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Maternal Effects in the Magpie







ABSTRACT

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Diss.

Maternal effects are suggested to be an efficient way to adapt offspring to variable and changing environments with consequent effects on variation in offspring fitness. In birds, mothers are able to affect the quality of their offspring through egg quality and through their incubation and parental care behaviour. Maternal effects via egg quality and hatching asynchrony, caused by incubation behaviour, may incur costs for both the mother and her offspring. For my thesis, I investigated the adaptive significance of maternal effects in an altricial passerine bird, magpie (*Pica pica*), in relation to variable environmental conditions among and within nests. The main question was whether the maternal transmission of egg components is only a reflection of maternal condition during egg laying, or, if the transmission could also be an optimal, evolutionary adaptive strategy. The question includes the effects of sibling hierarchy, induced by parental incubation behaviour, on offspring performance. Maternal transmission of different important compounds was affected both by the available resources, reflecting environmental conditions, and by maternal engineering within the clutch. Many of these egg quality factors such as antioxidants, hormones and immune factors had remarkable effect on offspring performance and they seem to function together with the effects of asynchronous hatching (age difference between hatching positions) as an optimal brood reduction mechanism in variable environmental conditions. In general, when resources are plentiful, parents are able to raise the whole clutch and the youngest nestlings can catch up in size to their older nest mates before fledging. However, if conditions are unfavourable, brood reduction happens quickly and the first hatched nestlings have enhanced possibilities for success. I also found that mothers can create, by differential allocation of egg quality compounds, different phenotypes which may have variable future reproductive strategies and fitness expectation depending on future conditions.

Key words: Antibodies; cell-mediated immunity; egg quality; hatching asynchrony; maternal effects; testosterone

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original studies which are referred to in the text by their roman numerals (I-VI). I am the main author of papers II-V, and I performed a significant part of the work of papers I and VI.

- I Siitari, H., Alatalo, R. V., Pihlaja, M., Hämäläinen, J., Blount, J. D., Groothuis, T. G. G., Hytönen, V. & Surai, P. Constraints and adaptivity in egg quality in the magpie (*Pica pica*). Manuscript.
- II Pihlaja, M., Siitari, H. & Alatalo, R. V. 2006. Maternal antibodies in a wild altricial bird: effects on offspring immunity, growth and survival. *J. Anim. Ecol.* 75: 1154-1164.
- III Pihlaja, M., Alatalo, R. V. & Siitari, H. Maternal manipulation of offspring performance in the magpie (*Pica pica*). Manuscript.
- IV Pihlaja, M., Siitari, H., Buchanan, K. L. & Morales, J. Testosterone, stress and maternal antibodies in nestlings of a wild passerine: maternal compensation by antibody allocation? Manuscript.
- V Pihlaja, M., Siitari, H., Alatalo, R. V. & Leppänen, S. Habitat, sex and hatching order effects on T-cell-mediated immunity in magpie (*Pica pica*) nestlings. Manuscript.
- VI Timonen, J., Pihlaja, M. & Siitari, H. Effects of breeding habitat on egg quality in the Magpie. Submitted manuscript.

1 INTRODUCTION

1.1 Maternal effects

"Cross-generational transmission of individual quality such that the offspring survival, growth and fecundity are affected by the properties of the environment in which the parents lived"

Ginzburg 1998

In addition to genetic quality, offspring performance can be influenced by propagule quality and by the environment in which the offspring developed. In mammals, an individual's early development is influenced by the environment inside the mother. In oviparous vertebrates, instead, the early developmental environment is mostly enclosed inside the egg. In addition, ovipositing parents can influence the environment of the developing embryo by their incubation or other egg caring behaviour, for example, adjusting the temperature of the eggs. After the birth or hatching parents can further shape the phenotype of their offspring by their caring behaviour until the offspring's independence.

In birds, which play the leading role in this thesis, mothers can affect their offspring's quality through egg quality, and indeed recent studies suggest strong maternal effects on several egg quality parameters (e.g. Royle et al. 2001, Saino et al. 2002a, Grindstaff et al. 2003, Groothuis et al. 2005b, I, II). This 'maternal engineering' can modify differences between siblings (Groothuis et al. 2005b, Eising et al. 2006, Rubolini et al. 2006, II, III, IV). Maternally transmitted substances such as hormones, antioxidants and antibodies can have major effects on offspring development and survival (for carotenoids: see Royle et al. 2001, Saino et al. 2002a; for hormones, see the recent review by Groothuis et al. 2005b, Socman & Schwabl 2000, Andersson et al. 2004; for immunoglobulins: Grindstaff et al. 2003, II, III), and also have long-term effects (Eising et al. 2006, Grindstaff et al. 2006). Often these egg quality parameters have significant systematic variation within a clutch by changing along the laying order (Blount

et al. 2002, Buechler et al. 2002, Saino et al. 2002a, Royle et al. 2003, Müller et al. 2004, Groothuis et al. 2005b, I, II).

In addition to egg quality *per se*, parents can create a size hierarchy within their brood by their incubation behaviour. This may be an important mechanism in some species in creating different phenotypes within clutch (see Laaksonen 2004). When incubation is started before the clutch is completed, the young hatching from the later laid eggs are younger, and thus, smaller than their older nest mates. Brood reduction, a phenomenon of partial termination of the clutch or brood due to production of a larger clutch than parents are able to raise, is quite common in asynchronously hatching broods. Last-hatched nestlings are the ones that suffer the most (e.g. Wiebe and Bortolotti 1995, Evans 1996, Viñuela 2000).

Maternal effects, through egg quality and caring behaviour, may provide an adaptive phenotypic response to environmental variation, creating additional variation in offspring fitness (Mousseau & Fox 1998). In this thesis, I investigated the adaptive significance of maternal effects in an altricial bird in relation to variable environmental conditions among and within nests.

1.2 Egg quality, hatching asynchrony and nestlings' phenotype

Egg size has commonly been used as an egg quality index (Lindström 1999), but egg size does not explain a considerably high variation in offspring performance. However, maternally transmitted substances in the eggs have a potentially significant role in offspring performance. A common suggestion is that maternal engineering is targeted to the survival probability of the last hatched chicks. For example, higher androgen levels in the last laid eggs may (via increased begging behaviour) decrease the effect of hatching asynchrony, which creates size hierarchies within clutch, and thus, enhance the competitive ability of later-hatched and therefore smaller chicks (Eising et al. 2001). Indeed, in lesser black-backed gulls (Royle et al. 2001) and black-headed gulls (Groothuis et al. 2006) mothers were able to improve the competitive ability of last hatched nestlings via egg quality.

