Chapter 6

Multiple pathways of maternal effects in black-headed gull eggs: Constraint and mutual adjustment

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Prenatal maternal effects can profoundly shape offspring fitness, with egg size and quality being the most important effects for oviparous animals. Birds transfer multiple protective compounds to their eggs, such as antioxidants and immunoglobulins, that enhance neonatal disease resistance and early immune defence. These partly compensate for the incomplete functioning of the chicks’ immune system early after hatching. Avian mothers also deposit substantial amounts of androgens in their eggs that enhance the chicks’ competitiveness but which might be at the cost of immune function. We investigated in the black-headed gull whether female deposition of antioxidants (carotenoids and vitamin E), Immunoglobulin G (IgG), testosterone and egg mass correlate. We further analyzed their relationship with hatching asynchrony and offspring sex, which both play a key role in determining offspring survival. Egg mass, yolk antioxidant and yolk IgG concentrations decreased over the laying order, while yolk testosterone concentrations showed the reverse pattern. This suggests that the last-hatched chicks are substantially handicapped in their immune defence, in addition to the age and size disadvantage caused by hatching in a last position, which could be mitigated by the competitive advantage conferred by high testosterone concentrations. The yolk antioxidant, IgG and testosterone concentrations and egg mass were neither associated with each other nor with the sex of the embryo, no matter whether we analyzed this for individual eggs or for clutch averages. The decrease in antioxidant levels across the laying sequence was greater when the increase in testosterone concentrations was greater. Furthermore, the decrease in antioxidants over the laying sequence was greater when mothers had a low body mass at the time of clutch completion. Female black-headed gulls seem to be constrained in the deposition of high levels of antioxidants and perhaps IgG in later-laid eggs. Mothers may compensate for the lower quality of their last eggs by enhancing testosterone levels in these eggs, which is possibly a less costly investment for the mother. We suggest that this enhanced transmission of immunosuppressive testosterone may represent an adaptive maternal strategy to re-allocate the chick’s investment from costly immune function to growth and competitive skills necessary to overcome the consequences of hatching late from an egg of reduced quality.
Furthermore, transmission of maternal immunoglobulins has been documented in a variety of wild bird species and is positively correlated with nidicolous ectoparasite levels (Gasparini et al. 2001, Buechler et al. 2002). In domestic birds, the ability of the chick to raise an antibody mediated immune response develops during the first and second week post-hatch (Apanius 1998). If yolk androgens suppress neonatal antibody responses, then mothers may compensate for this potential cost by transferring an increased quantity of their preformed immunoglobulins into the egg.

Maternally transmitted antioxidants (particularly carotenoids and vitamin E) are critical for embryonic development as they protect growing tissues from oxidative damage including lipid peroxidation (Surai et al. 1999, Surai 2002). Pertinent to this study, antioxidants are widely believed to enhance immune function. For example carotenoids have been shown to enhance T-cell-mediated immunity early post-hatching in barn swallow chicks *Hirundo rustica* (Saino et al. 2003, for similar effect in adult zebra finches see Blount et al. 2003, McGraw and Ardia 2003). Maternal transfer of antioxidants to the yolk may also entail a cost for the mother. Antioxidants can only be obtained in the mother’s diet and their availability may thus be suboptimal (reviewed by Brush 1990), while deposition into the yolk reduces their availability for the maternal self-maintenance (von Schantz et al. 1999).

The size (mass) of an individual egg is directly related to the quantity of nutrients available for embryonic and post-natal growth, which in turn influences sibling rivalry in concert with hatching asynchrony. These nutrients determine energetic resources for growth as well as the development and expression of the immune system, both of which are energetically costly (reviewed by Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000). Egg size is likely to be traded off against maternal body condition (reviewed by Williams 1994, Christians 2002).

The sex of the offspring can also play a pivotal role in the maternal allocation, and possibly rates of utilization, of these egg components. In many avian species, early nestling mortality differs between male and female offspring and subsequently influences their fitness value to the parents. In gulls, the relationship between nestling survival and egg size and quality varies with the sex of the offspring (Nager et al. 1999, 2000).

