg enough that the albumen is soft and the yolk still frozen.
- Take out the yolk and clean off the rests of the albumen by rolling the yolk on a paper towel
- Place the egg yolk in a 50 ml falcon tube and record the weight of the yolk
- Let the yolk thaw completely, then add the appropriate amount of PBS (1x)
    - Egg yolk 2-3 g → 4 ml PBS
    - Egg yolk 3-4 g → 6 ml PBS
    - Egg yolk 4-5 g → 8 ml PBS
    - Egg yolk 5-6 g → 10 ml PBS
- Add 6 glass beads to each falcon tube
- Shake manually for 1 min. and then multivortex repeatedly until the egg yolk is well mixed
- Multivortex setting 8, ~20 min.
- When the yolk is homogenized, pipette 1,5 ml yolkmix to two 2 ml Eppendorf tubes labeled "egg ID.1" and "egg ID.2"
- Freeze the two 2 ml Eppendorf tubes at -20 °C

IgY Isolation

- Take one 2 ml Eppendorf tube containing yolkmix sample out of the freezer and let it thaw
- Vortex before transferring 400 µl prepared yolkmix sample to a new 2 ml Eppendorf tube
- Add 200 µl PBS to the "egg ID + mix" samples to reconstitute the ratio of PBS to yolk from 1:1 to 2:1
- Vortex 30 sec.
- Add 600 µl chloroform to the "egg ID + mix" samples
- Vortex 30 sec.
- Centrifuge at 3.000 xg (5700 rpm in the small centrifuge) for 15 min. at 4 °C
- After centrifugation, transfer the upper water layer (containing proteins) to a new tube 2 ml tube
- Store "egg ID sup" at -20 °C in a box labelled with sample range, date, name

IgY ELISA

- Coating of the 96-wells plate
    - Add 12 ml carbonate buffer to a 15 ml falcon tube
    - Let [IgY #] thaw (30-60min) and write it down in the lab journal
    - Transfer 15 µl undiluted anti-chicken IgY to the 15 ml falcon tube to dilute 1:800
    - Vortex 30 sec.
    - Use the multipipet to transfer 100 µl of the solution to each well on the 96-wells plate
    - Use PlateSeal to seal of the plate
    - Use the platevortex for 1 min.

    - Incubate overnight at 4 °C
- Blocking of the plate
    - Wash the incubated plate 3x with PBS-T using a squirt bottle
    - Add 100 µl blocking solution (PBS-M (2,5%)) to each well
    - Put the seal back on platevortex 1 min.
    - Incubate 2 h. at 37 °C
- Standard dilution preparation
    - Unfreeze (±30min) a TA.x Eppendorf tube (1:100 standard)
    - Dilute to 40 ng/ml by transferring 10 µl TA.x to a new 1,5 ml tube and adding 1 ml PBS (1x)
    - Label the new tube "S1" and vortex 30 sec.
    - Serial dilute the S1-S8
        - Transfer 500 µl S1 to new 1,5 ml labelled S2 together with 500 µl PBS (1x) and vortex 30 sec etc.
    - Store S1-S8 at 4 °C
- Sample dilution
    - Unfreeze a 2 ml "egg ID sup" stock tube
    - Transfer 2 µl "egg ID sup" to a new 2 ml Eppendorf tube and add 998 µl PBS-M (0,2%) to dilute 1:500 and label new tubes "egg ID sup -1" and vortex 30 sec.
    - Transfer 10 µl "egg ID sup -1" to a new 2 ml Eppendorf tube and add 290 µl PBS-M (0,2%) to dilute 1:30 and label new tubes "egg ID IgY" and vortex 30 sec.
    - Store "egg ID IgY" until step 5.7 at 4 °C
- Addition of the samples and standards
    - Wash the incubated plate 1x with PBS-T using a squirt bottle
    - Follow a premade sample distribution table
    - Put the seal back on and platevortex for 1 min
    - Incubate 1 h. at 37 °C
- Addition of the conjugate
    - Wash the incubated plate 1x with PBS-T using a squirt bottle
    - Pour the 12 ml conjugated antibody diluted in TBS-M (0,2%) into a disposable reagent reservoir (25 ml)
    - Add 100 µl to each well (except the blnk → add 100 µl PBS (1x) to each blnk well)
    - Put the seal back on and platevortex for 1 min
    - Incubate 1 h. at 37 °C
- Colour reaction
    - Wash the incubated plate 3x with TBS-T using a squirt bottle
    - Add 200 µl diluted p-NPP to each well
    - Put seal back on and platevortex in the dark for 1 min.
    - Incubate in the dark for 15 min. at room temperature
    - Add 100 µl sodium hydroxide (2 M) to each well after incubation to stop the reaction
    - Put the unsealed plate into the ELISA reader and measure absorbance at 405 nm and 650 nm
    - Save both the output (.pda) and export (.txt)
    - Use Prism to constitute a standard curve and get measured concentrations

