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Ecology and Evolution of Parental Care in the Black-billed Magpie (*Pica pica*)

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이 원 영

Ecology and Evolution of Parental Care in the Black-billed Magpie (*Pica pica*)

Won Young Lee

Advisor: Professor Piotr G. Jablonski, Ph.D.

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Seoul National University

ABSTRACT

Lee, Won Young

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Seoul National University

This thesis concerns with parental care behaviors in black-billed magpies including incubation and food allocation by parents. First, I investigated how incubation affected microorganisms on eggs using real-time PCR and pyrosequencing methods. I found that incubation increased overall bacteria contrary to previous studies. Pathogenic species reduced while beneficial/commensal bacteria increased. The results suggest that avian incubation, potentially due to difference in sensitivity to dehydration on the egg surface among microbes or due to the presence of antibiotics that certain bacteria produce, promote the growth of harmless or benevolent bacterial taxa and suppress that of pathogenic ones and consequently reduce the diversity of microbes on the egg surface. Second, I questioned if mothers consistently allocate more antibodies and more parental care (e.g. feeding) to the same offspring. By analyzing video-recordings of parental visits to the nests, I found that female parents' feeding was related with the amount of maternal antibodies that they injected at egg-laying. Also, males and females had different feeding strategies implying the existence of sexual conflict. Third, I report prolonged brooding of females and behavioral conflict during the feeding period between the pairs. Within the same pair, the duration of the conflict period was shortened in the consecutive years and the duration of the conflict period was shorter for pairs with larger territories. The results suggest that this unique form of sexual conflict is more likely to exist related with the quality, age and/or the past experience of the pair and the pairs seemed to

adjust the conflict maintaining the pair-bonds over the years. Lastly, I manipulated brood sex ratio at hatching and estimated whether parental feeding affects the brood sex ratio adjustment. I found that parents who initially created male-biased broods provided more food to female nestlings and parents who created female-biased broods provided more to male nestlings. The sex-specific mortality of nestlings was affected by the original sex ratio that the parents created. The results suggest that magpie parents shape brood sex ratio by differential feeding to adjust the sex ratio bias which was initially induced at the early stage of parental investment.

Keywords: parental care, microorganism, offspring sex ratio, sexual conflict, magpie, *Pica pica*

Student number: 2010-30101

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Chapter 1.

General introduction

General Introduction

Parental care

Parental care is defined as any form of parental behavior that increases the fitness of a parent's offspring (Clutton-Brock 1991). Theory predicts that parents should increase their offspring's survival by providing efforts to protect them from predators, food shortages and environmental hazards, to maximize their fitness returns. In birds, parental care involves a serious of parental behaviors including nest building, egg incubation, and then defending and feeding the nestlings.

During the incubation period, as a parental behavioral mechanism, incubation was hypothesized to inhibit microbial growth on eggshells (Cook et al. 2005ab, Shawkey et al. 2009). Avian incubation is a process to keep the eggs at a regular temperature and humidity range, which is necessary for embryonic development (Deeming 2002). Since embryos in the eggs are highly dependent during the egg stage on the parental incubation to develop and hatch successfully, parents control temperature and humidity on eggshell surfaces (Deeming 2002) spending energetic costs of 10-20% weight loss (Rahn and Paganelli 1990). Recent studies showed that incubation behavior reduces microbial growth by drying eggs (D'Alba et al. 2010, Ruiz-de-Castañeda et al. 2011a) or by antibiotic-producing bacteria from oil glands (Soler et al. 2010). The effect of incubation on the bacterial loads on the eggshells was studied in tropical environment (Cook et al. 2005ab, Shawkey et al. 2009). However, it is still unclear whether it is applicable to the birds in temperate areas (Wang et al. 2009, Ruiz-de-Castañeda et al. 2011b).

Parental investment theory predicts that parents adjust their current expenditure to expected future benefits of parental care and this is in relation

to past investment (Trivers 1972, Maynard Smith 1977, see Clutton-Brock 1991). Since past investment reduces parents' capacy for prospective investment, the benefits of future expenditure are related with the past investment of parents (See Maynard Smith 1977). During the period of parental care, parents may favor certain nestlings in order to ensure the survival of the nestlings that give them greatest fitness return. During egg production, mothers may deliver materials (such as immune proteins and hormones; Rollier et al. 2000, for review see Groothuis and Schwabl 2008) that help the nestlings against pathogens into the favored eggs, whereas fathers' means for favoritism is highly limited before hatching. In order to perform maternal favoritism more efficiently, mothers are expected to feed more those nestlings to whom they earlier delivered more materials during egg production. Although the effect of maternally derived antibodies on nestling growth and survival has been rigorously studied (Heeb et al. 1998, Grindstaff 2003), the potential correlation of maternally derived antibodies with the provisioning rules of mothers after hatching has not been investigated yet. Considering that both, the maternal transmission of antibodies and the provisioning effort, are presumably costly (both are known to be condition-dependent), one can predict that these two aspects of breeding strategies of mothers are tightly linked to each other. Fathers' provisioning rule might be different from mothers' rule (e.g. blue tits, Dickens and Hartley 2007) which can imply sexual conflict between male and female parents.

Parents invest differentially to male and female offspring and thus to adjust offspring sex ratio in a way to maximize the parents' fitness (Fisher 1930, Trivers and Willard 1973, Charnov 1982). Fisher's equal allocation theory (1930) presented that male and female ratio is adjusted by selection because frequency-dependent selection stablizes the offspring sex ratio to near an equal state. Conversely, Fisher's theory means that parents would bias the sex ratio unless the resource investment is equal in the two sexes. Trivers and

Willard (1973) hypothesized that parents would bias the sex ratio of offspring to increase their fitness returns in different environmental conditions. Since Triver and Willard, many empirical studies supported the hypothesis that parents adjust their relative allocation to males and females (see Hardy 2002 and West 2009). In birds, sex ratio adjustment by parents can be achieved by controlling offspring's sex at birth (West and Sheldon 2002, Cassey et al. 2006) or inducing differential mortality of embryos (Krackow 1995, Palmer 2000) or nestlings (Wiebe and Bortolotti 1992, Torres and Drummond 1999, Lee et al. 2010a). If parents can regulate initial brood sex ratio, offspring sex ratio would be adjusted at an early stage (West and Sheldon 2002). On the other hand, if parents cannot afford to adjust initial brood sex ratio, for instance by random segregation of sex chromosomes (Charnov 1982), or if they are under the pressure of unpredictable environment throughout the nestling rearing period (Lee et al. 2010a), offspring sex ratio would be adjusted at a later stage. However, it is still unclear whether parents induce adaptive sex ratio adjustment by affecting sex-specific nestling mortality during the period of parental care.

In bi-parental care animals, male and female parents face a situation whether either parent invest more than their mates. When parents cares for offspring, sexual conflict over parental care occurs to exploit the other to pay more investment. In parental care of animals, male and female parents face a situation whether either parent invests more than their mates. Since parents pay the cost of their own investment but benefit from the total investment by both parents, each parent would benefit from larger investment by the other parent (Lessells 2012). Hence, each sex should prefer the other to invest more in sharing of parental care. In bi-parental species, there are also sexual conflicts over the relative amount of care during the period of parental care and this may lead to the evolution of parental behavior in both sexes to shift the load towards the other sex (Arnqvist and Rowe 2005).

Parental care in the Black-billed Magpie

In this thesis, I studied parental care in the Black-billed Magpie (*Pica pica*) focusing on incubation behavior and feeding behavior during the breeding season. For parents, it would be important to incubate eggs and feed nestlings effectively. In magpies, brood reduction is commonly observed (Birkhead 1991) and the parents cannot afford to raise all the eggs which were initially laid by females in most cases. Thus, I expected that parental strategies would be evolved to defend the eggs from microbial infection and. Moreover, after hatching, it would be important for parents to allocate food with limited resources efficiently.

Magpie is a socially monogamous bi-parental bird. A magpie population has been monitored since 1998 in Seoul National University (SNU) campus area as a part of long-term ecological monitoring project. On magpie eggshells, there are microbes which can harm embryos inside the eggs (Soler et al. 2011). Therefore, I expected that the eggshell microbes could affect the egg hatchability and parents would evolve to protect the eggs against microbial infection. In parental investment strategies, I predicted that maternal investment would be maintained from egg-laying to feeding. Because both transmission of maternal materials and feeding activity are costly, these two aspects of breeding strategies of mothers were expected to be tightly linked. Also, previous studies on magpies showed that nestlings with more maternal antibodies at hatching produced more antibodies de novo (Philaja et al. 2006). Thus I predicted that the nestlings with more antibodies at hatching were favored and acquired more provisioning effort by the parents. Although magpie parents seem to feed their nestlings differentially (Moreno-Rueda et al. 2007), a difference in feeding strategies between male and female parents has not been investigated yet. In previous studies in the same magpie population, parental feeding and brood sex ratio adjustment were studied (Lee

et al. 2010ab, 2012). Lee et al. suggested that parental feeding was responsible for the nestling mortatlity durint the feeding period. Considering that the mortality is mostly due to starvation in magpies, parental feeding is crucial for nestling survival. Also, magpies have relatively a long period of time for parental care (20-21 days for incubation and 30-35 days for feeding nestlings) and different sex roles in male and female parents (Birkhead 1991).

In this study, I investigated (1) how incubation affects microorganisms on eggs using real-time PCR and pyrosequencing method, (2) if mothers consistently allocate more antibodies and more parental care (e.g. feeding) to the same offspring, (3) if parental feeding affects the brood sex ratio adjustment by manipulating brood sex ratio at hatching by analyzing video-recordings of parental visits to the nests, and (4) prolonged brooding of females and 'behavioral conflict' during the feeding period between the pairs.

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Chapter 2.

Bacterial communities on magpie eggshells and the effects of incubation in a temperate zone

Abstract

Microbial infection causes mortality of embryos inside the egg and thereby greatly reduces the effectiveness of parental effort. Incubation behavior was hypothesized to inhibit the transmissible pathogenic microbial growth on eggshells. However, most researchers have focused on the effect of incubation on microbes, without proper evaluation of the causal effect of incubation on hatching success through suppressing microbial growth. Moreover, traditional culture-dependent techniques have been used to determine the presence and abundance of microbes, which requires a careful data interpretation. Using a culture-independent quantification method, real-time PCR, we investigated whether (i) incubation effort increases egg hatchability, (ii) incubation inhibits microbial growth on eggshell surface, and (iii) microbial presence and growth decreases hatching success. We sampled microorganisms from magpie eggshells at early incubation stage (at day 3 after laying) and late stage (at day 18) and quantified total bacteria and the expected microbial species, such as pathogenic microorganisms and anti-biotic producing bacteria. Here, we showed that female incubation increased total bacterial abundance while *Escherichia coli* abundance was suppressed by incubation. As female spent more time for incubation, egg hatchability increased. In our results, incubation increased overall bacteria contrary to previous studies. However, pathogenic species reduced while beneficial/commensal bacteria increased. This suggests that Furthermore, the changes of *E. coli* abundance at early incubation stage significantly decreased hatching success. Presence of *Candida albicans*, a fungal pathogen, affected hatching success. Our results provide evidence that the incubation increases hatching success by suppressing microbial growth on eggshells in wild birds.

INTRODUCTION

Bacterial infection on the eggshells of wild birds is an important factor that determines the fitness of both parents and offspring. Since pathogenic microorganisms on egg surface may decrease egg viability through trans-shell transmission (Bruce and Drysdale 1994, Pinowski et al. 1994, Cook et al. 2003, 2005ab), any defence mechanism against proliferation of microbes on the egg surface would be advantageous to ensure the viability of embryos inside the eggs. It has been suggested that parental behavioral mechanism, incubation behavior, inhibit microbial growth on eggshells by reducing the humidity on the egg surface (Shawkey et al. 2009, D'Alba et al. 2010, Ruiz-de-Castañeda et al. 2011a) or by the presence of antibiotic-producing bacteria from feathers (Peralta-Sanchez et al. 2009) or oil glands (Soler et al. 2008). In contrast to wet tropical environment where incubation seems to dry the eggs and the proliferation of general microbes on the egg surface is suppressed (Shawkey et al. 2009), recent studies conducted in temperate environments produced conflicting results. Whereas some found incubation decreases bacterial loads on the eggs (D'Alba et al. 2010, Ruiz-de-Castañeda et al. 2011a), others failed to find significant changes in bacterial loads on the incubated eggs (Wang et al. 2009, Ruiz-de-Castañeda et al. 2011b). Therefore, it is still unclear whether incubation in temperate environment inhibits microbial activities on eggs as it does in tropical environment.

Nearly all of previous studies on bacterial loads on the eggs have used culture-based methods (Cook et al. 2005a,b, Ruiz-De-Castañeda et al. 2011ab, 2012, Walls et al. 2012; except Shawkey et al. 2009). Although culture-based quantification of microbes has its own merits such as easiness to implement and low cost, it can only provide data on the abundance of specific target microbes and fails to provide any measures of diversity at a community level.

Moreover, less than 1 % of microbial species are culturable (Amann et al. 1995) and only some of the microbes have been under study. There also can be problems with left censoring in counting the colony numbers (Lee et al. 2013).

The effect of incubation on the bacterial loads on the eggshells in tropical environment was studied using both culture-dependent (Cook et al. 2005ab) and culture-independent (Shawkey et al. 2009) methods but the results were somewhat different. In their study using culture method, Cook et al. found that, although overall abundance of microbes decreased, the abundance of pathogenic bacteria decreased and that of harmless bacteria (Gram-positive rods) increased with incubation. However, in a study that employed culture-independent method on the same species, Shawkey et al. found that both microbial abundance and diversity decreased with incubation and they did not find any evidence on the selective proliferation of harmless bacteria by incubation. The reason why the two studies on the same species and same study population found somewhat different results remains unclear; it might be due to methodological difference or difference in sampling years (2002 for Cook et al.'s study and 2007 for Shawkey et al.'s study).