We may expect that the deposition of immuno-suppressive androgens, and immuno-enhancing IgG, antioxidants and nutrients are adjusted to each other. They should also be adjusted to the need to mitigate hatching asynchrony and the risk of infectious diseases. The expectation that mothers compensate for immuno-suppressing effects of elevated androgens in their last-eggs by transferring more IgG and antioxidants to these eggs has not been supported by previous studies of the lesser black-backed gull *Larus fuscus*. Levels of androgens increased while those of carotenoids, vitamin E and maternal IgG decreased
with laying order (Blount et al. 2002, Royle et al. 2001). This suggests a negative association between immuno-suppressing and immuno-enhancing factors. These trends at the population level may not accurately reflect the co-variation of these parameters within individual eggs. Females may be physiologically constrained in their ability to vary these yolk components across the laying sequence within a clutch, but individuals may still differ in the transfer of these compounds within the whole clutch, as well as in the rate of increase or decrease across the laying sequence. In addition, they might vary the transfer of these compounds depending on the sex of the offspring.

We ask whether several prenatal maternal effects exhibit mutual adjustment. We measure within and between clutch variation in egg mass, yolk testosterone, IgG, carotenoids and vitamin E, as well as the relations between these variables and with hatching asynchrony, offspring sex and maternal body mass in the black-headed gull *Larus ridibundus*. This is an appropriate model organism, producing eggs that contain high levels of maternal androgens, which vary systematically between and within clutches (Eising et al. 2001, Groothuis and Schwabl 2002), and their functional consequences for growth and behavior have been convincingly demonstrated (e.g., Eising et al. 2001, Eising and Groothuis 2003). In this species immune-relevant egg components such as IgG and antioxidants may be particularly important as it breeds in extremely dense colonies in which the aggregation of large numbers of birds during the breeding season enhances the risk of infectious diseases (Brown & Brown 1986, Loye and Zuk 1991, Tella 2002). Indeed, concentrations of maternal IgG in the yolk are positively related to breeding density and presumably to the potential risk of infection (Müller et al. in press). Finally, early nestling mortality in this species is related to hatching asynchrony, T-cell-mediated immunity, and sex of the offspring (Müller et al. 2003).

**Material and Methods**

**(a) Study species and data collection**

Black-headed gulls are monogamous, colonial breeders. The clutch typically consists of three eggs, which are laid over a three- to five-day period (Cramp and Simmons 1983). In 2001, nests of several neighbouring black-headed gull sub-colonies (300-1000 breeding pairs) along the northeast coast of the Netherlands were checked once a day for egg laying. Freshly laid eggs were marked with non-toxic ink referring to the position within the laying order and laying date. We collected 20 complete clutches on the day of clutch completion. The eggs were weighed to the nearest 0.1 g and subsequently placed in an incubator at 37.5 °C with 60 % humidity to allow embryonic development, and then frozen at minus 20 °C. Since some incubation takes already place before clutch completion, the eggs were incubated differentially according to their laying position to approximately equalize the total incubation time (60h in case
of the first laid egg, 72h for the second-laid egg and 84h in case of the last-laid egg). This also reduced a potential effect of incubation on testosterone (Elf and Fivizanni, 2002, Eising et al. 2003), IgG (Kowalczyk et al. 1985) and antioxidant levels. Dummy eggs were used to maintain female incubation behavior so that we could capture the females (within three days of egg removal) to obtain body measurements (N=7).

(b) Egg analyses
The collected eggs were defrosted and the yolk and embryo separated. A small tissue sample of the embryo was used for Chelex® resin-based DNA extraction (Walsh et al. 1991). Two µl of the resulting DNA solution was used in a polymerase chain reaction (PCR) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (for details see Griffiths et al. 1998). The reliability of this method has been established in earlier studies on this species (e.g., Müller et al. 2003).

The yolks were homogenized for the analysis of hormones, IgG and antioxidants. In all cases all eggs of a clutch were analyzed in the same assay.

I. Hormone analysis
For hormone analysis, about half of the homogenized yolk was diluted with one ml water per gram of yolk and about 150 mg of this emulsion was used for hormone analysis. We followed a standard procedure according to Schwabl (1993), with a slight modification. Briefly, samples were extracted twice with 4 ml petroleum ether/diethylether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently, the hormones were separated on diatomaceous earth chromatography columns. Androgen concentrations were measured in double competitive-binding radioimmunoassays (RIA) with tritiated hormone (NEN, the Netherlands) and hormone-specific IgG (Endocrine Science, USA). The average recovery was 49.4 %, the inter-assay intra-assay variation was 4.2 %.