(Okuliarova et al., 2014; Okuliarová et al., 2010, 2009)

2. Distribution tables set-up for the ten ELISA's

ELISA 1

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	3	3	28	28	3051	3051	105	105	Bo	S1
B	Blnk	S2	45	45	76	76	3070	3070	3017	3017	Bo	S2
C	B0	S3	95	95	77	77	3097	3097	3018	3018	Bo	S3
D	B0	S4	128	128	106	106	3122	3122	3050	3050	Bo	S4
E	TA	S5	129	129	107	107	10	10	3069	3069	IgY	S5
F	TA	S6	3005	3005	136	136	27	27	3096	3096	IgY	S6
G	Blnk	S7	3063	3063	158	158	53	53	3123	3123	IgY	S7
H	Blnk	S8	3088	3088	3016	3016	75	75	IgY	IgY	IgY	S8

ELISA 2

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	8	8	3093	3093	3033	3033	140	140	Bo	S1
B	Blnk	S2	25	25	3120	3120	3059	3059	161	161	Bo	S2
C	B0	S3	51	51	14	14	3081	3081	3023	3023	Bo	S3
D	B0	S4	73	73	37	37	32	32	3075	3075	Bo	S4
E	TA	S5	134	134	61	61	58	58	3101	3101	IgY	S5
F	TA	S6	156	156	86	86	81	81	3102	3102	IgY	S6
G	Blnk	S7	3014	3014	119	119	111	111			IgY	S7
H	Blnk	S8	3068	3068	145	145	112	112	IgY	IgY	IgY	S8

ELISA 3

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	19	19	3062	3062	159	159	68	68	Bo	S1
B	Blnk	S2	44	44	3085	3085	3015	3015	96	96	Bo	S2
C	B0	S3	67	67	3112	3112	3049	3049	130	130	Bo	S3
D	B0	S4	94	94	29	29	3095	3095	153	153	Bo	S4
E	TA	S5	127	127	54	54	3121	3121	3039	3039	IgY	S5
F	TA	S6	152	152	78	78	4	4	3086	3086	IgY	S6
G	Blnk	S7	3001	3001	108	108	20	20	3113	3113	IgY	S7
H	Blnk	S8	3038	3038	137	137	46	46	IgY	IgY	IgY	S8

ELISA 4

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	34	34	3028	3028	125	125	123	123	Bo	S1
B	Blnk	S2	83	83	3054	3054	150	150	148	148	Bo	S2
C	B0	S3	84	84	3077	3077	3037	3037			Bo	S3
D	B0	S4	113	113	3104	3104	3084	3084			Bo	S4
E	TA	S5	114	114	17	17	3111	3111			IgY	S5
F	TA	S6	141	141	42	42	40	40			IgY	S6
G	Blnk	S7	3026	3026	65	65	63	63			IgY	S7
H	Blnk	S8	3027	3027	92	92	90	90	IgY	IgY	IgY	S8