On the other hand, the effect of incubation on the microbial community on the eggs in the temperate regions was never investigated using culture-independent method. Here, we employed culture-independent methods to examine the effect of incubation on the structure of microbial community on the eggshell. First, we conducted quantitative PCR (or real-time PCR), which is known to be more sensitive than culture-based method (Klein 2002, Epsy et al. 2006), to estimate total bacterial abundance. Second, we performed pyrosequencing of the 16S rRNA gene to examine diversity and relative abundance of microbes on the eggshells. NGS (next generation sequencing) approaches, including pyrosequencing, allow researchers to find microbial community structures by reading 16S rRNA gene sequences of bacteria from

complex samples (Edwards et al. 2006). Among other culture-independent methods (e.g. microarrays, Shawkey et al. 2009), pyrosequencing technology has been a powerful tool to study microbial samples since this method is suitable to estimate unknown microbial community by searching bacteria-specific sequences in 16S rRNA (for review see Roh et al. 2010). To our knowledge, this is the first detailed study to quantify absolute numbers of the total bacterial abundance on avian eggshells by quantitative PCR and estimate microbial community structures by 16S rRNA gene pyrosequencing.

MATERIALS AND METHODS

Study population and microbial sampling

This study was conducted on the magpie population at the Seoul National University (SNU) campus (37°47' N, 126°95' W; Seoul, Korea). From early March in 2012, we regularly visited magpie nests to determine the date of egg-laying. Incubation lasts 21-22 days after first egg laying and it starts in several days after the first egg is laid (Birkhead 1991, mostly in 3-4 days in our population, see Lee et al. 2013). For our samples, magpies started laying eggs from 14th March continued until 8th May. The average daily temperature was 13.57 °C (±6.31, SD) and the relative humidity was 51.29 % (±14.57, SD) throughout the incubation period.

Microbial sample from the eggshell was collected twice per nest, at day 3 (before incubation started) and day 18 (before hatching) during the incubation period. We sampled 9 incubated nests and 3 non-incubated (control) nests, in total 24 eggs. At day 3, we chose one egg randomly, swabbed the surface by rubbing a sterile rayon swab (Yuhan Lab Tech Co., Korea) wetted with sterile sodium phosphate buffer (0.2 M, pH 7.2). We used

one swab for one egg in a clutch and measure the dimension of the egg and mark the egg ($S = 3 \times L^{0.771} \times W^{1.229}$, S is the surface area of eggs, L is length, and W is width; formula from Narushin 1997, for details, see Soler et al. 2011). The swab was put into the 1.5 ml tube containing 500 ul of buffer solution and the tube was placed in a handy container with icepacks until we finished field work. Then, the samples were stored in the deep freezer (-20 °C, preferably -50 °C). At day 18, we chose one egg randomly except the egg which was marked before and sampled the egg by the same method at day 3.

For a control treatment, we prepared three artificial nests in April, around the middle of time of egg-laying period, by putting the nest materials collected from abandoned nests. We then placed two quail eggs cleaned with bactericidal disinfectant 70 % isopropyl alcohol wipes (Yuhan-Kimberly, Korea) inside each artificial nest. There was no difference in the structure of microbial community on the eggshell surfaces between quail eggs and magpie eggs before incubation (Welch two sample t-test, $t = 0.87$, $df = 10.38$, $p = 0.40$). The artificial nests were put at a similar height of magpie nests and distributed around SNU campus. We sampled the quail eggshells twice as we did with magpie eggs (one egg at day 3 after putting the eggs and another at day 18). The average temperature and humidity between the two sample dates were 13.93 °C (± 3.04, SD) and 55.33 % (± 18.41, SD). From the swab samples, we extracted DNA with the MoBio PowerSoil DNA kit (MoBio laboratories, Carlsbad, CA, USA) as directed by manufacturer's instructions.

Quantitative PCR

Extracted DNA was amplified in a qPCR machine (Illumina Eco™, Diego, CA, USA) with a bacterial universal primer set (338F: 5'-ACT CCT ACG GGA GGC AGC AG-3', 518R: 5'-ATT ACC GCG GCT GCT GG-3'; see Fierer et al. 2005) to detect the abundance of total bacteria. PCR assays

were performed with 5 µl of QuantiMix SYBR 2× (PhileKorea Technology, Seoul, Korea), 0.25 µl of 10 pmol/L forward and reverse primer, 3.5 µl of ddH_2O, and 1 µl of DNA extract of total 10 µl cocktail solution. PCR thermal condition was as follows; initial polymerase activation for 10 min at 95 °C, PCR cycling for 10 sec at 95 °C and for 30 sec at 60 °C for 40 times, The melting curve was obtained by lowering the temperature from 95 °C to 55 °C. To generate standard curves, we designed recombinants of 16S rRNA target gene (Eub338F-518R region) in cloning vectors. By this method, we quantified the absolute numbers of total bacteria inferred from the reliable standard curves (for details see Lee et al. 2013). First, genomic DNA was extracted (Wizard Genomic DNA Purification Kit, Promega, Madison, USA) from an Escherichia coli K-12 MG1655 which was kindly donated from Dr. Chun's lab in Seoul National University. Then, the extracted DNA was amplified (GoTaq Green Master Mix, Promega, Madison, USA) with 338F-518R primers and the amplified species-specific gene fragments were inserted into cloning vectors (3003 bp, pGEM®-T Easy Vector, Promega, Madison, USA) transformed in competent cells (Hit-DH5α, RBC Bioscience, Taipei, Taiwan). Recombinants of cloning vector was selected in LB (Luria-Bertani) plates with ampicillin (100 µg/ml), IPTG (isopropyl beta-D-thiogalactoside, 0.5 mM), and X-Gal (5-bromo-4-chloro-3-indolyl β-d-galactoside, 80µg/ml). Then, plasmid mini prep kit (Hybrid-Q™, GeneAll Biotechnology, Seoul, Korea) was implemented to purify the recombinant plasmid DNA and the concentration of the plasmid DNA was measured with Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, Carlsbad, USA) and TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, USA). The corresponding DNA copy numbers were calculated using the following equation (Whelan et al. 2003, see Lee et al. 2006):

$$DNA copy number = \frac{6.02 \times 10^{23} (copy/mol) \times DNA amount (g)}{DNA length (bp) \times 660 (g/mol/bp)}$$

Standards were serially 10-fold diluted from 1×10^0 to 1×10^8 copies/µl. PCR was performed three times for each sample and the PCR results satisfied the following requirements: the efficiency of the standards were between 90 % and110 % , R^2 was higher than 0.99, and the standard deviation of PCR replicates was less than 0.167. PCR results were analyzed with Eco™ software (version 3.0). PCR results of Ct (threshold cycle) values were converted to the absolute DNA quantity by comparing the Ct values to the serially diluted standard curves (for details see Heid et al. 1996). Melt curves were checked by increasing temperature from 55 °C to 95 °C every 0.5 °C.

16S rRNA gene pyrosequencing and Data processing

Extracted DNA was amplified targeting the V1 to V3 region of 16S rRNA using primers (V1-9F: 5'-Adapter A-AC-GAG TTT GAT CMT GGC TCA G-3', V3-541R: 5'-Adapter B-barcode-AC-WTT ACC GCG GCT GCT GG-3'). The 9F primer included a 454 sequencing adapter A and a 2-bp linker sequence (AC) and the 541R incorporated a 454 adapter B, 8-10 bp barcodes specific to each sample, and a AC linker. PCR assays were performed with 0.25 µl of Pwo SuperYield DNA polymerase (Roche, Mannheim, Germany), 2 µl of 20 pmol/L forward and reverse primer, 2.5 µl of 1× BSA, 5 µl of 10×buffer, 1 µl of 10µM dNTPs, 38.25 µl of $dddH_2O$, and 1 µl of DNA extract of total 50 µl cocktail solution. PCR condition was as follows: initial denaturation for 5 min at 95 °C, 10 cycles of denaturation for 30 sec at 94 °C and annealing for 45 sec at 60°C to 55 °C (-0.5°C per cycle) and elongation

for 90 sec at 72 °C for 40 times, and additional 20 cycles of denaturation for 30 sec at 94 °C and annealing for 45 sec at 55 °C and elongation for 90 sec at 72 °C. The amplified PCR products were purified with the QIA-quick PCR purification kit (Qiagen, CA, USA) and then mixed in a pool. Pyrosequencing was performed by Macrogen Incorporation (Seoul, Korea) using 454/Roche GS-FLX Titanium Instrument (Roche, NJ, USA).

All the sequences were processed by trimming sequences to remove the unique barcode, linker, and primer sequences at both ends. In addition, we removed short sequences which were less than 300 base pairs, low-quality sequences (maximum homopolyer of 8 bp, minimum ambiguous base of 1, and minimum quality score of 25), and the sequences which had no matches to the 16S rRNA gene databases in BLASTn search (the expectation value was more than 10^{-5}) to reduce sequencing error (Unno et al. 2010). The resulting sequences were further denoised using single linkage preclustering (SLP) algorithm and putative chimeras were additionally removed by using UCHIME (Edgar et al. 2011) implemented in mothur (Schloss et al. 2009). Quality-checked sequences were taxonomically assigned using EzTaxon-e database (Kim et al. 2012).

Statistical methods and taxonomic analysis

We used paired t-tests to compare the total bacterial abundance (log transformation) and bacterial diversity (Shannon diversity index) at day 3 and day 18 in incubated and control nests, with SPSS version 20.0. Shannon index was calculated based on operational taxonomic units (OTUs), which were defined at 97% similarity cutoff of 16S rRNA gene sequence, by a randomly selected subset of 742 reads per sample in Primer v6 (Clarke and Gorley 2006).

We estimated Bray-Curtis dissimilarity between bacterial communities at day 3 and day 18 within nests to generate a non-metric multidimensional scaling (NMDS) using Primer v6 (Clarke and Gorley 2006). We compared Bray-Curtis dissimilarities between incubated and control nests by Welch two sample t-test in R (Roberts 2013). We used Analysis of Similarity (ANOSIM) analysis, a non-parametric method for estimating differences between groups based on 1,000 random permutations, to compare the bacterial community structures at day 3 and day 18 between incubated and control nests. NMDS and ANOSIM analysis were performed in Primer v6 program.

Compositional analysis (Aitchison 1986, see Aebischer and Robertson 1993) was used to examine the effect of incubation on the bacterial composition at the phylum and genus levels on the dominant taxa. This approach is based on the log-ratio analysis of compositions to overcome problems of proportional data (Aitchison 1986), for instance the proportions are dependent each other in a group and the sum of proportions is one (Aebischer and Robertson 1993, Cummins and O'Halloran 2002). We chose 4 major groups at the phylum level (>90% of all sequences) and 10 major groups at the genus level (>50 % of all sequences). Then, we divided the proportions of the major groups by the sum of the rest of groups and log-transformed the values. If the proportions are zero, we replaced the zeros with 0.0001 (Cummins and O'Halloran 2002). The log-ratio values were analyzed using repeated measures MANOVA in SPSS 20.0.

We performed a Dufrene-Legendre indicator species analysis to find bacterial taxa which are strongly associated to particular groups (Dufrene and Legendre 1997) using the 'indval' function in the 'labdsv' package in R software (version 3.0.1). This analysis provided an indicator value, which quantifies fidelity and specificity of species in a group, and a statistical significance value by permutation. Indicator value ranges from 0 to 1 and

provides a measure of the strength of the association between the taxon and the particular group.

RESULTS

Total bacterial abundance estimated by quantitative PCR highly increased (approximately 26 times on average) during the incubation period on incubated eggs while there was, but no significant increase on non-incubated eggs (Figure 2-1; Paired t-tests, $t = -2.67$, $df = 8$, $p = 0.03$ on incubated eggs and $t = -0.17$, $df = 2$, $p = 0.88$ on non-incubated eggs).

Using pyrosequencing data, we detected 4434 OTUs (operational taxonomic units), 1590 genera, 553 families, 221 orders, 90 classes and 32 phyla of bacteria in total. Bacterial diversity (Shannon index) was significantly reduced on incubated eggs but it did not on non-incubated eggs (Figure 2-2; Paired t-test, $t = 2.48$, $df = 8$, $p = 0.04$ on incubated eggs and $t = -0.13$, $df = 2$, $p = 0.91$ on non-incubated eggs). NMDS plots showed that, whereas the bacterial assemblages on non-incubated eggs remain unchanged between day 3 and 18 (Figure 2-3B), those on incubated eggs changed substantially (Figure 2-3A; Welch two sample t-test using Bray-Curtis dissimilarity, $t = 2.74$, $df = 5.64$, $p = 0.04$). Bacterial community structures did not differ between incubated eggs and non-incubated eggs either at day 3 (ANOSIM, $R = 0.22$, $p = 0.84$) or at day 18 ($R = 0.01$, $p = 0.55$).

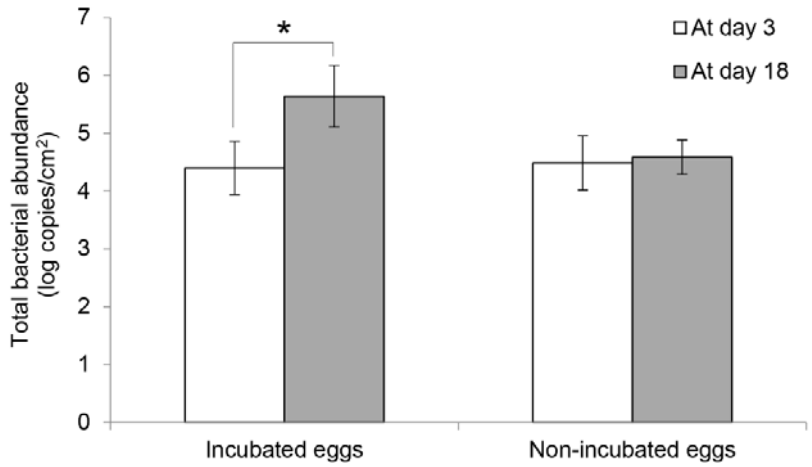

Figure 2-1. Bacterial abundance on eggshells on incubated and non-incubated eggshells. Total bacterial abundance (log copy numbers per square centimeters, ±SE) was measured by log copy numbers per square centimeter in 9 nests and 3 control nests at day 3 (before incubation) and day 18 (before hatching) after egg laying, using quantitative PCR with a bacterial universal primer set (333F-518R). Asterisk means a significant difference ($p<0.05$) by paired t-test between at day 3 and day 18.