II. Immunoglobulin Assay
Immunoglobulin concentrations in plasma and yolk homogenate were determined after 10-fold dilution (w/w) with an anionic detergent buffer (0.33 % sodium dodecyl sulphate in 0.5 M Tris-HCl pH 6.8 and 10% glycerol). This is the standard sample buffer for protein separation using polyacrylamide gel electrophoresis (Harlow and Lane, 1999), which we used to resolve the spectrum of egg-yolk proteins. Gull IgG was identified on the basis of molecular weight of the denatured molecule and of the molecular weight of the subunits produced under reducing conditions as outlined in Apanius et al (1983). IgG concentration was measured with a quantitative Coomassie G-250 staining protocol (Neuhoff, 1988) using a standard curve based on purified chicken IgG (Sigma I4881) and
Multiple pathways expressed in mg/ml of serum and mg/mg of yolk. Randomly chosen serum (N=23 individual females) and yolk (N=36 eggs) samples were analyzed twice and the repeatability of the method (intraclass correlation coefficient) was estimated to be 0.977 (F 22,23= 45.18, p<0.0001) and 0.683 (F 35,36= 2.46, p<0.0032), respectively. In one clutch only two of the three eggs could be measured successfully, hence we excluded the complete clutch from the analysis of IgG.

III. Biochemical assays for Carotenoid analysis

An aliquot of yolk (~ 200 mg) was mixed with 0.5 ml 5 % sodium chloride by vortexing. Next, 1 ml ethanol was added to the mixture and homogenized for 20 s, then 2 ml hexane was added and the mixture was homogenized for a further 20 s. After centrifugation the lipophilic hexane phase was collected. Extraction using hexane was performed once more. The combined hexane phase was evaporated to dryness under a stream of nitrogen gas, then redissolved in 0.3 ml dichloromethane-methanol (1:1) ready for HPLC. For analysis of total carotenoids, samples (10 µl) were injected into an HPLC system fitted with a Spherisorb type S5NH2, 5µ C18 reverse-phase column (25 cm x 4.6 mm) (Phase Separations, Clwyd, UK) and a mobile phase of methanol-distilled water (97:3) at a flow rate of 1.5 ml min⁻¹. Carotenoids were identified as a single peak at 445 nm, and the concentration determined using lutein (Sigma-Aldrich, Poole, UK) in methanol as a standard. For analysis of vitamin E (α- and γ-tocopherol), samples (10 µl) were injected into an HPLC system fitted with a Spherisorb type S30DS2, 3µ C18 reverse-phase column (15 cm x 4.6 mm) (Phase Separations, Clwyd, UK), and a mobile phase of methanol-distilled water (97:3) at a flow rate of 1.05 ml min⁻¹. Fluorescence detection of vitamin E involved excitation and emission wavelengths of 295 nm and 330 nm, respectively, and the concentration determined in relation to solutions of α-tocopherol and γ-tocopherol (Sigma-Aldrich, Poole, UK) in methanol. Tocol was used as an internal standard. Concentrations are given in µg/g of yolk. Nineteen complete clutches were successfully analyzed.

(c) Statistical analyses

None of the data sets deviate significantly from a normal distribution. In the first approach we analyzed the within clutch allocation pattern using hierarchical linear models in the MLwiN program 1.10 (Rasbash et al. 2000). This method allows analyses of variance and covariance taking into account the nested relationship of different eggs in a nest and controls for multiple (independent) variables. Significance was tested using the increase in deviance (Δdeviance), when a factor was removed from the model, which follows a χ²-distribution (Wald statistic). Position in the laying sequence, offspring sex and their interaction were included as categorical predictors. Since we were interested in within nest effects of offspring sex independently from sex differences at the
total clutch level, the clutch sex ratio was included in the model too (Snijders and Bosker 1999).

In a second approach we investigated the potential relationships between levels of testosterone IgG, antioxidants and egg mass in individual eggs. To this end we calculated for each of these components the residuals over the laying sequence in the MLwiN program and subsequently applied parametric correlation analyses (Pearson, SPSS). In a third approach we correlated total clutch levels among the different yolk components.