ELISA 5

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	6	6	3044	3044	88	88	3079	3079	Bo	S1
B	Blnk	S2	23	23	3066	3066	89	89	3109	3109	Bo	S2
C	B0	S3	49	49	3091	3091	122	122			Bo	S3
D	B0	S4	71	71	3115	3115	147	147			Bo	S4
E	TA	S5	99	99	1	1	118	118			IgY	S5
F	TA	S6	100	100	2	2	144	144			IgY	S6
G	Blnk	S7	155	155	16	16	3030	3030			IgY	S7
H	Blnk	S8	3008	3008	39	39	3057	3057	IgY	IgY	IgY	S8

ELISA 6

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	12	12	3105	3105	3076	3076	146	146	Bo	S1
B	Blnk	S2	35	35	3106	3106	3103	3103	3035	3035	Bo	S2
C	B0	S3	85	85	33	33	15	15	3058	3058	Bo	S3
D	B0	S4	115	115	59	59	38	38	3080	3080	Bo	S4
E	TA	S5	142	142	82	82	62	62	3110	3110	IgY	S5
F	TA	S6	3029	3029	162	162	87	87			IgY	S6
G	Blnk	S7	3055	3055	3024	3024	120	120			IgY	S7
H	Blnk	S8	3078	3078	3025	3025	121	121	IgY	IgY	IgY	S8

ELISA 7

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	9	9	3013	3013	151	151	3082	3082	Bo	S1
B	Blnk	S2	26	26	3046	3046	3002	3002			Bo	S2
C	B0	S3	52	52	3067	3067	3003	3003			Bo	S3
D	B0	S4	74	74	3092	3092	3036	3036			Bo	S4
E	TA	S5	104	104	3118	3118	3061	3061			IgY	S5
F	TA	S6	135	135	66	66	3083	3083			IgY	S6
G	Blnk	S7	157	157	93	93	3034	3034			IgY	S7
H	Blnk	S8	3012	3012	126	126	3060	3060	IgY	IgY	IgY	S8

ELISA 8

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	22	22	3090	3090	3032	3032	3098	3098	Bo	S1
B	Blnk	S2	48	48	13	13	3056	3056			Bo	S2
C	B0	S3	70	70	36	36	3107	3107			Bo	S3
D	B0	S4	98	98	60	60	3108	3108			Bo	S4
E	TA	S5	154	154	116	116	56	56			IgY	S5
F	TA	S6	3009	3009	117	117	79	79			IgY	S6
G	Blnk	S7	3043	3043	143	143	3019	3019			IgY	S7
H	Blnk	S8	3065	3065	3031	3031	3071	3071	IgY	IgY	IgY	S8

ELISA 9

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	7	7	3089	3089	3048	3048	3053	3053	Bo	S1
B	Blnk	S2	24	24	3116	3116	3094	3094	3073	3073	Bo	S2
C	B0	S3	50	50	102	102	3119	3119	3074	3074	Bo	S3
D	B0	S4	72	72	103	103	11	11	3100	3100	Bo	S4
E	TA	S5	101	101	133	133	30	30			IgY	S5
F	TA	S6	132	132	3010	3010	55	55			IgY	S6
G	Blnk	S7	3042	3042	3011	3011	109	109			IgY	S7
H	Blnk	S8	3064	3064	3047	3047	138	138	IgY	IgY	IgY	S8

ELISA 10

Well/number	1	2	3	4	5	6	7	8	9	10	11	12
A	Blnk	S1	41	41	69	69	57	57	3052	3052	Bo	S1
B	Blnk	S2	64	64	97	97	80	80	3072	3072	Bo	S2
C	B0	S3	91	91	131	131	110	110	3099	3099	Bo	S3
D	B0	S4	124	124	3007	3007	139	139			Bo	S4
E	TA	S5	149	149	3040	3040	160	160			IgY	S5
F	TA	S6	5	5	3087	3087	3020	3020			IgY	S6
G	Blnk	S7	21	21	3114	3114	3021	3021			IgY	S7
H	Blnk	S8	47	47	31	31	3022	3022	IgY	IgY	IgY	S8