Maternal hormones have major effects on offspring developmental rates (Schwabl 1996) and competitive ability (Schwabl 1996, Eising et al. 2001, 2006), even affecting their phenotypes later in life (Eising et al. 2006, Rubolini et al. 2006). Testosterone is an essential hormone in shaping offspring phenotype. It is transmitted to the offspring through the egg yolk and the levels of testosterone in eggs vary significantly among and within clutches (Lipar et al. 1999, Sockman & Schwabl 2000, Petrie et al. 2001, Tschirren et al. 2005, I). The growth of the young can be affected by testosterone (e.g. Schwabl 1996, Tschirren et al. 2005). Thus, a mother may manipulate the growth rate of her young by differential allocation of testosterone to her eggs. On the one hand, testosterone might have positive effects on growth, but on other hand it may have negative

effects on, for example, immunity (Ketterson & Nolan 1999, von Schantz et al. 1999, Andersson et al. 2004, Groothuis et al. 2005a, Müller et al. 2005) or survival (Sockman & Schwabl 2000). In black headed gulls, embryonic exposure to androgens suppressed cellular and humoral immunity in early life (Müller et al. 2005). It is also suggested that higher levels of testosterone in males may disturb the functioning of antioxidants (von Schantz et al. 1999), although testosterone alone has also been shown to be immunosuppressive without an antioxidant vector (Råberg et al. 1998). However, the negative effect of testosterone on immune function may not always be that straightforward. For example, in great tit (*Parus major*) nestlings, experimentally enhanced yolk testosterone levels did not have a negative effect on T-cell mediated immunity (Tschirren et al. 2005). Growth enhanced by testosterone may also cause stress which can be detected by high levels of stress induced heat shock proteins (HSPs) (Sørensen et al. 2003, Martínez-Padilla et al. 2004). HSPs protect individuals against protein damage caused by external or internal factors, but high HSP levels may represent a trade-off with other traits (see review of Sørensen et al. 2003).

Diet-derived antioxidants and embryonic exposure to antioxidants (e.g. vitamin A, E and carotenoids) have been shown to be important for reducing the negative effects of androgens and supporting immune function (Chew 1996, Haq et al. 1996, Gore & Qureshi 1997, von Schantz et al. 1999). Yolk levels of maternally transferred carotenoids showed decreasing trends along the laying order in gull species (Blount et al. 2002, Royle et al. 2001) and in magpies (*Pica pica*) (I). Birds cannot synthesize antioxidants, they must be gained from the diet. Thus, the availability of antioxidants during the egg laying period may influence the maternal transmission of antioxidants to the offspring and thus have an essential role in offspring performance (I).

Newly hatched young are dependent on innate immunity and maternal antibodies (mainly immunoglobulin G) for the protection against pathogens (Klasing & Leshchinsky 1998; see also Tella et al. 2002). In birds, immunoglobulins are transmitted to the offspring via the egg yolk (Deeming 2002, see Grindstaff et al. 2003 for a review). Maternal immunoglobulins protect the offspring during their early life-stages, when their own adaptive immunity is not yet fully developed, against pathogens that their mothers had been exposed to earlier (Klasing & Leshchinsky 1998, Rollier et al. 2000, Gasparini et al. 2001, Grindstaff et al. 2003, Grindstaff et al. 2006). Maternally 'inherited' immunoglobulins have been shown to be essential for nestlings' own antibody production and survival (II).

In addition to maternal antibodies, immune factors in the egg albumen, e.g. avidin and lysozyme, can be important for hatching success (Green 1990, Saino et al. 2002b) and there is within-clutch variation among laying positions in both compounds in magpies (*Pica pica*) (I).

Substantial variation in many egg quality parameters within clutches may be the result of optimal allocation by a mother to change the competitive ability of siblings within the brood or to secure the success of the nestlings high in the

sibling hierarchy (e.g. Groothuis et al. 2006, III), or, it may simply reflect a constrained allocation by the mother (for carotenoids, see Groothuis et al. 2006, I, but see Royle et al. 2001, Blount et al. 2002, VI). The question of whether the maternal transmission of egg components is only a reflection of resources available for maternal usage during egg laying, or, if the transmission is also an optimal, evolutionary adaptive strategy, has remained unsolved. The aim of my thesis was to find answers to these questions and, further, to investigate the effects of sibling hierarchy, induced by parental incubation behaviour, on offspring performance.

2 MATERNAL EFFECTS AS AN ADAPTATION

"Costs of reproduction and optimal resource allocation are the central themes in life-history evolution"

Roff 1992

2.1 Adaptation to unpredictable environmental conditions during the breeding season?

Raising young requires large amounts of food resources. Thus, birds usually breed at the time of the year when these resources are plentiful, mainly in the spring and early summer. In early spring, when many bird species start to breed, weather conditions can be highly variable. This leads to unpredictability in food resources, which usually, especially in the case of passerine birds, consist of invertebrate fauna. Thus, birds may start breeding with plenty of resources and invest in large clutches predicting a high number of successful offspring, but there is a serious risk of losing that investment if parents are not able to feed their young efficiently enough. The cost of losing a brood is suggested to be high in altricial birds where parents feed the young in their nest for some time after hatching. Thus, it is an advantage if parents can somehow secure their investment under unpredictable environmental conditions.

Brood reduction due to starvation under severe sibling competition caused by asynchronous hatching and size hierarchy, or even due to siblicide, is thought to be the main mechanism for securing enough resources to at least some of the offspring (e.g. Braun & Hunt 1983, Reynolds 1996, Stenning 1996). Then, if resources are plentiful, smaller siblings would also receive food and could survive even under heavy competition. However, bird nestlings grow extremely fast, and even with plentiful resources it can be difficult for the younger siblings to grow large enough to fledge with the older nest mates.

Thus, to be successful, in good conditions, these last hatched nestlings should be able to catch up in size to their siblings.