Finally, we correlated the change over the laying order among the different egg components. The increase in testosterone levels was calculated as the concentration of last egg minus that of the first egg. The decrease in other components was calculated as the concentration of the first egg minus that of the last egg. Since the biological effect of a certain absolute change is very likely to be stronger in case of low levels than of high levels of that compound in the clutch, we calculated relative changes over the laying order by dividing the changes over the laying sequence by the concentration of the first egg (see Müller et al. in press).

Levels of vitamin E and carotenoids very strongly correlated and showed similar trends in all analyses. Therefore, and because of their similar antioxidant function, we used the sum of both concentrations as a measure of combined antioxidant concentrations in all these analyses.

The relation between body mass of mother gulls and clutch levels and relative changes over the laying sequence of the four egg components (n=7 except for antioxidant levels where n=6) was analyzed by means of Pearson correlations.

Results
(a) Variation of the separate components within clutches

Egg mass declined slightly with the laying sequence (fig 1a; laying sequence: Δdeviance 12.08, df=2, p=0.002), independent of sex, or sex in interaction with laying sequence (table 1; sex: Δdeviance 0.79, df=1, 0.37; sex x laying sequence: Δdeviance 0.30, df=2, 0.86). Post-hoc tests revealed that the second-laid egg was significantly heavier than the other eggs (post hoc, first vs. second: Δdeviance 4.59, df=1, p=0.03; second vs. third: Δdeviance 11.76, df=1, p<0.001; first vs. third: Δdeviance 2.42, df=1, p=0.11).

Yolk IgG concentrations decreased across the laying sequence (fig. 1b; laying sequence: Δdeviance 11.90, df=2, p<0.005). There was neither a significant difference in yolk IgG concentrations between male and female eggs (table 1; sex: Δdeviance 0.04, df=1, p=0.84) nor an effect of sex in interaction with laying sequence (sex x laying sequence: Δdeviance 2.88, df=2, p=0.24).
### Table 1

Table 1: Mean egg mass (+/- se) and mean concentration (+/- se) of all egg components analyzed in this study, separated for the laying position of the egg and sex of the embryo.

Yolk antioxidant concentrations consistently decreased from first to last laid egg (fig. 1c; laying sequence: Δdeviance 13.20, df=2, p<0.001). Again, neither sex nor the interaction between sex and laying sequence contributed significantly to the
explained variation in antioxidant titers (table 1; sex: Δdeviance 0.45, df=1, p=0.50; sex x laying sequence: Δdeviance 0.69, df=2, p=0.71). The significant decrease in laying order was present in all antioxidant components measured (total carotenoids: Δdeviance 18.68, df=2, p<0.001; vitamin E α-tocopherol: Δdeviance 14.61, df=2, p<0.001; vitamin E γ-tocopherol: Δdeviance 18.22, df=2, p<0.001). The separated values for all three components are shown in table 1.

Consistent with earlier studies in Black-headed gulls, yolk testosterone concentrations increased across the laying sequence (fig. 1d; laying sequence: Δdeviance 13.00, df=2, p=0.002). There were no significant associations between sex of the egg or sex in interaction with laying order and yolk testosterone concentration (table 1; sex: Δdeviance 1.66, df=1, p=0.20; sex x laying sequence: Δdeviance 0.19, df=2, p=0.91).

Figure 1: (a) Mean egg mass (+/- se) and mean concentrations (+/- se) of (b) IgG, (c) testosterone and (d) antioxidants in relation to the position of the egg in the laying sequence (a-egg= first-, b-egg= second- and c-egg=third-laid egg)
(b) Covariation among components.
There was no statistically significant correlation among the residuals over the laying sequence of any of the four egg components presented in figure 1 (Table 2, first two columns). This was also the case for variation at the level of the total clutch (Table 2, column 3 and 4). The relative increase in testosterone concentrations was larger when the decrease in antioxidant concentrations was also larger (Table 2 last two columns).

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Table 2: Pearson correlation coefficients and related p-values for the correlations among the four egg components in the three levels of analyses. See text for further details.