References

Agnvall, B., Ali, A., Olby, S., Jensen, P., 2014. Red Junglefowl (Gallus gallus) selected for low fear of humans are larger, more dominant and produce larger offspring. Anim. Int. J. Anim. Biosci. 8, 1498–1505. https://doi.org/10.1017/S1751731114001426

Ainsworth, S.J., Stanley, R.L., Evans, D.J.R., 2010. Developmental stages of the Japanese quail. J. Anat. 216, 3–15. https://doi.org/10.1111/j.1469-7580.2009.01173.x

Al-Natour, M.Q., Ward, L.A., Saif, Y.M., Stewart-Brown, B., Keck, L.D., 2004. Effect of diffferent levels of maternally derived antibodies on protection against infectious bursal disease virus. Avian Dis. 48, 177–182. https://doi.org/10.1637/5319

Bayly, K.L., Evans, C.S., Taylor, A., 2006. Measuring social structure: a comparison of eight dominance indices. Behav. Processes 73, 1–12. https://doi.org/10.1016/j.beproc.2006.01.011

Boag, D.A., Alway, J.H., 1981. Heritability of dominance status among Japanese quail: a preliminary report. Can. J. Zool. 59, 441–444. https://doi.org/10.1139/z81-064

Bollen, L.S., Hau, J., 1999. Comparison of immunospecific antibody response in young and old chickens immunized with human IgG. Lab. Anim. 33, 71–76. https://doi.org/10.1258/002367799780578435

Borgh, V.D.H.M. van der, 2017. Social hierarchy and egg yolk testosterone deposition in Japanese quail (Coturnix Japonica). Master thesis Utrecht University.

Boulinier, T., Staszewski, V., 2008. Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol. Evol. 23, 282–288. https://doi.org/10.1016/j.tree.2007.12.006

Brambell, F.W.R., 1970. The transmission of passive immunity from mother to young. North Holl. Res. Monogr. Front. Biol. 18.

Bumstead, N., Reece, R.L., Cook, J.K., 1993. Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. Poult. Sci. 72, 403–410. https://doi.org/10.3382/ps.0720403

Cáceres, V.M., Strebel, P.M., Sutter, R.W., 2000. Factors determining prevalence of maternal antibody to measles virus throughout infancy: a review. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 31, 110–119. https://doi.org/10.1086/313926

Chandra, R.K., 1975. Antibody formation in first and second generation offspring of nutritionally deprived rats. Sci. USA 190, 289–290.

Chang, G.B., Liu, X.P., Chang, H., Chen, G.H., Zhao, W.M., Ji, D.J., Chen, R., Qin, Y.R., Shi, X.K., Hu, G.S., 2009. Behavior differentiation between wild Japanese quail, domestic quail, and their first filial generation. Poult. Sci. 88, 1137–1142. https://doi.org/10.3382/ps.2008-00320

Cheng, K., Kimura, M., 1990. Mutations and major variants in Japanese quail. Poult. Breed. Genet. 333–362.

Coakley, C.M., Staszewski, V., Herborn, K.A., Cunningham, E.J., 2014. Factors affecting the levels of protection transferred from mother to offspring following immune challenge. Front. Zool. 11, 46. https://doi.org/10.1186/1742-9994-11-46

Conrad, B., 2018. CV in ELISA: How to Reduce Them and Why They're Important [WWW Document]. URL http://www.enzolifesciences.com/science-center/technotes/2018/january/cv-in-elisa-how-to-reduce-them-and-why-they-re-important/ (accessed 4.26.18).

Dixon, L.M., Sparks, N.H.C., Rutherford, K.M.D., 2016. Early experiences matter: a review of the effects of prenatal environment on offspring characteristics in poultry. Poult. Sci. 95, 489–499. https://doi.org/10.3382/ps/pev343

Ficken, M.S., Weise, C.M., Popp, J.W., 1990. Dominance Rank and Resource Access in Winter Flocks of Black-Capped Chickadees. Wilson Bull. 102, 623–633.