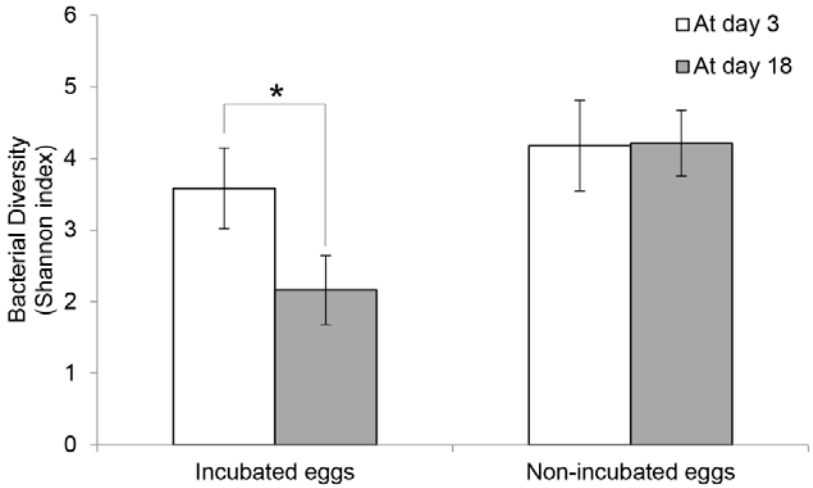

Figure 2-2. Bacterial diversity (Shannon index) on incubated and non-incubated eggshells. Bacterial diversity (±standard error) was estimated by Shannon index with 570 sequences per sample acquired from 16S rRNA gene pyrosequencing, at day 3 and day 18 after egg laying in 9 incubated nests and 3 control nests. Asterisk means a significant difference ($p<0.05$) by paired t-test between at day 3 and day 18.

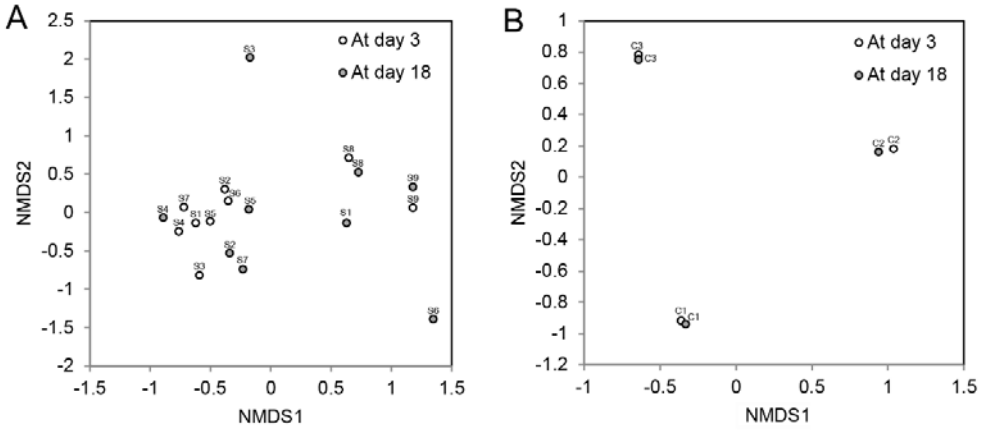

Figure 2-3. NMDS plots of bacterial community structure at day 3 and day 18 after first egg-laid, in 9 incubated nests (A) and three control nests (B).NMDS (Nonmetric Multidimensional Scaling) plots were generated with Bray-Curtis dissimilarities between samples in 9 incubated nests, S1-S9, in (A) and 3 control nests, C1-C3, in (B). NMDS plot is mapping the bacterial structure of each sample considering the relative distances with other samples, based on both bacterial composition and abundance.

Relative abundance of four major bacterial phyla (comprising more than 90 % of all sequences) is shown in Figure 2-4, and that of 10 major bacterial genera (comprising more than 50 % of all sequences) is given in Figure 2-5. At the phylum level, we did not find any significant differences in changes in microbe abundance between incubated and non-incubated eggs (Wilk's lambda = 0.78, F (4, 7) = 0.50, p = 0.74). However, at the genus level, the changes in microbe abundance between incubated and non-incubated eggs was marginally significant (Wilk's lambda < 0.01, F (10, 1) = 214.01, p = 0.05). Paired t-tests for comparison of relative abundance between at day 3 and 18 on each genus separately revealed that relative abundance of *Pseudomonas* significantly decreased (Paired t-test, t = 2.99, df = 8, p = 0.02) whereas that of *Bacillus* increased (t = -2.71, df = 8, p = 0.03) on incubated eggs. We further attempted to identify the species using EzTaxon-e database with more than 99% of 16S rRNA gene sequence similarities. We identified two pathogenic *Pseudomonas* spp., *P. oryzihabitans* (GenBank accession no. D84004) and *P. plecoglossicida* (accession no. AB009457), and three antibiotics-producing *Bacillus* spp., *B. subtilis* (accession no. ABQL01000001 and EU138467), *B. pumilus* (accession no. ABRX01000007), and *B. licheniformis* (accession no. AE017333). Conventionally sequences with ≥ 97% identity are considered to be from the same species (Peterson et al. 2008).

Dufrene-Legendre Indicator Species Analysis identified 8 indicator bacterial taxa (genus) which are strongly associated with the bacterial communities on incubated eggs at day 3 (Table 2-1). The indicator taxa were mostly soil bacteria, but it also included two opportunistic pathogenic genera (*Flavobacterium* and *Acinetobacter*) and one genus of antibiotic-producing bacteria (*Streptomyces*). No indicator taxa were found on incubated eggs at day 18 and on non-incubated eggs at day 3 and at day 18.

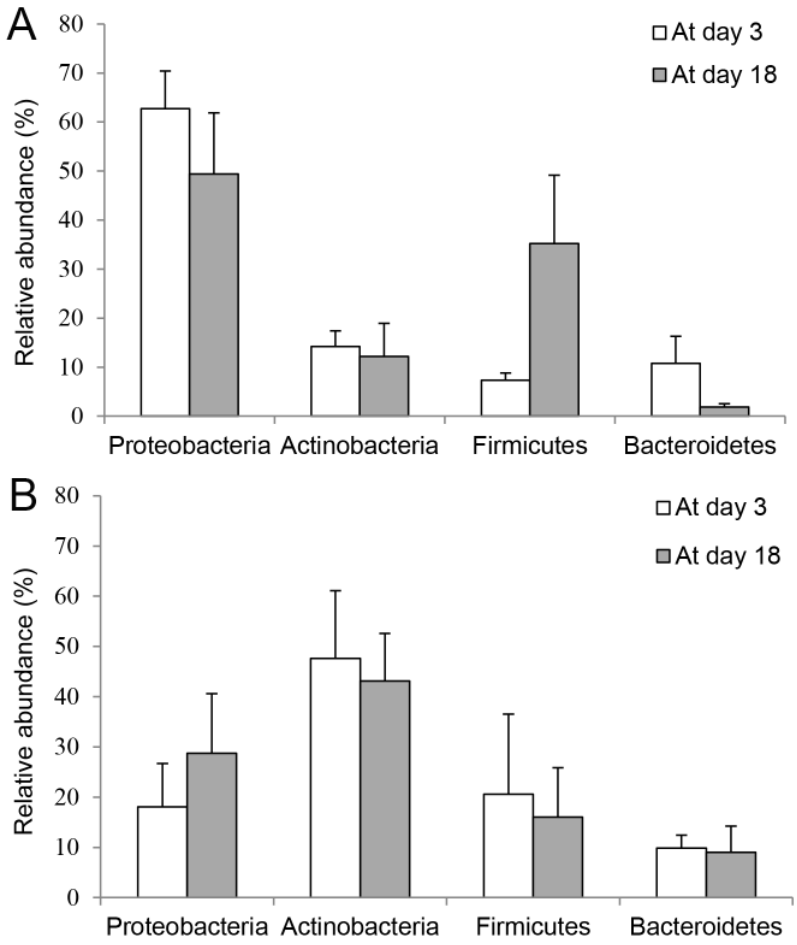

Figure 2-4. Relative abundance of four major bacterial phyla on eggshells at day 3 and day 18, in 9 incubated nests (A) and three control nests (B). 16S rRNA gene pyrosequecing revealed relative abundance (%, +standard error) of four major bacterial phyla among 32 explained more than 90% of sequences in 9 incubated nests (A) and 3 control nests (B) at day 3 and day 18.

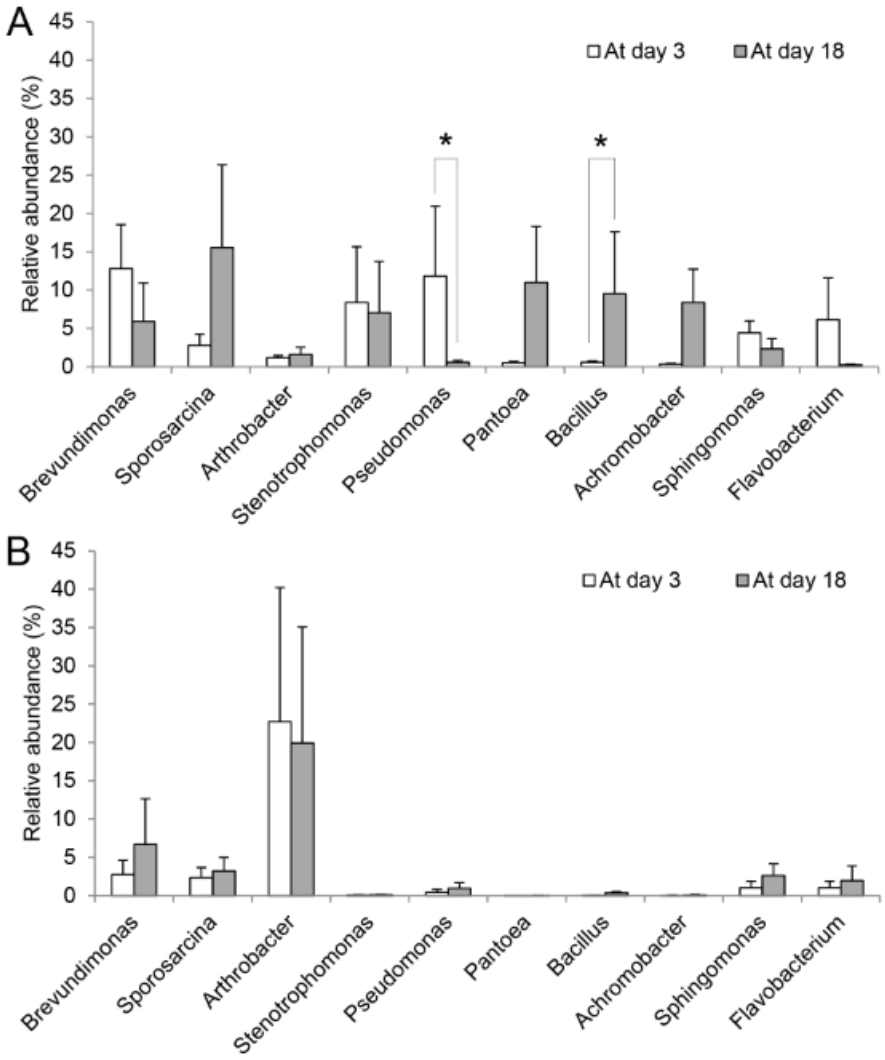

Figure 2-5. Relative abundance of 10 major bacterial genera on eggshells at day 3 and day 18, in 9 incubated nests (A) and 3 control nests (B). Relative abundances (%, +standard error) of 10 major bacterial genera among 1590 which explain more than 50% of sequences in 9 incubated nests (A) and 3 control nests (B) at day 3 and day 18. Asterix means a significant difference ($p<0.05$) between at day 3 and day 18 by paired t-tests.

Table 2-1. The result of Dufrene-Legendre indicator species analysis, which shows 8 indicator genera on incubated eggs at day 3.

Genus name	Indicator value	P value	Mean Percentage (standard error)	Characteristics (Reference)
Rhizobium	0.67	0.021	0.91 (0.17)	Nitrogen fixing in soil (Zahran 1999)
Arthrobacter	0.67	0.042	1.18 (0.29)	Found in soil (Hagedorn & Holt 1975)
Streptomyces	0.56	0.031	0.24 (0.06)	Antibiotic-producing (Malpartida & Hopwood 1984)
Flavobacterium	0.56	0.036	6.11 (5.46)	Found in soil and oppotunistic pathogens in fish (Bernardet et al. 1996)
Chryseobacterium	0.56	0.030	0.14 (0.05)	Found in soil and plants (Green et al. 2007)
Variovorax	0.55	0.047	0.83 (0.59)	Found in soil (Willems et al. 1991)
Microlunatus	0.55	0.035	0.16 (0.04)	Found in soil (Kämpfer et al. 2010)
Acinetobacter	0.50	0.024	1.12 (0.68)	Oppotunistic pathogens in human (Antunes et al. 2011)

DISCUSSION

We found that magpie eggshells harbor a diversity of bacterial taxa, perhaps even more diverse than those found by Shawkey et al. (2009) on tropical bird eggshells (553 families and 221 orders on magpie eggs in our study with pyrosequencing; 256 and 138 on thrasher eggs in Shawkey et al. 2009 with microarray). Although pyrosequencing is more commonly used than microarrays due to its cost efficiency and sensitivity (see Leoford 2008) , recent studies comparing the two methods provided strong correlations in terms of diversity and relative abundance (Claesson et al. 2009, van den Bogert 2011, Ahn et al. 2011).