Figure 2: Relative decrease in antioxidant concentration between the first-laid and last-laid egg against the relative increase in testosterone concentration between the first-laid and the last-laid egg.
(c) Maternal body mass and egg components.
Female body mass was not correlated with clutch mass (Pearson correlation coefficient $r=0.09, 9=0.85$), clutch yolk IgG concentrations ($r=0.66, p=0.11$), clutch yolk antioxidant concentrations ($r=0.36, p=0.49$) or yolk testosterone levels of the clutch ($r=0.25, p=0.59$). Female body mass was negatively correlated with the relative decrease in antioxidant concentration (fig. 2; $r=-0.94, p=0.006$) and was on the verge of a significant negative correlation with the increase in testosterone ($r=-0.75, p=0.05$). Female body mass was unrelated to the relative decrease in egg mass ($r=-0.03, p=0.94$) or IgG concentrations ($r=0.42, p=0.34$) across the laying sequence.

Discussion
We studied maternal deposition patterns of four substances relevant to the immune defence in black-headed gull eggs. We were interested to what extent these patterns relate to hatching asynchrony and sex of the embryo, and whether they show mutual adjustment. We hypothesized that avian mothers may compensate the possible immunosuppressive effects of elevated levels of testosterone, required for mitigating the negative consequences of hatching asynchrony for the last hatchlings, with higher levels of antioxidants and IgG, especially in later-laid eggs and eggs containing male embryos, the more vulnerable sex to early post-hatching mortality. The results do not support these predictions and reveal some interesting patterns that are contrary to our hypotheses.

Changes within the laying sequence
Maternally derived antioxidant and immunoglobulin (IgG) concentrations in the yolk decreased within the laying sequence (Figure 1b,c), as has been shown previously for the closely related lesser black-backed gull (Royle et al. 2001, Blount et al. 2002, Blount et al. 2004). The most parsimonious explanation for this pattern is maternal depletion.
Females must obtain antioxidants from the diet (Brush 1990). Depending on their availability, the foraging ability of the mother, and the requirements for the mother’s own antioxidant activity, mother gulls may face limitations in the amount of antioxidants they can transfer to their offspring. This is supported by the fact that under natural feeding conditions, the yolk of lesser black-backed gulls is not maximally provided with antioxidants. Supplemental feeding with a carotenoid enriched diet resulted in an almost twofold increase of the yolk carotenoid concentrations (Blount et al. 2002). Interestingly, yolk carotenoid levels still declined over the laying sequence despite the additional supplement that was available until clutch completion. This may reflect competition among oocytes, with higher rates of yolking of the oocytes that ovulate first (Meathrel 1991).
Like with the antioxidants, the transfer of maternal IgG to the yolk probably represents a cost to the mother, in particular during the period of rapid growth of the oocyte (Kowalczyk et al. 1985). However, the metabolic cost of immunoglobulin transfer to eggs is uncertain. It has been estimated that the fraction of the IgG pool that is transferred into oocytes is 10-20 % for chicken (Kowalczyk et al. 1985) and 1 % for turkey (Dohms et al. 1978). Furthermore, in barn swallows (Hirundo rustica), specific antibodies in eggs from immunized females did not vary across laying sequence (Saino et al. 2003). In this way, it is difficult to argue that declining immunoglobulin levels within the laying sequence are due to simple physiological depletion to systemic immunoglobulin production (Lochmiller & Deerenberg 2000). However, differences in physiology related to egg production between species should be considered here. In gull species egg production is relatively costly (Monaghan et al. 1998). In addition, immunoglobulin synthesis during egg production is under hormonal control (Barua et al. 1998), and the early onset of incubation in gulls may constrain the deposition of IgGs in last laid eggs.

The decline in egg mass over the laying sequence can be explained in terms of macronutrient depletion, i.e. later oocytes are disadvantaged during the process of yolk formation as less maternal nutrient reserves are available. The rate of the decrease in egg mass across the laying sequence may reflect the mother’s current foraging intake of nutrients. Thus later oocytes have a disadvantage as the process of yolk formation is not independent for each separate oocyte and maternal reserves deteriorate during laying. The steepness of the decrease in egg quality over the laying sequence may reflect the mother’s ability of compensatory antioxidant supply and potentially IgG synthesis. Indeed, clutches produced by more brightly colored females, indicating higher body carotenoid levels, showed a smaller decrease in carotenoid levels between second and third egg (Blount et al. 2002). To counteract this decline, females may prolong the laying interval between their eggs to provide them sufficiently with resources. This may entail the cost of increased hatching asynchrony since incubation starts already early after laying of the first egg possibly as a means to suppress egg predation and a decline in their viability (Brouwer and Spaans 1994, Webb 1987, Müller et al. in press).