Gasparini, J., McCoy, K.D., Haussy, C., Tveraa, T., Boulinier, T., 2001. Induced maternal response to the Lyme disease spirochaete Borrelia burgdorferi sensu lato in a colonial seabird, the kittiwake Rissa tridactyla. Proc. Biol. Sci. 268, 647–650. https://doi.org/10.1098/rspb.2000.1411

Gasparini, J., McCoy, K.D., Tveraa, T., Boulinier, T., 2002. Related concentrations of specific immunoglobulins against the Lyme disease agent Borrelia burgdorferi sensu lato in eggs, young

and adults of the kittiwake (Rissa tridactyla). Ecol. Lett. 5, 519–524. https://doi.org/10.1046/j.1461-0248.2002.00345.x
Goddard, R.D., Wyeth, P.J., Varney, W.C., 1994. Vaccination of commercial layer chicks against infectious bursal disease with maternally derived antibodies. Vet. Rec. 135, 273–274.
Graczyk, T.K., Cranfield, M.R., Shaw, M.L., Craig, L.E., 1994. Maternal antibodies against Plasmodium spp. in African black-footed penguin (Spheniscus demersus) chicks. J. Wildl. Dis. 30, 365–371. https://doi.org/10.7589/0090-3558-30.3.365
Grindstaff, J., 2013. IgG ELISA protocol.
Grindstaff, J.L., 2010. Initial levels of maternally derived antibodies predict persistence time in offspring circulation. J. Ornithol. 151, 423–428. https://doi.org/10.1007/s10336-009-0472-5
Grindstaff, J.L., 2008. Maternal antibodies reduce costs of an immune response during development. J. Exp. Biol. 211, 654–660. https://doi.org/10.1242/jeb.012344
Grindstaff, J.L., Brodie, E.D., Ketterson, E.D., 2003. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. Proc. R. Soc. Lond. B Biol. Sci. 270, 2309–2319. https://doi.org/10.1098/rspb.2003.2485
Grindstaff, J.L., Demas, G.E., Ketterson, E.D., 2005. Diet quality affects egg size and number but does not reduce maternal antibody transmission in Japanese quail Coturnix japonica. J. Anim. Ecol. 74, 1051–1058. https://doi.org/10.1111/j.1365-2656.2005.01002.x
Grindstaff, J.L., Hasselquist, D., Nilsson, J.-Å., Sandell, M., Smith, H.G., Stjernman, M., 2006. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proc. R. Soc. B Biol. Sci. 273, 2551–2557. https://doi.org/10.1098/rspb.2006.3608
Groot, D. de, 2017. The relation between dominance behaviours, body condition and egg characteristics in Japanese quail (Coturnix japonica). Master thesis Utrecht University.
Guibert, F., Richard-Yris, M.-A., Lumineau, S., Kotrschal, K., Guémené, D., Bertin, A., Möstl, E., Houdelier, C., 2010. Social instability in laying quail: consequences on yolk steroids and offspring's phenotype. PloS One 5, e14069. https://doi.org/10.1371/journal.pone.0014069
Hargitai R., Prechl J., Török J., 2006. Maternal immunoglobulin concentration in Collared Flycatcher (Ficedula albicollis) eggs in relation to parental quality and laying order. Funct. Ecol. 20, 829–838. https://doi.org/10.1111/j.1365-2435.2006.01171.x
Hasselquist, D., Nilsson, J.-Å., 2009. Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Philos. Trans. R. Soc. B Biol. Sci. 364, 51–60. https://doi.org/10.1098/rstb.2008.0137
Heller, E.D., Leitner, H., Drabkin, N., Melamed, D., 1990. Passive immunisation of chicks against Escherichia coli. Avian Pathol. J. WVPA 19, 345–354. https://doi.org/10.1080/03079459008418685
Hubrecht, R.C., Kirkwood, J. (Eds.), 2010. The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, 8 edition. ed. Wiley-Blackwell, Chichester.
Keller, L.F., Van Noordwijk, A.J., 1993. A Method to Isolate Environmental Effects on Nestling Growth, Illustrated with Examples from the Great Tit (Parus major). Funct. Ecol. 7, 493–502. https://doi.org/10.2307/2390037
Leitner, G., Melamed, D., Drabkin, N., Heller, E.D., 1990. An enzyme-linked immunosorbent assay for detection of antibodies against Escherichia coli: association between indirect hemagglutination test and survival. Avian Dis. 34, 58–62.
Lemke, H., Lange, H., 1999. Is there a maternally induced immunological imprinting phase à la Konrad Lorenz? Scand. J. Immunol. 50, 348–354.
Linder, N., Waintraub, I., Smetana, Z., Barzilai, A., Lubin, D., Mendelson, E., Sirota, L., 2000. Placental transfer and decay of varicella-zoster virus antibodies in preterm infants. J. Pediatr. 137, 85–89. https://doi.org/10.1067/mpd.2000.106902
Lundin, N., Dahlman-Höglund, N., Pettersson, N., Dahlgren, N., Hanson, N., Telemo, N., 1999. Antibodies Given Orally in the Neonatal Period Can Affect the Immune Response for Two