Previous studies suggested that bacterial abundance and diversity on the eggshell decrease (or at least does not increase) with incubation (Cook et al. 2005ab, Shawkey et al. 2009, Ruiz-De-Castañeda 2011a, 2012). In contrast, we found that total microbial abundance increased and microbial diversity decreased after incubation. This suggests that several bacterial taxa became dominant on the eggshells at the expense of others. Dufrene-Legendre indicator species analysis revealed that 8 significant taxa, which include both pathogenic and non-pathogenic bacteria that were mostly derived from soil, were present at day 3 on incubated eggs. However, no indicator species were detected on incubated eggs at day 18. This suggests that incubation promoted the bacterial assemblage to be more diversified. This is in accordance with the result from NMDS analysis that the bacterial assemblage on the eggshell changed substantially after incubation.

In our results, two out of 10 most abundant genera showed significant changes in abundance in almost all incubated eggs: *Pseudomonas* decreased and *Bacillus* increased. *Pseudomonas* includes opportunistic human pathogens, such as *P. aeruginosa* (Stieritz and Holder 1975) and *P. oryzihabitans* (Yu and Foster 2002, Levitski-Heikkila and Ullian 2005) and a fish pathogen, *P.*

plecoglossicida (Nishmori et al. 2000). On the other hand, the majority of *Bacillus* are harmless (Turnbull 1996) and some are reported to produce antibiotics (e.g. *B. pumilus, B. brevis, B. subtilis, B. firmus, B. licheniformis, B. mycoides, and B. circulars*; Tamehiro et al. 2002, Kuta et al. 2009, Awais et al. 2010). Although it needs further verification, some of the sequences that were obtained seemed to match those of antibiotics producing *Bacillus* (*B. sutilis, B. pumilus,* and *B. licheniformis*). Thus, our results suggest that incubation decreases potentially pathogenic bacteria by promoting the growth of non-pathogenic or antibiotics-producing bacteria. This selective effect of incubation on these two genera can result from three factors.

First is the antibiotics produced by *Bacillus* (Mannonov and Sattarova 2001), as a defense mechanism against pathogens (see Soler et al. 2010). If the antibiotics that *Bacillus* produced work on other microbes, both pathogenic and non-pathogenic, it would result in lower microbial diversity after incubation. For instance, if *Bacillus* spp. in feathers (Burtt & Ichida 1999, Gunderson 2008) produced antibiotic materials which suppress other pathogenic bacteria such as *Pseudomonas* spp. (Stieritz and Holder 1975), this could explain the compositional change in eggshell microbes that we observed in our study. We also detected *Enterococcus* spp., another bacteria that is known to produce bacteriocins and antibiotics and had been isolated from uropygial glands of hoopoes (Martin-Platero et al. 2006, Ruiz-Rodríguez et al. 2013) but they were found only in three nests at day 18 and the relative abundance was very low (0.20% ±0.18, mean± SE). Thus it is unlikely that this genus could have played a major role in bacterial compositional changes in our samples.

Second factor is the difference in sensitivity to the humidity changes on the eggshell. Eggshell surface becomes dry with incubation, which previous studies, conducted both in tropics (Shawkey et al. 2009) and temperate regions (D'Alba et al. 2010, Ruiz-de-Castañeda et al. 2011a),

suggested as the important function of incubation ("drying mechanism"). Similar to our results, previous studies also noticed that *Pseudomonads* decrease after incubation (Ruiz-de-Castañeda et al. 2011ab, 2012, Shawkey et al. 2009, D'Alba et al. 2010, Cook et al. 2003, 2005ab). If *Bacillus* and *Pseudomonas* differ in the sensitivity to the changes in humidity on the eggshell, this may cause the differential growth between these two throughout incubation. A comparison on the water activity values between *Bacillus subtilis* (0.90) and *Pseudomonas aeruginosa* (0.97) suggests that the growth of *Pseudomonas* may indeed be more hampered by desiccation than that of *Bacillus*. Although there is some possibility that our species identification is not correct (refer to Doolittle and Papke (2006) for the problem of "species" definition in microbes), incubation potentially promotes proliferation of selected microbes due to reduced humidity on the egg surface, which contrasts previous suggestion that reduction of humidity may negatively affect bacteria in general (Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2011a). Careful monitoring of the changes in relative humidity throughout incubation and the changes in microbial community as a whole (measuring changes in abundance in all taxa, not just a few of selected ones) would reveal the effect of dehydrating function of incubation on the microbial community.

Third factor is the competition among bacterial taxa. If incubation allows harmless bacteria to grow more rapidly than others and dominate the eggshell habitats, the growth of pathogenic bacteria may be inhibited. For instance, *Bacillus* and *Pseudomonas* were reported to be competitive with each other for nitrate and glucose (Nijburg et al. 1998, Simões et al. 2008). This bacterial antagonism might cause changes in the eggshell bacterial community throughout incubation. Even if the majority of *Bacillus* that we detected in our study are not the ones that produce antibiotics, the competition for resources between these two bacteria may explain the genus-specific growth patterns (increase of *Bacillus* and decrease of *Pseudomonas*).

Our results suggest that the incubation in magpies selectively promotes the growth of non-harmful or potentially beneficial *Bacillus*. *Bacillus* may have originated from three sources. First is uropygial gland. Secretions from uropygial gland contain microbes that produce antibiotics as well as waxes (Soler et al. 2008, 2011). The best studied example is the strains of *Enterococcus faecalis* from uropygial glands of hoopoes *Upupa epops* (Martin-Platero et al. 2006). However, so far there is no report of any species harboring *Bacillus* in the uropygial gland. In fact, antibiotics that are produced by *Enterococcus* are known to work against other microbes including *Bacillus* (Ruiz-Rodríguez et al. 2013). This does not seem to be confined to hoopoes, as it is known that secretions from uropygial gland inhibit *Bacillus* in other species as well (barn swallows, Møller et al. 2009; house finches, Shawkey et al. 2003). Thus, it seems not plausible that *Bacillus* which increased with incubation were originated from uropygial gland in magpies.

Second is the brood patch of the female. However, currently there is no evidence that avian brood patch contains special microbes. Third possibility is the white belly feather. *Bacillus* represents microbes that are derived from soil and some of them are well-known as feather-degrading bacteria (e.g. *Bacillus licheniformis*, Lin et al. 1992). White feathers are known to be susceptible to feather-degrading bacteria as they lack melanin granules that strengthen the feathers. Some birds use white feathers for nest lining so that the eggs can be surrounded by non-harmful (or potentially beneficial due to antibiotics or bacteriocins) feather-degrading bacteria (Peralta-Sanchez et al. 2009). Because magpies also have white belly feathers, we think that *Bacillus* that increased with incubation may originate from the belly feathers. This possibility implies that, depending on the ecological environment (such as the colors of belly feathers of the females or nest lining materials etc.), each species may utilize different microbes to maintain the

microbial community more benevolent to ensure the survival of the eggs. This warrants further studies on the changes of microbial communities on the eggshell in different species that exhibit a variety of ecological environments.

In summary, we suggest that avian incubation in a temperate zone would promote the growth of harmless or antibiotic-producing bacterial taxa and reduce pathogenic ones rather than inhibit the total bacterial growth. The function of incubation as selectively promoting the growth of non-pathogenic microbes and suppressing the proliferation of potentially pathogenic ones would possibly involve the resistance to desiccation or production of antibiotics of the former.

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Chapter 3.

Incubation increases hatching success by affecting microbial growth on eggshells

ABSTRACT

Microbial infection causes mortality of embryos inside the egg and thereby greatly reduces the effectiveness of parental effort. Incubation behavior was hypothesized to inhibit the transmissible pathogenic microbial growth on eggshells. However, most researchers have focused on the effect of incubation on microbes, without proper evaluation of the causal effect of incubation on hatching success through suppressing microbial growth. Moreover, traditional culture-dependent techniques have been used to determine the presence and abundance of microbes, which requires a careful data interpretation. Using a culture-independent quantification method, real-time PCR, we investigated whether (i) incubation effort increases egg hatchability, (ii) incubation inhibits microbial growth on eggshell surface, and (iii) microbial presence and growth decreases hatching success. We sampled microorganisms from magpie eggshells at early incubation stage (at day 3 after laying) and late stage (at day 18) and quantified total bacteria and the expected microbial species, such as pathogenic microorganisms and anti-biotic producing bacteria. Here, we showed that female incubation increased total bacterial abundance while *Escherichia coli* abundance was suppressed by incubation. As female spent more time for incubation, egg hatchability increased. In our results, incubation increased overall bacteria contrary to previous studies. However, pathogenic species reduced while beneficial/commensal bacteria increased. This suggests that Furthermore, the changes of *E. coli* abundance at early incubation stage significantly decreased hatching success. Presence of *Candida albican*s, a fungal pathogen, affected hatching success. Our results provide strong evidence that the incubation increases hatching success by suppressing microbial growth on eggshells in wild birds.

INTRODUCTION

Avian eggs have various microorganisms on their surfaces and pathogenic species, among the microbes, decrease egg viability through trans-shell infection the microbes can affect embryonic death through eggshells (Bruce and Drysdale 1994, Pinowski et al. 1994, Cook et al. 2003, 2005ab). Hence, birds have evolved several mechanisms to prevent the microbes; Physical defense of the eggshell cuticle (De Reu et al. 2005, 2006) and antimicrobial protein activity in the outer eggshell and cuticle (Wellman-labadie et al. 2008) or in the egg albumen (Shawkey et al, 2008, D'Alba et al. 2010b) were revealed by previous studies. As a parental behavioral mechanism, incubation behavior was suggested to inhibit microbial growth on eggshells (Cook et al. 2005ab, Shawkey et al. 2009). Avian incubation is a process to keep the eggs at a regular temperature and humidity range, which is necessary for embryonic development (Deeming 2002). Since embryos in the eggs are highly dependent during the egg stage on the parental incubation to develop and hatch successfully, parents control temperature and humidity on eggshell surfaces (Deeming 2002) spending energetic costs of 10-20% weight loss (Rahn and Paganelli 1990). Recent studies showed that incubation behavior reduces microbial growth by drying eggs (D'Alba et al. 2010a, Ruiz-de-Castañeda et al. 2011a) or by antibiotic-producing bacteria from oil glands (Soler et al. 2010). Hence, harmful microbes (e.g. Escherichia coli and Staphylococcus epidermidis in Pinowski 1994) were hypothesized to increase and/or beneficial bacteria (e.g. Enterococci in Ruiz-Rodríguez et al. 2012), based on the assumption that incubation increases hatching success.

However, no studies could approach species-specific or family-specific absolute quantification on eggshell bacteria to evaluate the bacterial species composition and changes (relative abundance was measured by Shawkey et al. 2009). Most studies in field behavioral studies have used

traditional culture methods, thereby people could detect less than 1 % of microbial species by culture because most of them are unculturable (Amman et al. 2005). Moreover, previous studies focused on the incubation effects on microbes without consideration of incubation effects on hatching success and microbial effects on hatching success (e. g. Shawkey et al. 2009). Contrary to previous studies in tropical forests (e. g. Cook et al. 2005a, Shawkey et al. 2009), recent studies in temperate areas found no significant effects of incubation on bacterial loads and hatching success (Wang et al. 2009, Ruiz-de-Castañeda et al. 2011b). Thus, it is still unclear whether incubation increases hatching related to the function of microbes on eggs even in temperate zones.

To our knowledge, this is the first study supporting the three previously suggested hypothetical relationships simultaneously among incubation effort, hatching success, and microorganisms on eggshell surfaces in a wild avian field study (Figure 3-1), and quantifying absolute numbers of the microbes on eggshells in the species level using a culture-independent method. In this study, we examined the effect of incubation effort on egg hatching success by suppressing the eggshell microorganisms in the Black-billed magpie (*Pica pica*) using a culture-independent method, real-time PCR, which is one of the most reliable methods for microbial quantification (Burgos et al. 2002, Klein 2002, Epsy et al. 2006). Magpie incubation is performed by female only during the whole incubation period, 21-22 days on average, and the incubation behavior starts in several days after the first egg is laid (Birkhead 1991; mostly in 3-4 days in our population, Lee personal observation). The number of egg produced was 5.88 ± 1.54 (mean ± SD) and the number of hatchlings was 3.71 ± 1.60 among 51 nests in 2011 (on average 52% of hatching success); Our magpie population had a lower hatching rate, compared to other avian populations in natural conditions (approximately 10% of egg fails; Koenig et al. 1982, Spottiswoode et al. 2004), and even

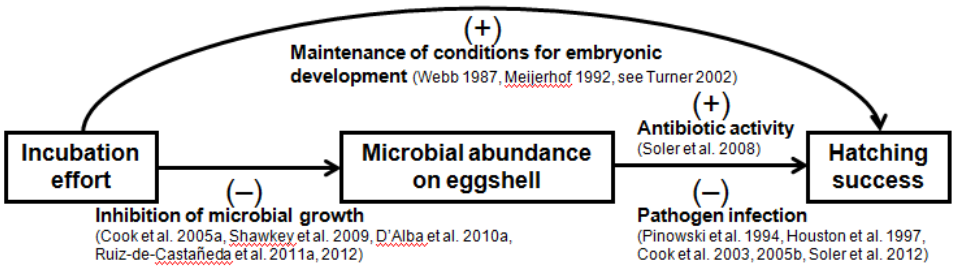


Figure 3-1. Hypothetical causal relationships of incubation effort, microorganisms on eggshells, and hatching success ('+' means positive effect and '–' means negative).

lower than other magpie populations (Dr. Soler, personal communication). Therefore, we suspected that the presence of pathogenic microbes killing embryos through the eggshells may be one of the main reasons for the low hatchability when we consider previous studies on magpie eggshell bacteria (Soler et al. 2011).