Mothers can affect the growth rate of their young by hormone allocation. Testosterone allocation into eggs has been shown to have growth enhancing properties (Schwabl 1996, Tschirren et al. 2005, III). Testosterone may have a negative affect on immunity (Ketterson & Nolan 1999, von Schantz et al. 1999, Andersson et al. 2004, Groothuis et al. 2005a, Müller et al. 2005, IV), and if immune defence is critical for the survival of the nestlings, mothers should respond to the need and somehow compensate for the reduced defence of the last hatched nestlings. Response may include the allocation of higher levels of maternal antibodies to later laid eggs (II). Moreover, when resources turn out to be limited, high levels of testosterone in the last hatched nestlings (Sockman & Schwabl 2000, Verboven et al. 2003, I), together with reduced levels of maternal antioxidants compared to their older nest mates (Blount et al. 2002, Royle et al. 2001, I), can accelerate the death of the last ones. This is an advantage to the rest of the brood and to the parents, since fewer resources are wasted on nestlings that are going to be sacrificed anyway.

The focus of the research on maternal allocation has been the survival probability of the last hatched chicks. However, the quality of the first laid egg may also be very important. Hatching asynchrony varies both between and within species. For example, in the magpie, hatching asynchrony between broods varies from less than a day to as long as five days (personal observation). Especially in cases where the rate of hatching asynchrony is low, the nestlings from first laid eggs should have better survival rates than their siblings hatched from last laid eggs over the phase of brood reduction and intense sibling competition. Otherwise, they may be in competition with or even lose out to their siblings from last laid eggs growing faster (Schwabl 1996, Tschirren et al. 2005, III) and possibly begging more intensively (Schwabl 1993). In unfavourable conditions, this would lead to the starvation of the whole brood.

2.2 Adaptation against parasites, brood-parasitism or predation?

Nest predation, parasites and interspecific brood parasitism are important evolutionary forces affecting the growth strategies of bird species (Bosque & Bosque 1995, Møller 2005, Remes 2006). However, within-species maternal effects may provide a fast way to adapt populations to different conditions created by varying parasite or predator densities, or the presence of brood-parasitism. So far, adaptation to these external factors via maternal effects has been unknown. However, in a recent comparative study of house finches (*Carpodacus mexicanus*), Badyaev et al. (2006) showed that in male nestlings', which were more vulnerable to ectoparasites than females, growth rate was enhanced in a population where ectoparasites were more abundant. A higher

growth rate of male nestlings in more parasitized broods can be induced by maternal allocation of higher levels of androgens, e.g. testosterone, to male eggs (Schwabl 1996, Tschirren et al. 2005, III). More studies and an experimental approach are needed to explore whether maternal effects can be an adaptive way to respond to parasite or predation pressure.

2.3 Adaptation of offspring to an unpredictable future?

Years are different and in the Northern Hemisphere, for example, wintering conditions may vary greatly from year to year in terms of weather conditions and in the amount of food available (Laaksonen et al. 2004). Survival over the winter to the next breeding season may vary according to an individual's phenotype with interaction to the wintering conditions. Moreover, the fitness of different phenotypes may depend on the conditions during the breeding season. For example, the basic metabolic rate (BMR), which is related to an individual's energy consumption, can be dependent on the size of the individual in interaction with current conditions (Jan-Åke Nilsson, personal communication).

Maternal antibodies can have long term effects on offspring immunity (Grindstaff et al. 2006, II) and within nest variation in maternal antibody deposition may generate individuals with different phenotypes. Furthermore, their future success could be related to parasite or pathogen prevalence. In addition to humoral immunity, the within-nest differences in cell-mediated immunity (CMI) (V) could be important later in life in respect to parasite abundance and the costs of using CMI.

Androgens transferred to the offspring through the egg yolk may not only affect offspring phenotype during early growth, as do antibodies, but also have effects later in life. In black-headed gulls (*Larus ridibundus*), maternal androgens affected offspring nuptial plumage and sexual displays almost a year after hatching (Eising et al. 2006). Thus, maternal hormone deposition may affect offspring's future reproductive success. In addition, in a study in zebra finches (*Taeniopygia guttata*), the early rearing conditions affected future reproductive strategies, having possible effects on population dynamics (Alonso-Alvarez et al. 2006).

However, there are only a very few studies trying to test these hypotheses, and, thus, they should be further studied. In future, it would be interesting to investigate the effects of different maternal substances in the egg on metabolic rates, winter survival and future reproductive success in different environmental conditions.

3 APPROACHES TO INVESTIGATING MATERNAL EFFECTS

3.1 Model species

Maternal effects in birds have mainly been studied in different gull species (e.g. Nager et al. 2000, Gasparini et al. 2001, Eising et al. 2001, 2006, Blount et al. 2002, Verboven et al. 2003, Müller et al. 2004, 2005, Groothuis et al. 2006), domestic galliformes (e.g. Haq et al. 1996, Klasing & Leshchinsky 1998, Yasuda et al. 1998, Müller et al. 2002, Koutsos et al. 2003, Grindstaff et al. 2005), and in some passerines (e.g. Buechler et al. 2002, Saino et al. 2002, 2003, Royle et al. 2003, Soler et al. 2003, Tschirren et al. 2005, Badyaev et al. 2006, Dubiec et al. 2006, Kilpimaa et al. 2006) and birds of prey (e.g. Korpimäki et al. 2000, Blanco et al. 2003, Martínez-Padilla et al. 2004). Most of the studies in wild living birds have been made in gulls, which are colonial breeders and have relatively small clutches, usually three or fewer eggs, hatching asynchronously (e.g. Graves et al. 1984, Gasparini et al. 2001, Müller et al. 2004), and in *Parus* species having large clutches with hatching asynchrony between some hours and a couple of days (personal observation).

For my thesis I used the magpie (*Pica pica*) as a model species. Magpie is a socially monogamous, medium-sized passerine. The advantage of this species for this study is the relatively large clutch size (mean 7.5 eggs) and highly asynchronous hatching among passerines, which creates a high degree of size hierarchy within clutches. Adult males are about 10 % larger than females (Birkhead 1991), but in my study population size differences do not begin to appear until the nestlings are more than one week old (Pihlaja et al. unpublished data). Furthermore, magpies breed in various habitat types, from urban city centres to rural farmlands, and territory quality varies a lot. Breeding pairs use the same territory all year around and keep the territory for several years. However, the nest is usually rebuilt every year, and that may be the

reason for extremely low densities of ectoparasites in their nests (personal observation) compared to many cavity breeding passerines.

3.2 Experimental approach

Maternal effects include many parameters (Mousseau & Fox 1998) and thus it is necessary to use experimental approaches to be able to investigate the importance and causes of different parameters.