Generations: Evidence for Active Maternal Influence on the Newborn's Immune System. Scand. J. Immunol. 50, 651–656. https://doi.org/10.1046/j.1365-3083.1999.00651.x
Masure, R.H., Allee, W.C., 1934. The Social Order in Flocks of the Common Chicken and the Pigeon. The Auk 51, 306–327. https://doi.org/10.2307/4077659
Meer, E. van der, Oers, K. van, 2015. Gender and Personality Differences in Response to Social Stressors in Great Tits (Parus major). PLOS ONE 10, e0127984. https://doi.org/10.1371/journal.pone.0127984
Michalek, S.M., Rahman, A.F., McGhee, J.R., 1975. Rat immunoglobulins in serum and secretions: comparison of IgM, IgA and IgG in serum, colostrum, milk and saliva of protein malnourished and normal rats. Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y. N 148, 1114–1118.
Mills, A.D., Crawford, L.L., Domjan, M., Faure, J.M., 1997. The behavior of the Japanese or domestic quail Coturnix japonica. Neurosci. Biobehav. Rev. 21, 261–281.
Muggli, N.E., Hohenboken, W.D., Cundiff, L.V., Kelley, K.W., 1984. Inheritance of maternal immunoglobulin G1 concentration by the bovine neonate. J. Anim. Sci. 59, 39–48.
Munhoz, L.S., Vargas, G.D., Fischer, G., Lima, M. de, Esteves, P.A., Hübner, S. de O., 2014. Avian IgY antibodies: characteristics and applications in immunodiagnostic. Ciênc. Rural 44, 153–160. https://doi.org/10.1590/S0103-84782014000100025
Noble, G.K., 1939. The Rôle of Dominance in the Social Life of Birds. The Auk 56, 263–273. https://doi.org/10.2307/4079047
Nol, E., Cheng, K., Nichols, C., 1996. Heritability and phenotypic correlations of behaviour and dominance rank of Japanese quail. Anim. Behav. 52, 813–820. https://doi.org/10.1006/anbe.1996.0226
Okuliarova, M., Kankova, Z., Bertin, A., Leterrier, C., Mostl, E., Zeman, M., 2014. Maternally derived egg hormones, antibodies and antimicrobial proteins: common and different pathways of maternal effects in Japanese quail. PloS One 9, e112817–e112817. https://doi.org/10.1371/journal.pone.0112817
Okuliarová, M., Šárniková, B., Rettenbacher, S., Škrobánek, P., Zeman, M., 2010. Yolk testosterone and corticosterone in hierarchical follicles and laid eggs of Japanese quail exposed to long-term restraint stress. Gen. Comp. Endocrinol. 165, 91–96. https://doi.org/10.1016/j.ygcen.2009.06.007
Okuliarová, M., Skrobánek, P., Zeman, M., 2009. Variability of yolk testosterone concentrations during the reproductive cycle of Japanese quail. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 154, 530–534. https://doi.org/10.1016/j.cbpa.2009.08.012
Otis, R. E. Social organisation in the Japanese quail (Coturnix coturnix japonica): appetitive and consummatory components. Michigan State University: Unpublished Ph.D. dissertation; 1972.;
Panda, B., Singh, R.P., 1990. Developments in processing quail meat and eggs. World39s Poult. Sci. J. 46, 219–234. https://doi.org/10.1079/WPS19900022
Pardue, S.L., Ring, N.M., Smyth, J.R., 1990. Autosomal albinism affects immunocompetence in the chicken. Dev. Comp. Immunol. 14, 105–112.
Roulin, A., Heeb, P., 1999. The immunological function of allosuckling. Ecol. Lett. 2, 319–324. https://doi.org/10.1046/j.1461-0248.1999.00091.x
Sahin, O., Luo, N., Huang, S., Zhang, Q., 2003. Effect of Campylobacter-Specific Maternal Antibodies on Campylobacter jejuni Colonization in Young Chickens. Appl. Environ. Microbiol. 69, 5372–5379. https://doi.org/10.1128/AEM.69.9.5372-5379.2003
Saino, N., Ferrari, R.P., Martinelli, R., Romano, M., Rubolini, D., Møller, A.P., 2002. Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner. Proc. Biol. Sci. 269, 1005–1009. https://doi.org/10.1098/rspb.2002.1992
Sezer, M., Tekelioglu, O., 2009. Quantification of Japanese quail eggshell colour by image analysis. Biol. Res. 42, 99–105. https://doi.org//S0716-97602009000100010
Shanaway, M.M., 1994. Quail Production Systems: A Review. Food & Agriculture Org.