We aimed to determine whether (i) incubation effort increases egg hatchability in our magpie population, (ii) incubation affects microbial growth of expected harmful bacteria including a fungal pathogen (*Candida albicans*; Hubalek and Balat 1974, Hubalek 1978, Velasco 2000) that may contribute to this low hatching success and/or beneficial microbes (e.g. *Enterococcus faecalis*; Soler et al. 2008, 2010) that produce antibiotics to kill the pathogenic bacteria, and (iii) pathogenic microbial presence and growth decreases hatching success.

MATERIALS AND METHODS

Study site, incubation recording and microbial sampling

This study was conducted on SNU campus (37°47' N, 126°95' W; Seoul, Korea) in 2011. Magpies started laying eggs from 3rd March 2011 till 28th April. The average daily temperature was 4.44 ± 4.67°C and the relative humidity was 51.85 ± 14.27% during the incubation period. We regularly monitored 51 breeding magpie pairs to determine the date of egg-laying. Because female magpies maintained incubation attentiveness to the end of hatching (Birkhead 1991), we chose one day in the middle of incubation period for recording at day 10 or 11 after first-egg laying. We avoided rainy days for recording not to include weather effects on incubation. Totally, 35 nests were video-recorded near the nest trees for four hours (0700 – 1100) using camcorders (SONY HDR-SR1). From the video-recordings, we

assumed that females were incubating if females entered the nest and stay inside. Percentage of female incubation time was quantified during the recording time by the video analysis. Among the 35 nests, one nest was excluded for the analysis because the laying date (13th May) was extremely out of the range (median of the rest was 27th March) and the hatchability was zero.

Microbial sampling from the eggshells was conducted twice during the incubation period, at early incubation stage (at day 1-3 after first-egg laying) and late stage (at day 16-18), by rubbing a sterile swab wetted with sterile 1 × PBS buffer: half of the hemisphere was swabbed before the full incubation occurs and the other half of the same eggs was swabbed a few days before egg hatching, to estimate the effect of female incubation on the microbial growth. One swab was used for a clutch (1-3 eggs) in each nest. The size of the eggs, length (L) and width (W), was also measure to estimate the surface area (S) of the eggs ($S = 3 \times L0.771 \times W1.229$; Narushin 1997). Among the 35 nests video-recorded for incubation, we determined laying dates in 19 nests at early incubation; 13 incubated nests and 6 from non-incubated nests. Six breeding pairs abandoned their nests after laying before incubation, possibly by human disturbance or other reasons. Thus, we treated the six nests as a control group. To make sure that the eggs in abandoned nests were not incubated, we continuously monitored the breeding pairs and confirmed that the birds re-nested in other nest trees or moved to other territories.

Detection and quantification of expected microorganisms by real-time PCR method

DNA was extracted from the swabs using the MoBio PowerSoil DNA kit (MoBio laboratories, Carlsbad, CA, USA), and then amplified with a real-

time PCR system (Illumina Eco™, Diego, CA, USA). Each specific primer set to detect and quantify expected microbial species on eggshells was referred from the previous studies (see Table 3-1). All Real-time PCR assays were performed with 5 μl of QuantiMix SYBR 2× (PhileKorea Technology, Seoul, Korea), 0.25 μl of 10 pmol/L forward and reverse primer, 3.5 μl of ddH2O, and 1 μl of DNA extract of total 10 μl cocktail solution. PCR thermal condition was as follows; initial polymerase activation for 10 min at 95°C, PCR cycling for 10 sec at 95°C and for 30 sec at 60°C for 40 times, and melting curve was obtained by lowering temperature from 95°C to 55°C.

To generate standard curves of each species, we designed recombinants of species-specific target gene in cloning vectors. By this method, we quantified the absolute numbers of each microbial species inferred from the reliable standard curves using PicoGreen-based method (Rothrock 2009). First, genomic DNA was extracted (Wizard Genomic DNA Purification Kit, Promega, Madison, USA) from cell lines which were kindly donated from KACC (Korean Agricultural Culture Collection) and Dr. Chun's lab in Seoul National University; Candida albicans (KACC 30003), Staphylococcus epidermidis (KACC 13234), Escherichia coli (KACC 16630), Klebsiella pneumonia (KCTC 2208), Staphylococcus aureus (KCTC 1916), and Enterococcus faecalis (KACC 11304). Second, the extracted DNA was amplified (GoTaq Green Master Mix, Promega, Madison, USA) with species-specific primers (Table 3-1). Third, the amplified species-specific gene fragments were inserted into cloning vectors (3003 bp, pGEM®-T Easy Vector, Promega, Madison, USA) transformed in competent cells (Hit-DH5α, RBC Bioscience, Taipei, Taiwan). Finally, recombinants of cloning vector and each microbial species-specific DNA fragment were selected in LB (Luria-Bertani) plates with ampicillin (100 μg/ml), IPTG (isopropyl beta-D-thiogalactoside, 0.5 mM), and X-Gal (5-bromo-4-chloro-3-indolyl β-d-galactoside, 80μg/ml). Then, plasmid mini prep kit (Hybrid-QTM, GeneAll

Biotechnology, Seoul, Korea) was implemented to purify the recombinant plasmid DNA. The concentration of the plasmid DNA was measured with Quant-iTTM PicoGreen® dsDNA assay kit (Invitrogen, Carlsbad, USA) and TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, USA). The corresponding DNA copy number was calculated using the following equation (Whelan et al., 2003):

$$\mathrm{DNAcopynumber} = \frac{6.02 \times 10^{23}(\mathrm{copy/number}) \times \mathrm{DNAamount(g)}}{\mathrm{DNAlength(dp)} \times 660(\mathrm{g/mol/dp})}$$

PCR results were analyzed with EcoTM software (version 3.0). PCR results of Ct (threshold cycle) values were converted to the absolute DNA quantity by comparing the Ct values to the serially diluted standard curves (for details see Heid et al. 1996). Standards were serially 10-fold diluted from 1 × 100 to 1 × 108 copies/μl. PCR was performed three times for each sample and the PCR results satisfied the following requirements: the efficiency of the standards were between 90% and110% , R2 was higher than 0.99, and the standard deviation of PCR replicates was less than 0.167. Melting temperature ranges were finally checked in order to exclude PCR noises (Table 3-1).

Statistical analysis

For statistical analysis, incubation time (duration of female incubation per hour) was arcsine-square root transformed and bacterial abundance were log-transformed. Laying date was in Julian days from 3rd March 2011. We conducted logistic regression with SAS PROC LOGISTIC (SAS ver 9.3, SAS Institute) to examine the effects of incubation time or bacterial abundance on hatching success (brood size as the numerator and clutch size as the denominator): incubation time, laying date, and clutch size were included as

explanatory variables for the effect of incubation time on hatching success; total bacterial abundance or *E. coli* abundance (at day 3 or at day 18 or the abundance change in the meantime), laying date, and clutch size were explanatory variables for the effect of total bacterial abundance or *E. coli* abundance on hatching success; presence or absence of *C. albicans*, laying date, clutch size were explanatory variables for the effects of presence of *C. albicans* on hatching success. The minimal models were selected through back-ward elimination from the full model with two-way interactions, following Johnson and Omland (2004), by lowering the Akaike information criterion (AIC) value. SAS PROC REG was used when analyzing laying date effects on incubation time or bacterial abundance. We used paired t-test in R 2.15.1 (http://www.r-project.org) to compare the bacterial abundance between at day 3 and at day 18 in both incubated and non-incubated nests. In addition to that, we compared the bacterial abundance of different variances between in incubated and non-incubated nests at day 3 with Welch two sample t-test in R 2.15.1.

Table 3-1. Target microbes, the primer sequences, and the melting temperature to specify the microbes.

Target	Primer sequences	Reference	Melt T (°C)
Universal bacteria (338F and 518R)	F: ACTCCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	Einen et al. (2008)	83.5–86.8
Escherichia coli (pathogen)	F: ATGGAATTTCGCCGATTTTGC R: ATTGTTTGCCTCCCTGCTGC	Hijnen and Medema (2006)	87.9–88.9
Staphylococcus aureus (pathogen)	F: GCGATTGATGGTGATACGGT R: AGCCAAGCCTTGACGAACTAAAGC	Brakstad et al. (1992)	81.0–82.0
Staphylococcus epidermidis (pathogen)	F: TCAGCAGTTGAAGGGACAGAT R: CCAGAACAATGAATGGTTAAGG	Iwase et al. (2008)	75.1–77.8
Klebsiella pneumoniae (pathogen)	F: TGCCCAGACCGATAACTTTA R: CTGTTTCTTCGCTTCACGG	Sun et al. (2009)	85.5–85.7
Candida albicans (fungal pathogen)	F: ATTGCTTGCGGCGGTAACGTCC R: TCTTTTCCTCCGCTTATTGATATGC	Khan et al. (2009)	79.0–79.6
Enterococcus faecalis (antibiotic-producing)	F: CCGAGTGCTTGCACTCAATTGG R: CTCTTATGCCATGCGGCATAAAC	Sedgley et al. (2005)	83.0–83.7

RESULTS

Female incubation time of percentage for 4 hours varied among nests from 66.97 % to 87.51 % (n= 34, mean = 82.49; SD = 4.16). However, incubation time did not change significantly as laying date delayed (Linear regression, n = 34, P = 0.3803). The minimal model for hatching success in different incubation times among nests showed that hatching success significantly increased as females spend more time for incubation and laying date was earlier (Wald chi-square = 4.9298, P = 0.0264 in Figure 3-2A; estimate = -0.0274, SE = 0.0124; Wald chi-square = 4.8731, P = 0.0273 in Figure 3-2B).

Using real-time PCR, total bacteria and *E. coli* were detected in all microbial samples in both incubated and non-incubated nests (n=20). In addition, *C. albicans*, a fungal pathogen, was found in five incubated nests and three non-incubated nests. *S. epidermidis*, a pathogenic bacteria (gram positive coccus), was detected in one incubated nest. Once a microbial species was detected from eggshells at day 3, it was continuously found on the same eggs at day 18 although the abundance differed. *S. aureus* and *K. pneumonia* (pathogenic bacteria) and *E. faecalis* (antibiotic-producing bacteria) were not detected in all eggs in this study.

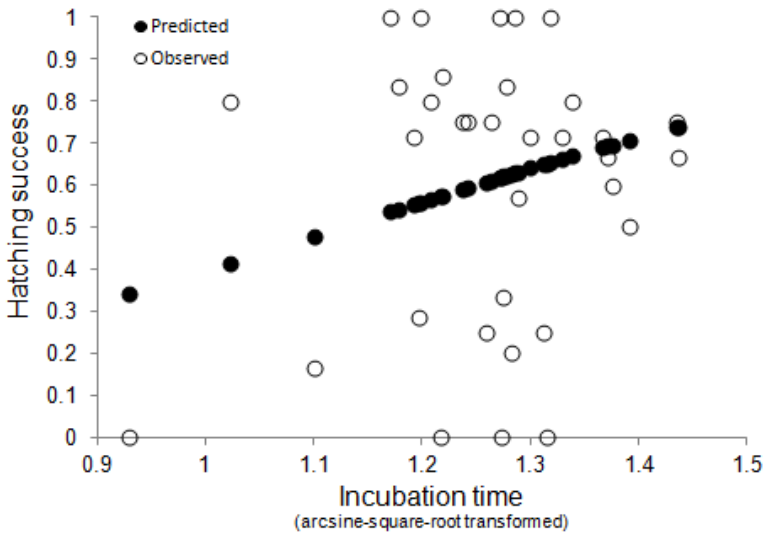
When we compared the abundance differences of total bacteria between at day 3 and at day 18, total bacteria significantly increased in incubated nests (Figure 3-3A; paired t-test, t = -2.427, P = 0.0382,) but it did not change in non-incubated nests (t = 0.762, P = 0.5016). On the other hand, *E. coli* abundance increased in non-incubated nests (Figure 3-3B; paired t-test,

t = -3.655, P = 0.0217) while there was no significant changes in incubated nests (t = 1.135, P = 0.2858). *C. albicans* seemed to increase after incubation period in both incubated and non-incubated eggs, but statistically significant changes were not found possibly due to the low sample size (Figure 3-3C; n = 5 in incubated eggs, n = 3 in non-incubated eggs; paired t-test, all P > 0.05). The microbial abundance at early incubation stage were not significantly different between on incubated and non-incubated eggs (Figure 3-3; Welch two sample t-test, t = -0.6102, df = 13.12, P = 0.5522 in total bacteria, t = 1.6003, df = 9.278, P = 0.1430 in *E. coli*, and t = 1.2636, df = 3.29, p-value = 0.2885 in *C. albicans*). Laying date did not influence total bacterial abundance at early incubation stage, at late incubation, and bacterial abundance changes between at early and late stage (Linear regression, n = 14, all P > 0.05).

Among the 14 incubated eggs, we analyzed the effect of bacterial abundance or presence on hatching success with laying date and clutch size together. For total bacterial abundance, all explanatory factors were deleted in the model reduction. Total bacterial abundance at day 3 and at day 18, the difference between the two measurements (abundance at day 18 – at day 3), laying date, clutch size did not remain in the minimal model. In *E. coli*, the minimal model includes the early incubation stage abundance (at day 3); Hatching rate was significantly lower in the eggs with more *E. coli* detected at laying (Figure 3-4A; Logistic regression, estimate = -2.2077, SE = 0.7810, Wald chi-square = 7.9908, P = 0.0047). *E. coli* abundance at day 18 and the changes between two measurements were erased during each model selection with laying date and clutch size. Due to the low sample size (n = 5) of *C. albicans* detected, we estimated the effect of presence of *C. albicans*, instead

of abundance, on the hatching success. Hatching success was significantly lower when *C. albicans* was detected (Logistic regression, Wald chi-square = 6.723, P = 0.0095; shown as boxplots in Figure 3-4B).