In this thesis I used three different experimental approaches to investigate the effects of resource limitation, maternal engineering via egg quality and incubation behaviour causing hatching asynchrony (I followed the nests during egg laying and observed initiation of incubation, which started in full effort usually after first two or three eggs were laid) on offspring performance. In the first experiment carried out in 2002 I studied the effect of resource limitation and laying order on egg quality, and on nestling immunity (supplemental feeding experiment) (I & II). In the second experiment I investigated the effects of egg quality of nestlings in different hatching positions and hatching order on the growth and survival of magpie nestlings by cross-fostering hatchlings between different hatching positions (Fig. 1) (cross-fostering experiment) (III). Hatching asynchrony (age difference between siblings) may have stronger effect on brood reduction than hatching order itself, which I have been using as explaining variable in my studies. However, in my studies both hatching order and hatching asynchrony had about the same effect (both significant or both not significant) and thus I have used only the hatching order as explanatory variable to make different studies more comparable.

In the third experiment I experimentally enhanced maternal testosterone levels by injection into the eggs just after laying to see how maternal testosterone affects nestlings' growth (III) and adaptive immunity (immunoglobulins) (testosterone injection experiment) (IV). All three experiments were conducted in the surroundings of Jyväskylä in central Finland (62°N 25°E) during the breeding seasons of 2002 to 2005.

3.2.1 Supplemental feeding experiment

In supplemental feeding experiment I used half of the nests as controls (same amount of disturbance, but no feeding) and half of the nests were supplementally fed before and during the egg laying. Breeding pairs were offered raw chicken eggs (5 eggs for each pair on every second day, amounting to 70% of daily energy needs, as calculated by Högstedt 1981). The chicken eggs were placed in dummy bird nests (1 to 3 m from the nest of an experimental pair) covered with hay. Both experimental and control nests were

visited every second day to check on the initiation of egg laying and monitor the consumption of supplemental food (fed nests only) and nest failures. Supplemental food was given before and during the whole egg laying period for an average of 19 days (range 15-23 days) from late March to late April 2002 and the supplemental feeding was conducted for a minimum of 7 days before the onset of egg laying for all the supplemented nests. Eggs were collected for laboratory analyses after they were laid and replaced with wooden dummy eggs to prevent artificially enlarged clutches. Detailed methods can be found in papers I and II.

3.2.2 Cross-fostering experiment

In my cross-fostering experiment I chose two first- or last-hatched nestlings from separate broods with known hatching rank to be cross-fostered to another nest. The effect of fostering was controlled by changing two first hatched nestlings with two first hatched ones in another nest with the same hatching date (we allowed a maximum of 24 hours difference in hatching) (first to first nestlings = controls in 22 nests). It would have been ideal to have had another control group, where two last hatched nestlings would have been swapped with two other last hatched ones. However, the mortality rate of last hatched nestlings was so high and hatching success of the last egg so uncertain, that I had to use all available last hatched nestling pairs as experimental pairs. As the first hatched control nestlings did not differ in any parameters from non-manipulated first hatched nestlings (Pihlaja et al. unpublished data), I used a group of non-manipulated last hatched nestlings in comparisons the with experimental groups. In the experimental groups, two first hatched nestlings were exchanged with two last hatched nestlings of another nest with the same hatching date (± 24 hours). Fifteen nests had two last hatchlings swapped to the position of first nestlings (last to first nestlings) and 15 nests had two first hatchlings swapped to the position of last nestlings (first to last nestlings) (Fig. 1). The cross-fostering was conducted within one day of hatching of the nestlings to be fostered. We followed the growth and survival of the nestlings until fledging age. Detailed methods can be found in paper III.

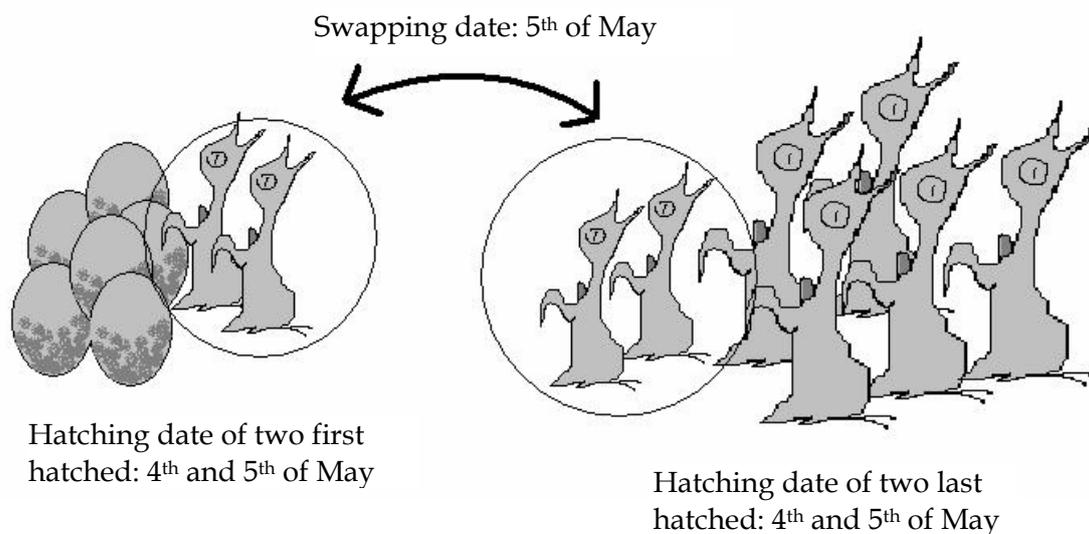


FIGURE 1 An example of how hatchling swapping was conducted in the cross-fostering experiment. Two first hatched nestlings in one nest were swapped with two last hatched ones in another nest.

3.2.3 Testosterone injection experiment

In the testosterone experiment I injected, after initiation of egg laying, testosterone diluted with sesame oil into the egg yolks. As a control treatment I injected sesame oil only. The testosterone and control injections were given to every second egg in each clutch. I planned the experiment in this way to be able to control for differences among nests and for different laying positions. Maternal testosterone levels naturally increase along the laying order and with the injection, the testosterone levels of earlier laid eggs were enhanced to the natural levels of the last laid eggs, while the levels of the last laid eggs were increased to higher than natural levels. The injection order (whether a clutch was started with a testosterone or sesame oil injection) was decided with a coin toss. All injections were conducted within 48 hours of egg laying. The amount of testosterone to be injected was estimated from egg yolk samples (I & IV), and adjusted to the natural testosterone level of the given population. After the nestlings hatched, I followed their growth and survival (III) and measured their levels of maternal and self-produced antibodies (IV). Detailed methods can be found in papers III and IV.