Gulls produce large eggs and we suggest that the decline of egg quality over the laying sequence is the result of a shift in the optimum for the mother. Since black-headed gulls rarely rear the full brood, the third egg is probably an insurance for loss of the first or second egg (Graves et al. 1984, Forbes et al. 1997, Stoleson and Beissinger 1995). In case of no loss, chicks of third eggs usually die in the first week after hatching, as a consequence of hatching asynchrony. Therefore, chicks of last-laid eggs have a much lower survival probability than those of earlier laid eggs (Müller et al. 2003), and the lower quality of the last less valuable egg may therefore reflect an adaptive maternal strategy in the light of
the value of the antioxidants and IgGs for herself. The low quality of the last-laid egg of a clutch explains why its chick has a lower survival even when corrected for hatching asynchrony and egg weight (Parson 1975). Yolk testosterone concentrations significantly increased with laying order, as has been shown in earlier studies on gulls, including the black-headed gull (Figure 1d, Eising et al. 2001, Royle et al. 2001, Groothuis and Schwabl 2002, Verboven et al. 2003). In contrast to maternal deposition of antioxidants and macronutrients, androgen deposition is likely to be less costly for the female. Production of steroids is not costly in itself. In case deposition of androgens in the egg requires elevated circulating levels of testosterone in the female, exposure to this hormone might be costly to the mother. However, evidence for such passive transfer is ambiguous (Schwabl 1997, Verboven et al. 2003) and enhanced testosterone deposition would require elevated maternal levels of the hormone for only a relatively short time span. The finding that females of low body condition allocated more androgens to the yolk compared to females in good condition (Verboven et al. 2003) suggests no important cost of androgen deposition for the female.

**Sex allocation**

We expected that male embryos, because of their higher sensitivity to egg quality (Nager et al. 1999), would receive greater maternal investment. This expectation has been supported by several studies (e.g. steroids: Petrie et al. 2001, Müller et al. 2002; egg size: Cordero et al. 2000, 2001; IgG: Saino et al. 2003) but not in others (steroids: Schwabl 1993, Verboven et al. 2003, carotenoids: Saino et al. 2003). We did not find any indication for sex specific allocation in this study.

**Mutual adjustment**

As a consequence of the different deposition patterns across the laying sequence, an indirect positive association between yolk concentrations of immunoglobulins and antioxidants, and a negative association between these two and yolk concentrations of testosterone was found. However, nor at the individual egg, nor at the level of the clutch, nor at the level of within clutch variation the deposition of antioxidants and antibodies were related. This may be due to the fact that antioxidants are markers for diet quality and foraging ability while IgG levels integrate exposure to infectious agents. Such covariation would have been adaptive, as antioxidants may protect the maternally derived IgG against catabolism in vivo (Haq et al. 1996). Our results indicate that mothers cannot interactively allocate these yolk compounds. In contrast there was a direct positive association between the rate of decrease in antioxidants and the rate of increase of the testosterone concentrations over the laying order. This will likely handicap the immune function of the last hatching chick even further.
Such a steep decrease of yolk antioxidants over the laying order may stem from a limited maternal antioxidant availability, which is supported by our finding that such mothers have a relatively low body mass. Such antioxidant limitation could be amplified in mothers that adaptively increase testosterone synthesis because the anabolic effects of testosterone may promote oxidative stress (von Schantz et al. 1999). The elevated levels of immunosuppressive testosterone and low levels of carotenoids in last laid eggs have been interpreted as a means of adaptive brood reduction in case of low food availability, while under food conditions that are sufficient for a proper development of immune function enhanced levels of maternal testosterone might help to overcome the disadvantage in sibling competition of the last hatched chick leading to enhanced survival (Royle et al. 2001). This elegant hypothesis does not take into account the high cost of egg production in gulls (Monaghan and Nager 1997, Monaghan et al. 1998) or the importance of the third egg as an insurance against failure of earlier eggs (see above). We suggest that the decrease in antioxidants with laying order is a constraint of the laying female, whereas enhanced testosterone allocation to last laid eggs serves as a mechanism to enhance competitiveness (Schwabl 1993, Eising and Groothuis 2003), which is especially relevant in eggs of poor quality. In addition we would like to suggest that the possible immunomodulatory effects of yolk testosterone may be interpreted as an adaptive maternal effect. For a growing chick the costs of raising an immune response should be traded against the resulting reduction in growth and the potential loss of a size advantage in the sibling rivalry (Soler et al. 2002, Brommer 2003). As a consequence of the allocation of high amounts of energy to growth the last-hatched chicks have a higher vulnerability to infectious diseases, but without this biased allocation they would probably have died anyway. Therefore, under the constraints that mother gulls face both in their ability to maintain egg quality over the laying sequence and in the necessity of early incubation, leading to hatching asynchrony, our results suggest that the allocation of testosterone reflects an adaptive maternal strategy.