A



B

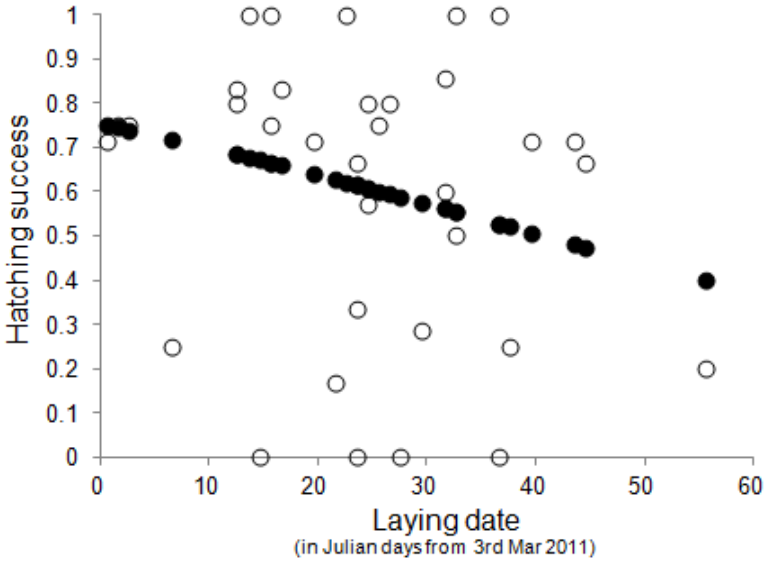
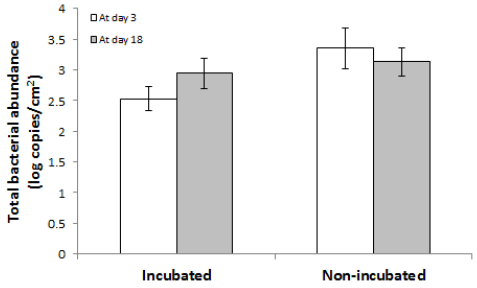
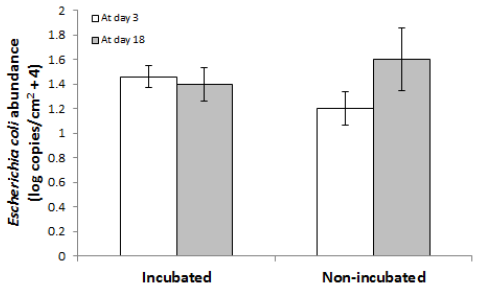


Figure 3-2. Effects of (A) incubation time, at day 10-11, and (B) laying date, from 3rd March till 28th April in 2011, on hatching success (Logistic regression, both $P < 0.05$). Filled circles are predicted values and empty circles are observed values.

A



B



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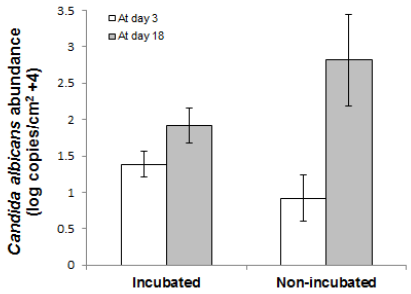
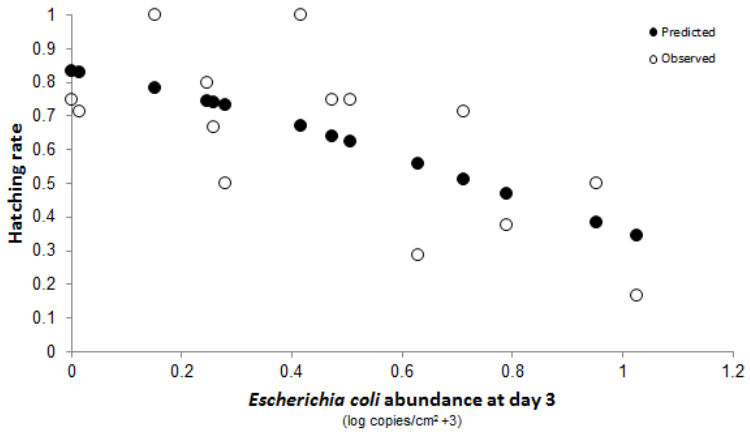


Figure 3-3. Bacterial abundance in incubated and non-incubated eggs at day 3 after first egg laying (white bars) and at day 18 (grey bars). 14 incubated eggs and 6 non-incubated eggs were compared in (A) total bacteria and (B) *E. coli*; 5 incubated eggs and 3 non-incubated eggs in (C) *Candida albicans*.

A



B

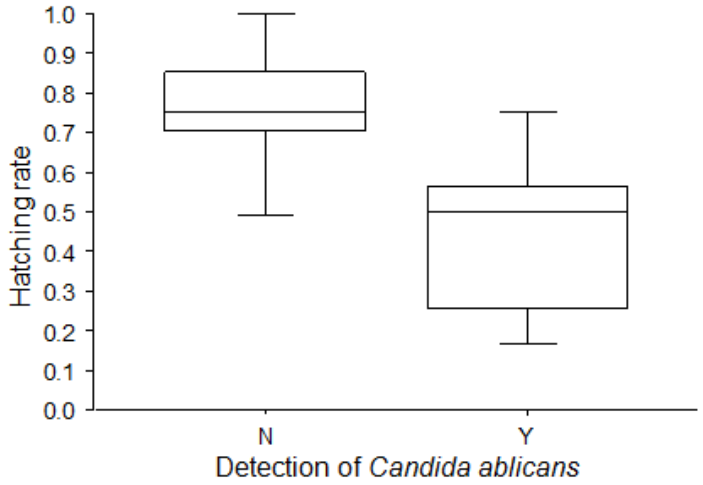


Figure 3-4. (A) The effect of *Escherichia coli* abundance at day 3 on egg hatchability. (B) Hatching success with presence or absence of *Candida albicans* (*C. albicans* was detected in 5 nests among 16 nests).

DISCUSSION

We investigated the hypothetical relationships among incubation effort, hatching success, and microorganisms on eggshell surfaces in a wild avian species. Our results support that the incubation effort increases hatching success, possibly by suppressing the pathogenic microbial growth since pathogens reduce hatching success. We found that hatching success was higher as females spend more time for incubation and lay eggs earlier. We expected that incubation would increase hatching success through several mechanisms, such as controlling temperature. Interestingly, eggs hatched better in earlier nests despite the much lower temperature, approximately 10°C difference (0.5°C on 3rd March 2011 and 10.4°C on 28th April), and the similar incubation effort of females. Transmissible pathogenic bacteria on eggshells may be one of the reasons for the low hatching rate in the late nests since bacterial growth highly depends on temperature (e.g. Rosenshine 1996). However, the bacterial abundance and growth were not affected by laying date in our results.

We found that incubation increased the growth of total bacteria and suppressed *E. coli* abundance. In comparison of bacterial abundance between on incubated and non-incubated eggs, the growths of total bacteria were encouraged on incubated eggs while it did not change on non-incubated eggs. However, *E. coli* was different from the pattern of total bacterial abundance; the growth *E. coli* of was inhibited by incubation. *E. coli* is one of the transmissible pathogens affecting embryos inside the eggshells (Bruce and Drysdale 1991, Pinowski et al. 1994). This result implied that incubation behavior increased the overall bacterial growth but pathogenic species on eggshells decreased as previous studies suggested in tropical forests (Cook et al. 2005a). We also showed that *E. coli* abundance on eggs before female

incubation reduced hatching success. This suggests that *E. coli* on eggshells influence egg hatching directly through eggshells or indirectly by other pathways. As a pathogen species, *E. coli* was found in all eggs at laying and the abundance was negatively related to hatching success. We think that a large number of *E. coli* at laying could affect embryos inside and egg hatchability eventually by infection. Although *C. albicans* was detected in a few nests, the impact of this seems quite strong since the presence of the pathogen greatly decreased hatching success. Indeed, infection of *C. albicans* can cause embryo death in birds (Meyer and Ordal 1946, Foley and Winter Jr. 1949, Pinowski 1994, Velasco 2000) by invading the chorioallantoic membrane inside the eggs (Gow et al. 2003).

Possible microbial sources on eggshells would be maternal cloaca, nest materials, parental body, or airbones (see Ruiz-de-Castañeda et al. 2011a). At laying, we observed white dirt materials on eggs, seems like feces derived from the maternal gut (Lee, personal observation). Considering that avian gut flora has a large number of *E. coli* and it can be transmitted to the egg surface through cloaca (Barrow 1994, Amit-Romach et al. 2004), we predicted that *E. coli* would widely exist at laying and it indeed found on all eggs and had a negative effect on hatching success. Therefore, we strongly suspect that maternal gut would be one of the main sources for microbes.

Total bacteria greatly increased by incubation on eggshells while it did not change on control eggs. Incubated eggshells provided a better condition for microbial growth when we consider cold temperature during the incubation period in our study site (mean 4.44°C). Our results were contrary to the tropic studies that incubation inhibited total bacterial growth (e.g. Shawkey et al. 2009). However, our results were in accordance with the previous studies conducted in tropical area that pathogenic species reduced by incubation. This implies that the previous hypothesis of incubation effects on microbial growth on eggshells is still valid in a cool area although the exact

mechanism is unclear. Several mechanisms under the incubation effects on microbes have been suggested so far. First, drying eggs would have antimicrobial effects through evaporation on the egg surfaces (D'Alba et al. 2010a, Ruiz-de-Castañeda et al. 2011a). Wet eggs would make a better environment for microbes to grow rapidly because bacteria require water for basic cellular metabolism to multiply (Madigan et al. 2005), so parental incubation behavior may contribute to the antimicrobial effects. Considering that our study area is much drier than our tropical regions in previous studies (51.85 ± 14.27% in our study, mean ± SD; 97.5 ± 0.5% in Cook et al. 2003; 98.2 ± 0.1% in Cook et al. 2005a), we think that the humidity would not be an important factor for incubation effects in our area. Secondly, physical abrasion (Shawkey et al. 2009) or antimicrobial function of nest materials through rolling the eggs during incubation were suggested (Clark et al. 1988, Peralta-Sanchez 2009, 2011). Removing the dirt materials like feces derived from maternal gut at laying would decrease the chance of microbial growth, considering that eggshells surfaces are not nutrient-rich environment (Bruce and Drysdale 1994). Thirdly, antibiotic-producing bacteria could be one of the mechanisms to prevent trans-shell egg infection (reviewed in Soler et al. 2010). Soler et al. (2008) demonstrated that European hoopoes harbor symbiotic enterococci in the uropygial gland that produces bacteriocins to inhibit pathogenic bacterial growth. Although an antibiotic producing bacteria, *E. faecalis*, was not found on our magpie eggshells, this can explain our results why *E. coli* was inhibited when total bacteria multiplied. If there was a symbiotic relationships among the magpie parents and antibiotic-producing bacteria, magpie parents could reduce pathogenic microbes by harboring antibiotic producing bacteria or at least by increasing commensal bacteria species.

Here, we demonstrated the causal relationships among incubation effort, hatching success, and microorganisms on eggshell surfaces in a wild

avian field study (see Figure 3-1). By detecting and quantifying total bacteria and several expected microbial species on eggshells, we showed that the microbes affected hatching success and incubation would increase hatching success by suppressing the microbial growth. Our quantification method using real-time PCR would be applied to various field studies of microbial quantification, replacing traditional culture counting. Even though we selected several expected microorganisms which were previously studied for the analysis, uncultured microbial taxa are also available using this method in future researches. Also, detection and quantification in a broad range of microorganisms would reveal not only the specific microbial abundance and presences but also the microbial composition and diversity on eggshells. We expect that behavioral studies with microbial analysis by newly developed techniques (e. g. Next-generation sequencing, Schuster 2008) would enable us to test long-standing hypotheses in behavioral ecology (Archie and Theis 2011).

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Chapter 4.

Do mothers have consistent investment strategies? Maternally derived immunity and parental food allocation in the Black-billed magpies (*Pica pica*)

ABSTRACT

In birds, young nestlings are largely dependent on antibodies transmitted from the mothers for immunity. This creates the possibility of differential allocation of antibodies by mothers among their offspring. We questioned if mothers consistently allocate more antibodies and more parental care (e.g. feeding) to the same offspring. By analyzing video-recordings of parental visits to the nests, we examined maternal antibodies and feeding toward a certain offspring. Furthermore, we questioned whether males also differentially allocate parental care according to the levels of maternal antibodies in the nestlings. Our results showed that maternal immunoglobulin levels were affected by brood size and hatching order. Early hatchlings in small brood size nests got more immunoglobulin. During the feeding period, nestlings with more immunoglobulin were favored by parents, especially in male nestlings. Female parents fed more to the late hatchlings with more immunoglobulin whereas male parents' feeding was not affected by nestlings' immunoglobulin levels. This suggests that female parents maintain consistent investment strategy during the feeding period towards offspring which were favored at egg laying stage with more maternal antibodies. Also, considering that males and females have different feeding strategies, the parental favoritism may not correspond to each other.

INTRODUCTION

In altricial birds, protection against pathogens is important for nestling survival in early stages. Before the generation of own antibodies, nestlings are dependent on innate immunity and maternal antibodies (mainly immunoglobulin; Tella et al. 2002). Maternally derived antibodies may directly help nestlings' survival or growth by killing pathogens (reviewed in Grindstaff et al. 2003) or indirectly by decreasing the usage of more costly innate immune factors (Grindstaff 2008). Hence, maternal antibodies transmitted to the eggs strongly affect the immune function of offspring for the first week after hatching, and even may have long-term effects (see Grindstaff et al. 2003, 2005, Ardia and Schat 2008). On the other hand, recent studies reported that there were no siginificant effects of maternal antibodies on nestlings' immune function (King et al. 2010) or on adult immune response (Addison et al. 2010). Costs of maternal antibody involves potential problems in autoimmune systems interfered by maternal antibodies during the development (Carlier and Truyens 1995, Greeley et al. 2002, Lemke et al. 2009). Thus, it is still unclear whether maternal antibodies increase nestlings' immunity and consequently survival.