3.3 Non-manipulated data

As emphasized earlier, well planned and conducted experiments are needed in the study of maternal effects. However, we should not forget the value of non-manipulated data as we need to know the natural patterns to be explained with experimentation. Non-manipulated data, when data collection is carried out carefully and data analysed properly, can be very valuable. I have used non-manipulated data analysis to investigate the development of adaptive immunity in magpie nestlings and the effects of maternal antibodies on the offspring's own antibody production, growth and survival (II), to study the relationship between nestlings' testosterone levels, maternal and self-produced antibodies, growth, hatching order and stress levels (IV), to find out if the strength of cell-mediated immune response is related to habitat, hatching order, sex or growth (V), and finally to compare egg quality in relation to habitat type, rural or urban, and laying order in those two different environments (urban = Jyväskylä city centre and/or within 50 m of a highway/major road and without physical barriers, rural = countryside and suburban with only small roads, fields and forest present) (VI). The last two studies (V and VI) provided important background information for the other studies.

One very important aspect in using these data has been the proper tool for statistical analysis. For analysing my data I mainly used hierarchical multivariate linear and binary logistic regression models (Goldstein 2003) with the program MLwiN 1.10 (Centre for Multilevel Modelling, University of Bristol), because my data have two or more levels: mainly within-nest (between siblings) and between-nest levels. In addition, a third (or more) level(s) can be added, for example the habitat as a higher level, if there is significant variation between the units at this third level, or repeated samplings within a nestling as a first level before individual and nest levels. In many cases, I was particularly interested in the within-nest effects, because the nest is the environment where nestlings are competing with their siblings before fledging. The program I used was developed for purposes like mine, to be able to analyse data on many levels and separate the relationships between different levels. For example, I could separate the opposite effects within nests and between nests (e.g. negative relation between nests and positive within nests). If the hierarchical levels are not taken into account, the regression of analysed factors may be flattened and important information is lost (Fig. 2). If only the aggregate variables of the lower hierarchical level are used, all the information about within-lower-level effects is lost (Fig. 2). Another advantage of the statistical tool I used, for example compared to Repeated Measures ANOVA, is that the units of different hierarchical levels do not require equal sample sizes (e.g. variable clutch sizes are not a problem). Furthermore, data from one unit is not lost if some measurements are missing (e.g. the value of one nestling or some measurement of a single individual). Thus, with this powerful statistical tool I have been able

to gain more and better information from my data than I would have had I used more simple statistical methods.

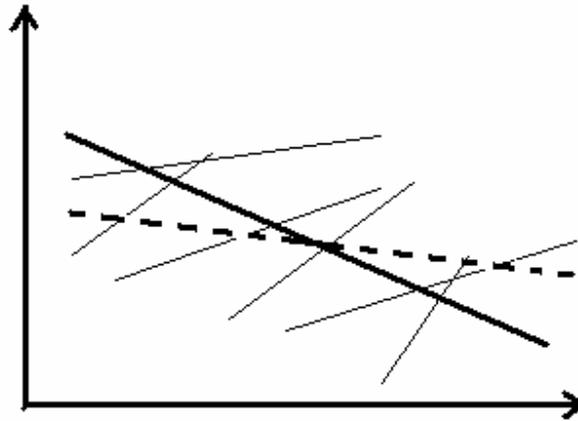


FIGURE 2 A schematic example of the meaning of the statistical method used for data analysis. Solid lines show regression in two different hierarchical levels, for example within nests (narrow lines) and between nests (thick line). The dashed line is a regression line produced from the same data, but without taking into account the two hierarchical levels.

4 RESULTS AND DISCUSSION

4.1 Between nest differences: effect of food resources and habitat

4.1.1 Limiting food resources

In the first study (I) food-supplemented females laid heavier eggs with heavier albumen compared to the controls, but there was no difference in clutch size between treatments. By increasing egg size, females may increase the future fitness of their chicks, since egg size is often positively related to hatching probability, chick size at hatching, early growth and survival (Williams 1994, Lindström 1999). More importantly, many egg substances (yolk immunoglobulins, albumen lysozyme and avidin, see discussion below) changed differentially along the laying order in the experimental and control groups.

The feeding treatment did not affect yolk testosterone levels but, interestingly, high testosterone levels were associated with larger clutches. There was some indication of the effect of food supply on yolk carotenoids, but this was not significant. In a study by Blount et al. (2002) carotenoid supply positively affected female carotenoid-based coloration and plasma and egg carotenoid levels, but the effect of carotenoid supply on yolk immunoglobulin levels was negative. However, the pathogen environment of colonial birds may differ from that of more solitary magpies and other passerines, and high levels of immunoglobulins in gulls may instead reflect increased pathogen exposure rather than their immune defence capacity.

Maternal immunoglobulins in the egg yolk increased over the laying order in the fed group, but remained at the same level through the laying order in the control group. This result indicates that immunoglobulin transfer is costly for females. This is in line with the study of Kilpimaa et al. (2006) in which female pied flycatchers (*Ficedula hypoleuca*) handicapped for feeding performance before egg laying, produced offspring with lower immunoglobulin levels

during the nestling period compared to controls. In addition, females in better body condition in the collared flycatcher (*Ficedula albicollis*) laid eggs with higher antibody levels, and immunoglobulin concentrations increased over the egg laying order, possibly enhancing the survival probability of the last hatched chicks (Hargitai et al. 2006). Furthermore, in my second study (II) higher levels of egg immunoglobulins in fed magpies were reflected in high immunoglobulin levels in their offspring: nestlings hatched from the eggs laid by fed magpie females had higher plasma immunoglobulin levels than the control group. In contrast, Grindstaff et al. (2005) did not find any effect of protein supply on immunoglobulin levels in egg laying females and in their eggs. However, in my study supplemented food also contained carotenoids and other compounds. Furthermore, birds in the Grindstaff et al. (2005) study were kept in captivity and fed *ad libitum*, and thus their energy gain was not constrained.