In conclusion, we documented multiple pathways for maternal effects on offspring phenotype that suggest few opportunities for the mother to adjust the different maternal effects to each other. In particular possible immunomodulatory effects of maternal testosterone are not compensated by increased deposition of maternal antioxidants and IgGs. This may in part be explained by maternal constraints. The only indication for mutual adjustment of maternal effects is found in the deposition of testosterone, possibly the less costly pathway for the mother, as a compensation for low egg quality reflected in low levels of antioxidants.
Introduction

Parents influence the fitness of their offspring by transferring to them other resources than their genome only. The advantage of such parental or maternal effects (Mousseau and Fox 1998) is that they can be adjusted to the prevailing post-hatching conditions, since the effects may arise from the environment as the mother experiences it. Different maternal resources may in principle be adjusted towards each other to maximize maternal fitness. Birds are excellent models for studying maternal effects, producing relatively large eggs which represent a substantial maternal investment that markedly influences post-hatching development and survival (reviewed by Williams 1994, Christians 2002). This maternal resource allocation takes place in a short time window, and no further adjustments of the egg components are possible once the egg is laid.

One important component of bird eggs that is present in substantial levels are maternally derived yolk androgens. Increased levels of maternal yolk androgens can influence hatching time (Sockman and Schwabl 2000, Eising et al. 2001), enhance begging behavior and post-natal growth (Schwabl 1993, 1996, Eising et al. 2001, Eising and Groothuis 2003), the first two probably by strengthening of the neck muscle (Lipar and Ketterson 2000). In this way the maternal hormones mitigate the negative consequences of hatching asynchrony for the last-hatched chick (Schwabl 1993, Eising et al. 2001). On the other hand maternal yolk androgens may entail costs for the offspring. In several bird species testosterone suppresses immune function (Ketterson and Nolan 1999, Peters et al. 2000, Duffy et al. 2001, but see Ros et al. 1997, Hasselquist et al. 1999), as does experimental elevation of yolk androgens in chicks (Hirota et al. 1976, Groothuis et al. subm.).

Experimental elevation of yolk androgens is widely recognized to suppress antibody responses via premature regression of the bursa of Fabricius (Hirota et al. 1976) but can also enhance the T-lymphocyte compartment by promoting thymic hyperplasia (Marsh 1992). Furthermore, androgens may promote oxidative stress resulting from accelerated growth (von Schantz et al. 1999).

The effect of maternally derived androgens on immunity may indeed be of great importance for a young chick. At hatching the chick leaves the sealed environment of the egg and is confronted with a spectrum of infectious agents that can cause morbidity and mortality while their immune system is not yet fully developed (Apanius 1998). Therefore, avian mothers provide the egg, and thus enhance the immune defence of their offspring, with maternal Immunoglobulin G (IgG) (Gasparini et al. 2001, Buechler et al. 2002, Saino et al. 2003) and antioxidants such as carotenoids and vitamin E (Royle et al. 2001, Blount et al. 2002, Saino et al. 2003). IgG, deposited in the egg yolk, provide protection during the vulnerable period between hatching and maturation of endogenous immune function (reviewed by Grindstaff et al. 2003). The protective role of maternally derived immunoglobulins has for example been demonstrated by experimental infections of domestic birds (Kariyawasam et al,