During the period of parental care, parents may favor certain nestlings in order to ensure the survival of the nestlings that give them greatest fitness return. However, how to exert such favoritism can depend on the stages of the breeding and the sex of the parents. During egg production, mothers may deliver materials that aid the immunity of nestlings against pathogens (such as immune proteins and hormones; Rollier et al. 2000, for review see Groothuis and Schwabl 2008) into the favored eggs, whereas fathers' means for favoritism is highly limited before hatching. After hatching, mothers can ensure the favoritism by selectivley provisioning those nestlings whom they delivered more materials during egg production. This may not be

the same nestlings that fathers favor; without any previous investment made (e.g. Dickens and Hartley 2006), fathers' favoritism may purely depend on the survival prospects of the nestlings.

Although the effect of maternally derived antibodies on nestling growth and survival has been rigorously studied (Heeb et al. 1998, Grindstaff 2003, Philaja et al. 2006, Hasselquist and Nilsson 2009), the potential correlation between maternally derived antibodies and the provisioning rules of mothers has not been investigated. Considering that both, the maternal transmission of antibodies and the provisioning effort, are costly (both are known to be condition-dependent; Philaja et al. 2006, Boulinier and Staszewski 2008), one can predict that these two aspects of breeding strategies of mothers are tightly linked to each other. On the father's side, it is still in question whether their favoritism coincides to the maternal favoritism towards nestlings.

Most studies on maternal effects have focused on the relation between maternally derived materials and the survival and growth of offspring (for review see Grindstaff 2003). However, we still do not know if nestlings with different levels of maternally-derived antibodies are fed preferentially by the mothers during the nestling period, and how much of such differential provisioning contributes to the positive effect of maternal antibodies on nestling growth, condition and survival. Here, we investigated the effect of not only the maternal antibodies on the breeding success, but also the effect of parental feeding preferences on the breeding success and on nestling's growth and survival.

In this study, we studied the correlation between the level of maternally derived antibodies in the hatchlings and food allocation of the parents in the Black-billed Magpie (*Pica pica*), a monogamous and bi-parental provisioning species. It has been assumed that magpie nestlings produce their own immunoglobulin rigorously at 8-10 days after hatching

(Philaja et al. 2006) so it is likely that early hatchilngs are largely dependent on maternally derived antibodies for protection against pethogens. If other things being equal, mothers would preferentially provision those nestlings who received more maternal antibodies as they already invested more in those nestlings. On the other hand, if the nestlings who recieved more maternal antiboidls are generally in good condition and the parental favoritism may be more based on the immediate condition of nestlings rather than the amount of investment that had been made in earlier breeding stages (i.e. delivery of maternal antibodies), then parents would provide baised provisioning to the nestlings in good conditions with more maternal antibodies.

Although magpie parents seem to feed their nestlings differentially (Moreno-Rueda et al. 2007; Lee et al. 2012), whether any difference exists in the feeding strategies between male and female parents has not been investigated yet. In magpies, brood reduction often occurs and it is mainly due to starvation during the feeding period (Birkhead 1991). Lee et al. (2010), based on the data collected from our magpie population, proposed that maternal effects and parental feeding strategies may play a major role in nestling survival. Hence, we examined how females allocate antibodies to their offspring and whether males and females allocate more food resources to those nestlings with high level of maternal antibodies.

MATERIALS AND METHODS

Study population and antibody measurement

We monitored approximately 40 pairs of magpies in the campus of Seoul National University (Korea) during the breeding season in 2009. From early March, we visited the magpie nests twice a week to determine the laying

and hatching dates of each breeding pair. We sampled the blood from the nestlings twice during their growth; right after hatching (i.e. post-hatching day 0 – 1, hence noted as "day 0"), and between 18-20 post-hatching days (hence noted as "day 20"). We sampled the blood (10 – 15 ul) from their medial metatarsal vein (at day 0) or brachial vein (at day 20) with capillary tubes (heparinized, Chase Scientific Glass, Rockwood, TN, USA) and centrifuged the blood samples within 5 hours to separate blood cells and plasma. Using red blood cells, we extracted DNA and determined sex of nestlings by molecular sexing (Griffths et al. 1998). With plasma, we measured immunoglobulin levels of the nestlings (see below).

Maternally derived antibodies in the plasma were detected with ELISA (Enzyme-Linked Immunosorbent Assay), which is a main immunological technique to measure the concentration of antibodies (Ardia and Schat 2008). Females transfer immunoglobulin as IgY type (avian prototype of IgG) in the yolk (Ardia and Schat 2008) and nestlings begin to produce their own immunoglobulin as IgM type (transforms to IgY later) in several days after hatching (Philaja et al. 2006). Hence, we assumed that antibodies of nestlings measured right after hatching are derived from their mothers (Apanius 1998, Ardia and Schat 2008). Immunoglobulin data was obtained from 55 nestlings (22 nests).

We followed the protocol of Philaja et al.'s (2006) for ELIZA with a few modifications. We prepared anti-chicken IgG antibody (C-6409, Sigma Chemical Co) in 50mM carbonate buffer (pH 9.6, adjusting Na_2CO_3 1.515 grams and $NaHCO_3$ 3 grams to one liter of distilled water) and put the solution into a microplate with an overnight incubation at 4°C. After emptying the plate, we masked the wells with 1% BSA-PBS solution and washed the plate three times with PBS-Tween 20 (0.05%). Then, we put diluted plasma samples (1:1000 dilution at day 0 and 1:20000 dilution at day 20 with 1% BSA-PBS solution) onto the wells in microplates, incubated the

plates for one hour at room temperature, and washed them with PBS-Tween 20 (0.05%) three times. Alkaline phosphatase conjugated anti-chicken IgG antibody (1:20000 dilutions, A-9171, Sigma Chemical Co.) was added and incubated overnight at 4°C. Finally, we put P-nitrophenyl phosphate (1mg/mL, Sigma 104 Phosphatase substrate, Sigma Chemical Co.). After 3 hours of incubation at room temperature, stop buffer (3M NaOH) was added to the plates and the absorbance at 405 mm was read using Tecan's Infinite M200 with i-control 1.6 software.

Video-recording

To monitor parental feeding and nestling begging behaviors, we installed small bullet-shaped video cameras in magpies' nests when the eggs hatched and recorded all the activities within the nests during day 6 and day 15, 4 hours daily during 0700 – 1100 (for details see Lee et al. 2010). Among these dates, we chose to analyze the recordings between day 6 and day 10 because this stage corresponds to the rapid growth of nestlings and parental feeding at this stage is the most critical for the growth and survival of nestlings. In total, the behavior of 23 nestlings from 9 nests and parental responses to them were successfully observed and included in the analyses. Each nestling was identified by the marking on the head and bill, which was a combination of color and shape made with nontoxic nail polish (lacks di-butyl phthalate, formaldehyde, and toluene; Kiss Products Co., USA). From the video, we recorded feeding events of each parent to nestlings. Begging intensity of nestlings was ranked from one (the weakest) to five (the strongest) based on their begging posture (See Redondo and Castro 1992). Hunger level of nestlings was defined as the time interval (minutes) after their last food acquisition from either parent and categorized into four levels by the four quartile values between the parental feeding events (based on time passed

without parental feeding; level 1: less than 8 minutes, level 2: between 8 and 13 minutes, level 3: between 13 and 24 minutes, and level 4: more than 24 minutes).

Statistical analysis

A generalized linear mixed model (SAS 9.3, PROC MIXED) was used to elucidate ecological factors that are related to maternally derived immunoglobulin levels of nestlings. Brood size, relative laying date (relative rank of laying date divided by total number of nests), relative hatching order (relative rank of hatching order divided by total number of nestlings in a brood), and sex of nestlings were included as explanatory variables and the identity of the nest was treated as a random factor. We selected a minimal model by stepwise backward elimination process (P value criteria = 0.05) from the initial model containing all the main effects and two-way interactions.

To identify the factors affecting the intensity of begging of nestlings, generalized linear mixed model was implemented using SAS version 9.3 (PROC GENMOD). The response variable was the presence of parental feeding (binary response) at each begging event of a focal nestling. The explanatory variables contained two sets of variables; as for the brood variables, brood size, relative laying date, and sex ratio of the brood were included; as for the nestling variables, immunoglobulin level at hatching, intensity of the begging at the event of begging, relative hatching order, and sex of the focal nestling were. As each nestling was measured many times, nestling identity was treated as the nested factor (nestlings within each nest). The initial models contained the main effects and two-way interactions and backward elimination was conducted. One-way analysis of variance (ANOVA) was used to compare the between-nests and within-nest variances of the

maternal immunoglobulin levels at hatching. We used linear regression to evaluate the effect of immunoglobulin at day 0 on immunoglobulin at day 20.

RESULTS

Maternal immunoglobulin level

The mean level of immunoglobulin of nestlings was 7.20 (±11.97, SD) U/mL at day 0 and 170.72 (±94.91, SD) U/mL at day 20. The nestlings from the same brood had similar levels of maternally derived immunoglobulin (i.e. immunoglobulin at day 0) than the ones between the nests (One-way ANOVA, $F = 2.518$, $p = 0.013$). Similar to results from a previous study (Pihlaja et al. 2006), nestlings who had higher level of immunoglobulin at day 0 had higher level of immunoglobulin at day 20 (Figure 4-1; Linear regression, coefficient $= 0.1599 \pm 0.0681$ (SE), $df = 1$, $t = 2.3458$, $p = 0.0370$).

The immunoglobulin level at day 0 was affected by brood size, relative laying date, and relative hatching order (Table 4-1). Nestlings produced from late breeding parents had relatively lower level of immunoglobulin (Figure 4-2). Nestlings that hatched later than others had lower level of immunoglobulin and this was more pronounced in smaller broods (Figure 4-3).

Table 4-1. Minimal model for maternal immunoglobulin levels (IgG at day 0).

Effect	df	F value	P value	β (±SE)
Brood size	1	4.24	0.054	-0.175 (0.085)
Laying date	1	4.45	0.049	-0.395 (0.187)
Hatching order	1	10.28	0.003	-2.147 (0.667)
Brood size × Hatching order	1	7.11	0.013	0.408 (0.153)

The initial model included main effects and two-way interactions of brood size, relative laying date, relative hatching order, and sex of the focal nestlings.

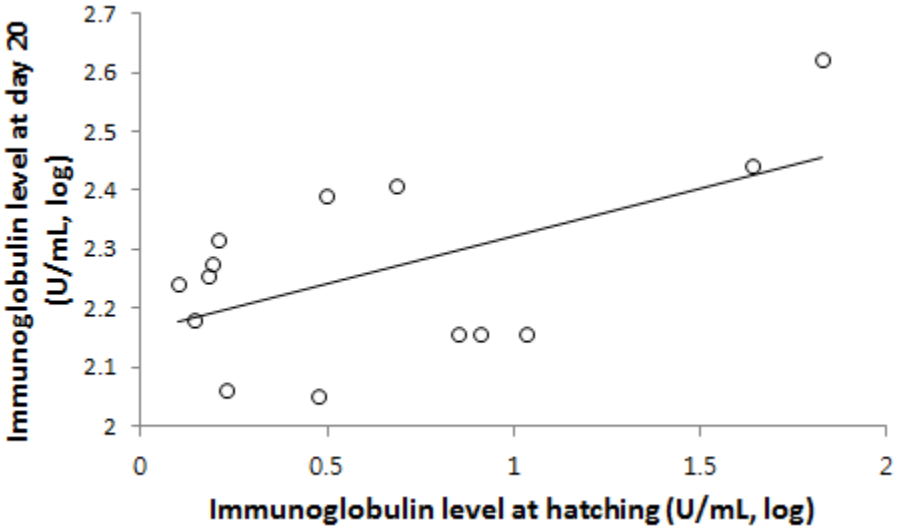

Figure 4-1. Immunoglobulin level (log-transformed) of nestlings at day 0 and at day 20 of 14 nestlings in 8 nests. The fitted line is produced from linear regression (y = 0.160x + 2.163).

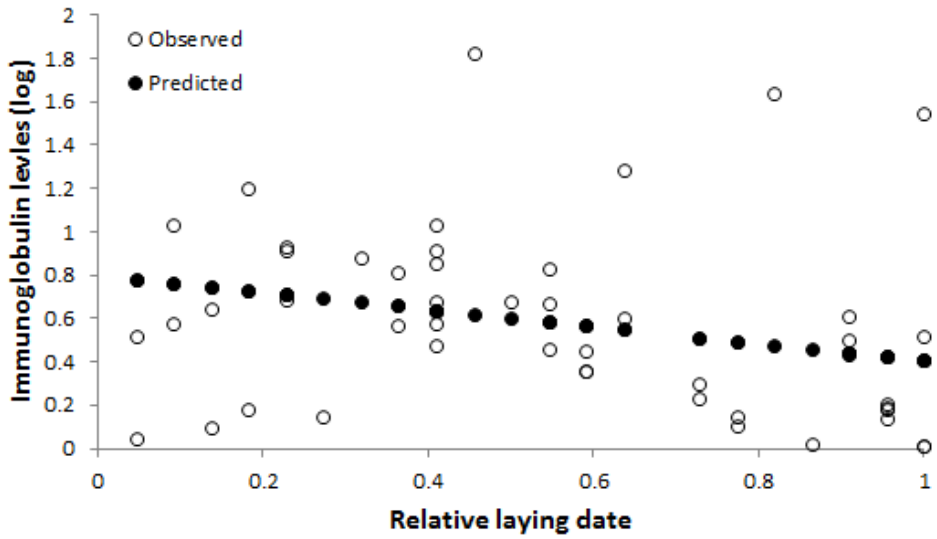

Figure 4-2. Immunoglobulin level of hatchlings decreased with laying date. Predicted values were obtained from a generalized linear model.