Albumen composition has been investigated in only a few studies so far (I). Saino et al. (2002b) investigated the albumen lysozyme activity in barn swallows (*Hirundo rustica*). In their study, higher plasma lysozyme activity was related to better hatching success, and the first laid eggs contained more lysozyme than the last laid eggs. Furthermore, nestlings from earlier hatching positions had higher lysozyme activity at five days old than hatchlings of the same age from later hatching positions (Saino et al. 2002b). The importance of albumen avidin, however, has rarely been studied in an ecological context. Avidin has the remarkable capacity of binding biotin, and biotin in turn enhances bacterial growth in the eggs. Thus, high levels of avidin in the egg albumen may inhibit bacterial growth and be beneficial for the growing embryo. I found that avidin allocation to the eggs may involve energy costs. Fed females laid clutches where avidin levels remained constant from the first to the last laid eggs, whereas in the control group the avidin levels decreased over the laying sequence. Lysozyme levels, however, had just the opposite pattern, suggesting that lysozyme transfer is not that costly for females. Indeed, lysozyme levels were negatively related to albumen size, which in turn was heavier in the eggs of supplementally fed females. Thus, the decreasing trend of lysozyme levels in the eggs of fed females may be a consequence of increased egg size. On the other hand, higher levels of avidin in the last laid eggs of supplemented females compared to the last eggs of control females may compensate for the effects of the lysozyme. Investing in avidin transfer may be more efficient, but if resources are limited, cheaper lysozyme may be the right solution. Albumen composition may be crucial for embryo development and successful hatching, and needs further investigation.

4.1.2 Habitat effects and between nest variation

Magpies breeding in rural habitats produced eggs of higher quality than magpies breeding in urban habitats (VI). Yolk carotenoids decreased from the first to the last laid egg in urban, but not in rural habitats. This result supports the indication of the feeding experiment that carotenoids are resource limited. On the other hand, these two results are in contrast in that in the feeding experiment there was a decrease with laying order in both groups. In any case, since carotenoids are acquired from food, the environment of a mother may play a major role in the carotenoid concentrations in the eggs. Royle et al. (2001) as well as Blount et al. (2002) found that carotenoid-fed females in the lesser black-backed gull (*Larus fuscus*) had higher plasma carotenoid concentrations, and carotenoid concentrations in the eggs they laid decreased along the laying order in both supplemented and control groups. This supports the idea of parental favouritism; a mother favours the chicks hatched from the first laid eggs by investing more carotenoids in them. In our study, higher carotenoid concentrations in the last laid eggs in rural habitats compared to urban ones may be a result of good food quality, reflecting only the fact that a mother has been able to invest more into her last eggs. In urban habitats mothers may be able to invest more carotenoids only in the first laid eggs leaving the last laid eggs and offspring under higher brood reduction pressure. Furthermore, in the study of Blount et al. (2002), the decrease in carotenoid levels with the laying order was not as steep in the eggs of carotenoid supplemented mothers as in the control nests. This provides some support for my finding that rural mothers could be able to invest more antioxidants in the youngest nestlings. Furthermore, egg weight followed the same pattern as carotenoids (VI) indicating that laying order may have an adaptive role. Asynchronously hatching broods with consequent size hierarchy are susceptible to brood reduction. Thus, it is reasonable for the mother to invest more in the first laid eggs in order to have at least a few viable chicks. On the other hand, in a favourable environment where all the chicks have a good chance of survival, a mother has an opportunity to compensate for lesser egg quality by providing enough food for all her offspring.

Immunoglobulin levels in eggs were higher in the rural area than in the urban one (VI). In some studies, breeding density (Müller et al. 2004) or parasite prevalence and infections (Gasparini et al. 2001, Buechler et al. 2002) have been shown to increase immunoglobulin levels. These results come from colonial breeders. However, magpie breeding density in my study area was higher in urban areas where total immunoglobulin levels were lower than in rural habitats (VI). Still, the breeding density is low compared to colonial seabirds. The transfer of passive immunity to chicks may enhance offspring resistance to pathogens (Grindstaff et al. 2003, II), and may be costly. Our results suggest that immunoglobulin levels in this case are constrained by some external factors like food quality, rather than caused by infection. Indeed, in the first and second studies (I & II) I showed that transmission of maternal immunoglobulins was limited by the resources available during the breeding season. In rural areas

food quality during the breeding season is likely to be better than in urban areas, even though urban areas are better during the winter.

Albumen lysozyme concentrations did not differ between the rural and urban eggs, but there was some indication that the rural eggs contained more avidin than the urban eggs. This is again in the line with the result of the feeding experiment (I), where the avidin concentration in the last laid eggs of food supplemented females was higher than in the last laid eggs of control mothers. However, lysozyme concentration decreased along the laying order in both habitats (VI), while in the feeding experiment it was constant in the control group and decreasing only in the fed group (I).

In addition to egg quality, nestling quality also differed between the two habitats. Nestlings in rural habitats had stronger cell-mediated immune response (CMI) at the age of one week than nestlings in urban habitats (V). In addition, nests with higher CMI had better survival until fledging (V). In the second paper (II), I found significant variation between habitats in maternal immunoglobulin levels. The same data indicated that survival was lower in nests with on average higher levels of maternal immunoglobulins (II). This may result from, e.g., a virus epidemic in some areas.

There was considerable variation between the nests in nestlings' mean testosterone levels measured from plasma in the middle of the nestling period, which was negatively related to average maternal antibody levels in the nestlings after hatching (IV). This was just the opposite of the within-nests relationship. The variation in testosterone levels among the nests might be caused by, for example, territory quality and/or density (Birkhead 1991) or by a mother's phenotype. Thus, it would be interesting in the future to investigate how parasite or predation pressure affects testosterone levels among the nests and also how this might be related to nestling immunity and growth rates. Following Badyaev et al.'s (2006) study which suggested that the higher growth rate of male house finch (*Carpodacus mexicanus*) nestlings in one population compared to another population, was directly due to a higher rate of ectoparasitism in the first population, I would expect to find some effects of immunity and growth on mean testosterone level between the nests. In great tits (Tschirren et al. 2005) and in magpie nestlings (III) increased yolk testosterone levels led to higher growth rates. In common lizards (*Lacerta vivipara*) also, growth rate was enhanced by testosterone, but on the other hand testosterone-treated individuals were also more susceptible to parasites (Uller & Olsson 2003).

4.2 Differential allocation within the clutch and hatching asynchrony

Environmental conditions during the breeding season are important in shaping maternal investment. However, mothers may create phenotypic variation within their brood by differential allocation of egg components. In all the studies included in this thesis I found significant systematic variation within clutches in egg quality or nestlings phenotype. I will concentrate on those that I have found most interesting: variation in carotenoid, testosterone and immunoglobulin concentration in the eggs (I & VI), variation in testosterone, immunoglobulin and stress protein levels in the nestlings (II & IV), and variation in the cell-mediated immune response of the nestlings (V).