Shawkey, M.D., Kosciuch, K.L., Liu, M., Rohwer, F.C., Loos, E.R., Wang, J.M., Beissinger, S.R., 2008. Do birds differentially distribute antimicrobial proteins within clutches of eggs? Behav. Ecol. 19, 920–927. https://doi.org/10.1093/beheco/arn019

Sheppard, J.K., Walenski, M., Wallace, M.P., Velazco, J.J.V., Porras, C., Swaisgood, R.R., 2013. Hierarchical dominance structure in reintroduced California condors: correlates, consequences, and dynamics. Behav. Ecol. Sociobiol. 67, 1227–1238. https://doi.org/10.1007/s00265-013-1550-5

Smith, N.C., Wallach, M., Miller, C.M., Morgenstern, R., Braun, R., Eckert, J., 1994. Maternal transmission of immunity to Eimeria maxima: enzyme-linked immunosorbent assay analysis of protective antibodies induced by infection. Infect. Immun. 62, 1348–1357.

Smits, S., 2016. Egg preparation for embryo sexing and yolk hormone measurement. University of Utrecht protocols. November 2016.

Smits, S., 2017. Identifying female dominance structures in Japanese quail (Coturnix Japonica) and their impacts on egg characteristics. Master thesis Utrecht University.

Starck, J.M., Ricklefs, R.E., 1998. Avian Growth and Development: Evolution Within the Altricial-precocial Spectrum. Oxford University Press.

Wakabayashi, K., 2010. ELISA A to Z from introduction to practice.

Weiß, B.M., Kotrschal, K., Foerster, K., 2011. A longitudinal study of dominance and aggression in greylag geese (Anser anser). Behav. Ecol. 22, 616–624. https://doi.org/10.1093/beheco/arr020

Yannakopoulos, A.L., Tserveni-Gousi, A.S., 1986. Quality characteristics of quail eggs. Br. Poult. Sci. 27, 171–176. https://doi.org/10.1080/00071668608416870