An increase of androgens (to which testosterone belongs) over the laying sequence has been described in many previous studies both in semi-precocial and altricial species (gulls: Eising et al. 2001, Royle et al. 2001, Groothuis & Schwabl 2002, Verboven et al. 2003, Groothuis et al. 2006, for both, see the review by Groothuis et al. 2005). My results are in line with these studies (I). This thesis supports the hypothesis of the optimal allocation function of testosterone on offspring quality (Groothuis et al. 2006) (I, II & III), as does the study of Verboven et al. (2003) in which females in better physical condition actually laid eggs of lower testosterone concentration, but with an increasing trend over the laying sequence. Although Groothuis et al. (2006) suggested that carotenoids are constrained rather than optimally allocated to decrease over the laying sequence, my thesis supports the adaptive allocation of carotenoids over the laying sequence (I, III & VI). This is because carotenoid levels decreased in a similar way in both the fed and control clutches, but tended to be higher in the clutches laid by the fed group (I). Thus, higher testosterone and lower carotenoid levels in the last laid eggs might be a result of optimal allocation, since they might affect the rate of brood reduction, as Royle et al. (2001) suggested. Indeed, the experimental study III strongly supports the idea; first hatched nestlings survived longer when they were swapped to last hatching positions compared to non-manipulated last hatched nestlings. Instead, these originally first ones grew more slowly in the last hatched positions than naturally last hatching nestlings. Furthermore, experimentally enhanced testosterone levels of the first laid eggs increased the growth rate of those nestlings (III). In addition, testosterone treatment negatively affected the survival probability of nestlings originating from last laid eggs, but not nestlings from first laid eggs. Thus, I suggest that higher levels of carotenoids enhance the survival of the first hatched nestlings and testosterone instead accelerates brood reduction by the death of the last hatched nestlings. However, testosterone enhances growth to some extent but, in high doses, as in last laid eggs with experimentally enhanced testosterone levels, it can also reduce growth (III). This can be due increased physiological stress induced by a testosterone metabolism (von Schantz et al. 1999, Sørensen et al. 2003). Stress

induced heat shock proteins (HSPs) protect the body against protein abnormalities caused by external or internal stressors, but in high levels can themselves be costly (Sørensen et al. 2003). I found that last hatched nestlings, without any manipulation, have higher stress levels than their earlier hatched nest mates (IV), thus increased testosterone may increase metabolic costs to the level that it trade-offs with growth.

In environmental conditions that turn unfavourable after hatching, egg constituents may induce brood reduction. Asynchronous hatching further speeds up the process (III). When last hatched nestlings were manipulated to grow in first hatching positions, they had as good survival as the first hatched controls. In addition, even if first hatched nestlings survived longer when manipulated to grow in last position, their survival until fledging was significantly lower than the survival of the first hatched controls. Thus, it is not just the egg quality or hatching asynchrony, but these two mechanisms together, which produce the adaptive phenomenon, brood reduction, in magpies. Thus in magpies, in contrast to gulls (e.g. Graves et al. 1984, Forbes et al. 1997), the last laid eggs may not be considered as insurance eggs. Instead, in good post-hatching environmental conditions, the negative effects of testosterone metabolites may be compensated for by antioxidant supply by feeding parents. Moreover, mothers compensate for the negative effect of testosterone on maternal antibodies (IV) by allocating more immunoglobulins to last hatching nestlings (I, IV & V). This may be important for the survival of the last hatched nestlings (II) when brood reduction is not needed. The amount of maternal immunoglobulins positively affects the levels of immunoglobulins produced by a nestling. This may compensate for the other more costly immune defences (Klasing & Leshchinsky 1998). For example, I found that females in the last hatching position had weaker cell-mediated immune response (CMI) at the age of one week than females in earlier hatching positions, while in males such a relationship did not exist (V). This contrasts with the results of Dubiec et al. (2006), where male blue tit (*Cyanistes caeruleus*) nestlings were more sensitive to poor rearing conditions than females. Also in Eurasian kestrel (*Falco tinnunculus*) male nestling in nest with starvation had weaker CMI than females (Fargallo et al. 2002), but in kestrels males are smaller than females. Moreover, immunoglobulin levels measured in magpie nestlings a few days later after CMI challenge were higher in females than in males (V). Egg carotenoid content has been shown to have a significant positive effect on offspring CMI in the barn swallow (*Hirundo rustica*) (Saino et al. 2003). Moreover, higher levels of testosterone in the last laid eggs (I) can be one factor weakening the efficiency of the CMI of the last hatched female nestlings, especially if females are more sensitive to the effects of high levels of testosterone than males. CMI was also related to growth; a nestling growing well in a time of immune challenge had high level cell-mediated immune response (V). This result is supported by González et al. (1999) who found that nutrient rich food enhanced the CMI of juvenile house sparrows (*Passer domesticus*). It is also in line with the result of better CMI in rural bred nestlings compared to urban bred ones (V).

The effects of egg quality on the individual's future fitness are poorly known. However, the very recent study of Alonso-Alvarez et al. (2006) indicates that early growth conditions affected the individual's resistance to oxidative stress in interaction with age. Birds sensitized to stress at a young age were more resistant to it at an older age. This early sensitisation was found to affect a nestling's future reproductive strategy and even suggested to have an effect on population dynamics (Alonso-Alvarez et al. 2006). Furthermore, both maternal androgens (Eising et al. 2006) and maternal antibodies (Grindstaff et al. 2006) can have long lasting effects on offspring performance.

5 CONCLUSIONS

These results provide evidence that allocating maternal factors differentially into eggs may be an adaptive evolutionary mechanism to create within-clutch sibling hierarchies, but may be constrained by environmental factors such as food availability and quality (I, II, III, IV, V & VI). The within-clutch variation in egg components, together with asynchronous hatching, serves as an adaptive physiological mechanism for brood reduction, and might be a basis for parent-offspring conflict, since mothers create hierarchies in their clutches through optimal allocation of resources into their eggs (I, III, IV & VI). Furthermore, some of these egg components are costly for the mother (I & II). Albumen constituents and their costs for the mother are still poorly understood but may have major impact on hatching success (I & VI). Androgen deposition into eggs may instead have indirect costs: allocation of antibodies in to eggs is costly (I & II), and mothers had to compensate the loss of antibodies in later laid eggs caused by higher levels of testosterone included toward the end of the laying sequence. I was able to show that maternally allocated testosterone increases the costs to the mother as she has to compensate for the lost of antibodies, which are resource limited, caused by enhanced testosterone levels in later laid eggs (IV). Furthermore, the clutch size is relatively large (5 to 11 eggs), which does not support the view of having one or two insurance eggs in the clutches. If brood reduction is working properly, females can, without major extra costs, lay large clutches, since the costs of raising extra young are probably greater than those of laying some extra eggs. However, there is some contrasting evidence in gulls, since producing one extra egg in black-backed gulls reduced parental ability to rear their brood (Monaghan et al. 1998). Still, the risk of loosing the investment put in couple of eggs may pay off, if there is a possibility that young are succeeded to be raised, since the future of the same parents is also risky. Thus, the quality and age of the parents, in the manner of their expected future reproductive success should be related to their clutch size and brood reduction risk.

There was some evidence for differences in egg quality between the rural and urban areas (VI). Moreover, the urban centre in the study, Jyväskylä, is relatively small and closely connected to rural areas, and thus even stronger effects are expected when the urban habitats of larger cities are compared to rural habitats. This study, (VI), together with study V, provides important information on the effects of habitat quality on several egg quality parameters (VI).

Higher maternal immunoglobulin levels at hatching positively affected the development of adaptive immunity in this species (II). This may be important for the offspring in future, since nestlings' total antibody levels have been found to have a significant relationship to general antibody responsiveness (Kilpimaa et al. 2006). In mammals, specific maternal antibodies against a common pathogen (PUUV) are related to delayed infection and earlier maturation (Kallio et al. 2006). Moreover, adaptive immunity is less costly to maintain through out the life time than innate immune functions (Klasing & Leshchinsky 1998), even though the development of adaptive immunity traded-off with growth early in life in magpie nestlings (II). In long living species, investment in adaptive immunity should pay its cost back later in life (Klasing & Leshchinsky 1998).

To conclude, maternal effects may provide an adaptive phenotypic response to environmental variation, with consequent effects on variation in offspring fitness (Mousseau & Fox 1998) and even on population dynamics (Alonso-Alvarez et al. 2006). Future research should focus on the egg components and their relationship to chick performance and life history decisions. Furthermore, it is still unclear how maternally modified phenotypes are related to future success, e.g. winter survival and reproductive success.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Harakan äitivaikutukset

Äitivaikutuksiin kuuluvat ympäristön aiheuttamat piirteet äidin ilmiasussa, jotka siirtyvät äidiltä jälkeläisille, ja jälkeläisen ilmiasun muovaaminen emon jälkeläisilleen jakamien resurssien avulla. Äitivaikutukset voivat olla tehokas keino sopeuttaa jälkeläiset erityyppisiin ja vaihteleviin ympäristöoloihin ilman suoraa perinnöllisen sopeuman syntymistä, ja niillä voi olla merkittävä vaikutus jälkeläisen kelpoisuuteen. Linnuilla emo voi vaikuttaa poikastensa laatuun jo munan laadulla. Poikanen saa munassa kaikki ensimmäisiin kehitysvaiheisiin ennen kuoriutumista tarvitsemansa resurssit. Tämän lisäksi emo voi muokata poikastensa ilmiasua sekä haudonta- että hoivakäyttäytymisellä. Munan laadun kautta tulevat äitivaikutukset, kuten myös haudontakäyttäytymisellä aikaan saatu poikasten eriaikainen kuoriutuminen voivat aiheuttaa kustannuksia sekä emolle että poikaselle.

Väitöskirjassani olen tutkinut äitivaikutusten merkitystä sopeumana vaihteleviin ympäristöoloihin sekä pesän sisällä että pesien välillä pesäviipyisiin varpuslintuihin kuuluvalla harakalla (*Pica pica*). Pääasiallisena tavoitteena oli selvittää peilaako munassa olevien yhdisteiden määrä vain emon kuntoa pesimäkauden alussa ja muninnan aikana, vai voiko näiden poikaselle tarjolla olevien yhdisteiden määrän vaihtelun takana olla myös evolutiivisesti merkittävä sopeuma. Tämä lähtökohta sisältää myös vanhempien haudontakäyttäytymisen aiheuttaman pesän sisäisen sisarusten välisen hierarkian vaikutuksen.

Havaitsin väitöskirjatyössäni, että tärkeiden yhdisteiden siirtoon jälkeläiselle munassa vaikutti sekä saatavilla olevien resurssien määrä, joka heijastaa ympäristöolosuhteita, että emon aiheuttama manipulointi pesyeen sisällä. Monet näistä munanlaatutekijöistä, kuten antioksidantit, hormonit ja vasta-aineet vaikuttivat merkittävästi jälkeläisen ilmiasuun ja selviytymiseen. Ne myös toimivat yhdessä eriaikaisen kuoriutumisen kanssa optimaalisena poikuekoon pienennysmekanismina vaihtelevissa ympäristöolosuhteissa. Ympäristöolojen ollessa hyvät ja ravinnon määrän runsas vanhemmat voivat kasvattaa onnistuneesti koko poikueen. Tällöin viimeisenä kuoriutuneet poikaset voivat saamiensa korkeampien hormonipitoisuuksien ja maternaalisten vasta-aineiden ansiosta kasvaa kiinni vanhemmat sisarensa ennen pesästälähtöä. Jos olosuhteet muuttuvatkin heikoiksi pesinnän aikana, poikuekoon pienennys tapahtuu nopeasti hormonimetabolian ja kovan sisaruskilpailun aiheuttaman fysiologisen stressin vuoksi. Tällöin ensimmäisenä kuoriutuneilla poikasilla, jotka ovat saaneet emoltaan enemmän suojaavia antioksidantteja ja ovat kuoriutumisen eriaikaisuuden vuoksi suurempikokoisia, on parantuneet mahdollisuudet selviytyä. Tutkimuksessani havaitsin myös, että emot voivat munan laatua muuttamalla luoda erilaisia ilmiasuja jälkeläisilleen. Näillä vaihtelevilla ilmiasuilla voi olla erilainen lisääntymisstrategia tulevaisuudessa ja myös eri kelpoisuusodotus riippuen tulevista oloista.

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