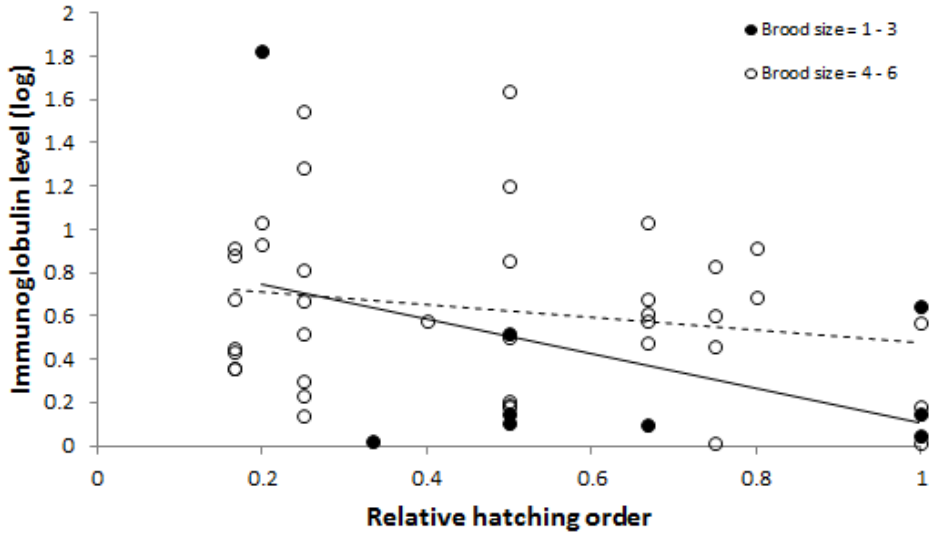

Figure 4-3. Immunoglobulin level (log) of hatchlings decreased with hatching order of nestlings. For convenience, we divided the brood size into two categories and presented the results. Dotted line (y = -0.801x + 0.903) is for smaller broods (brood size 1-3) and solid line (y = -0.290x + 0.766) is for larger broods (brood size 4-6).

Food allocation by parents

Factors affecting the probability of being fed by parents

From video-recordings, 1635 begging events and 1137 feeding events were observed totally. Begging intensity of nestlings did not contribute to the probability of being fed by parents while other factors were included in the minimal model (Table 4-2). Male nestlings with more antibodies at hatching had more chances to be fed by parents (Figure 4-4). Core chicks had a higher probability of being fed in females but lower probability in males (Figure 4-5). In relation to brood sex ratio, nestlings in male-biased broods got more chances to be fed (Figure 4-6). Probability of being fed by parents increased with relative laying date (Figure 4-7).

Factors affecting the probability of being fed by female parents

Contrary to what can be expected, female parents provided less feeding in larger broods (Table 4-3). Feeding activity of the female parent was differentially influenced by the level of maternally derived immunoglobulin and relative hatching order of the focal nestling (Table 4-3). Late hatching nestlings with more maternal antibodies had a higher probability of being fed by the female parent, but core chicks with more antibodies had less (Figure 4-7).

Factors affecting the probability of being fed by male parents

Feeding of the male parent was positively influenced by relative laying date only (Table 4-4, Figure 4-9). Neither begging intensity nor maternal immunoglobulin levels of nestlings remained in the minimal model.

Begging intensity of nestlings

Maternal immunoglobulin levels and brood size influenced begging intensity of nestlings (Table 4-5). Interestingly, hunger level was removed in the process of model selection. Begging intensity increased with brood size and interaction of brood sex ratio and immunoglobulin level. Nestlings who received more maternal antibodies did not beg more intensively than the ones with less antibodies in both male and female nestlings (Figure 4-10).

Table 4-2. Minimal model for for the probability of being fed (binary response) by either parent.

Effect	df	χ^2	P value	β (±SE)
Brood size	1	7.26	0.007	-0.677 (0.165)
Immunoglobulin level	1	4.64	0.031	3.051 (0.625)
Sex	1	4.99	0.027	2.764 (0.957) for female
Brood sex ratio	1	9.28	0.002	2.132 (1.058)
Laying date	1	5.20	0.023	3.166 (0.667)
Hatching order	1	0.58	0.447	1.855 (1.027)
Immunoglobulin level × Sex	1	4.80	0.029	-3.074 (0.692) for female
Hatching order × Sex	1	4.70	0.030	-2.975 (1.119) for female

The initial model included main effects and two-way interactions of brood size, brood sex ratio, relative laying date of the nests; immunoglobulin level at day 0, sex, relative hatching order, and begging intensity of the focal nestling.

Table 4-3. Minimal model for the probability of being fed by female parents.

Effect	df	χ^2	P value	β (±SE)
Brood size	1	4.14	0.042	-0.442 (0.147)
Immunoglobulin level	1	3.23	0.072	-2.194 (0.777)
Hatching order	1	3.79	0.052	-2.204 (0.952)
Immunoglobulin level × Hatching order	1	4.56	0.033	4.978 (2.277)

Explanatory variables included in the initial model were the same as in Table 4-2.

Table 4-4. Minimal model for the probability of being fed by male parents.

Effect	df	χ^2	P value	β (±SE)
Laying date	1	5.31	0.021	2.458 (0.640)

Explanatory variables included in the initial model were the same as in Table 4-2.

Table 4-5. Minimal model for begging intensity of a focal nestling.

Effect	df	F value	P value	β (±SE)
Brood size	1	7.26	0.020	0.144 (0.053)
Brood sex ratio	1	0.83	0.379	-0.350 (0.383)
Immunoglobulin level	1	60.39	<.0001	-4.258 (0.569)
Sex	1	3.99	0.069	-0.492 (0.247) for female
Hatching order	1	2.47	0.142	0.354 (0.225)
Brood sex ratio × Immunoglobulin level	1	45.82	<.0001	6.409 (0.947)
Brood sex ratio × Hatching order	1	24.71	0.000	-2.682 (0.540)
Immunoglobulin level × Sex	1	13.02	0.004	1.341 (0.372) for female

Explanatory variables included in the initial model were the same as in Table 2 except for hunger level of the focal nestling.

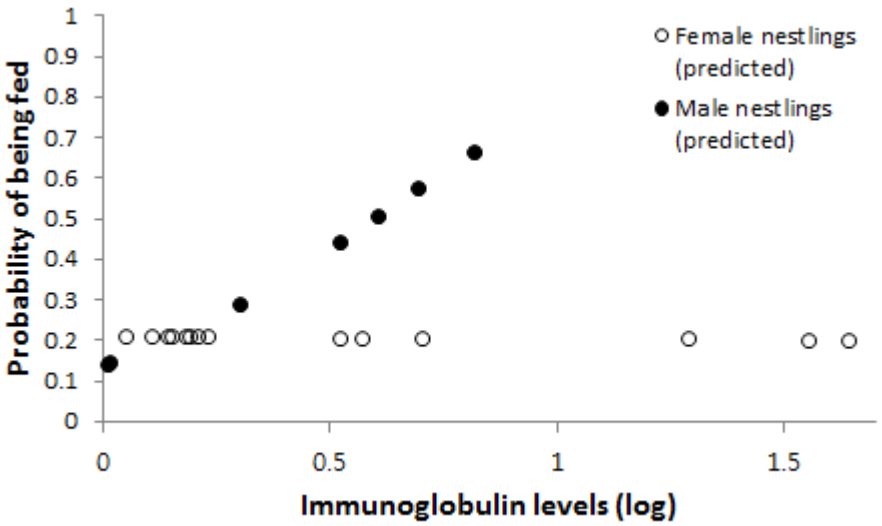

Figure 4-4. Probability of being fed by either parent increased with maternal immunoglobulin levels (log) in male nestlings but not in female nestlings.

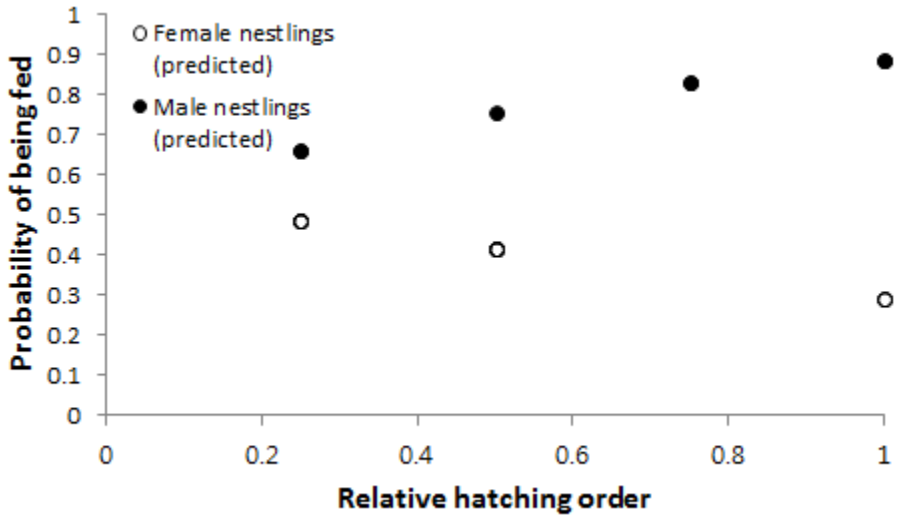

Figure 4-5. Probability of being fed by either parent increased with hatching order in male hatchlings but decreased in female nestlings.

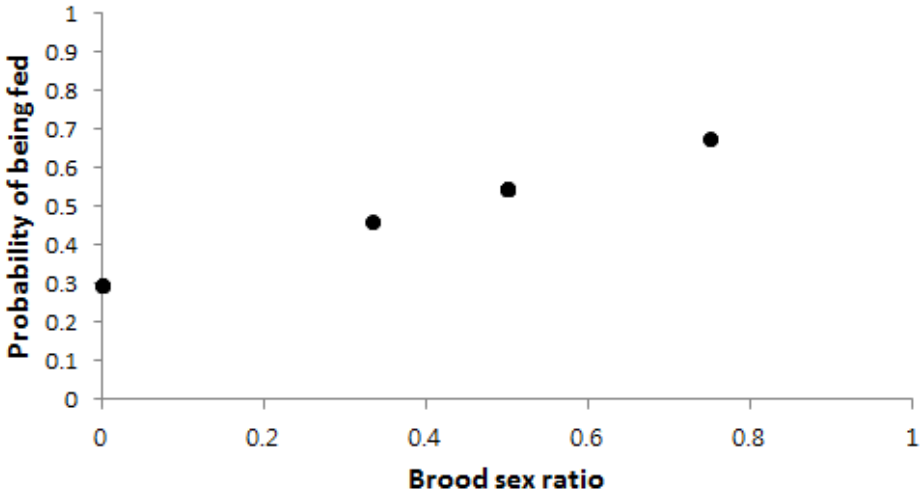

Figure 4-6. Probability of being fed by either parent increased with brood sex ratio (number of male nestlings per total number of nestlings in a brood).

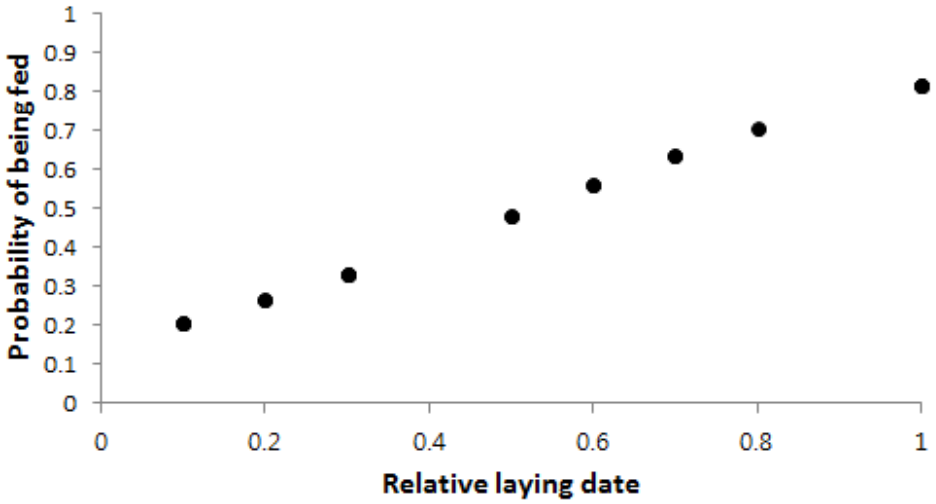

Figure 4-7. Probability of being fed by either parent increased with laying date of the parents.

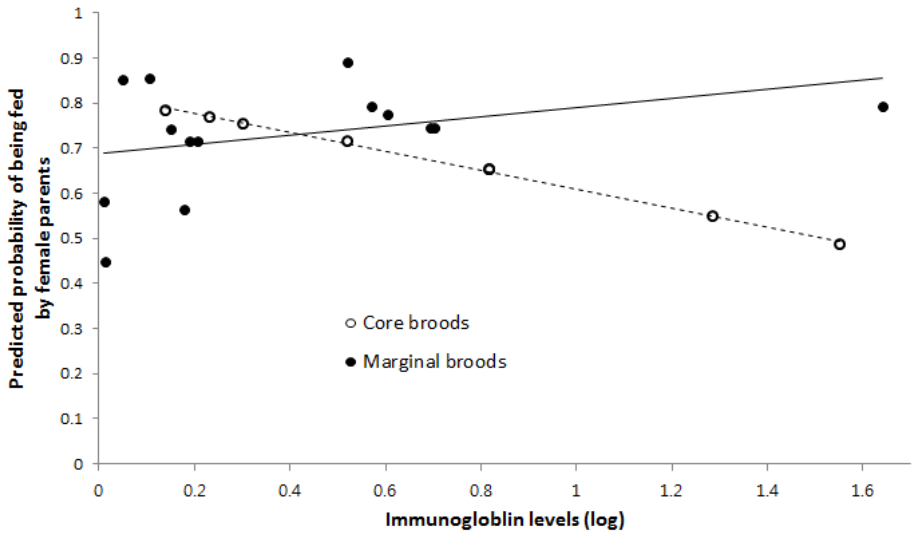

Figure 4-8. Probability of being fed by female parents was affected by immunoglobulin levels and hatching order of the focal nestling. For convenience, we divided core brood (early hatching nestlings with hatching order < 0.5) and marginal brood (late hatching nestlings with hatching order ≥ 0.5) and presented the results. Solid line is for core broods and dotted line is marginal broods.

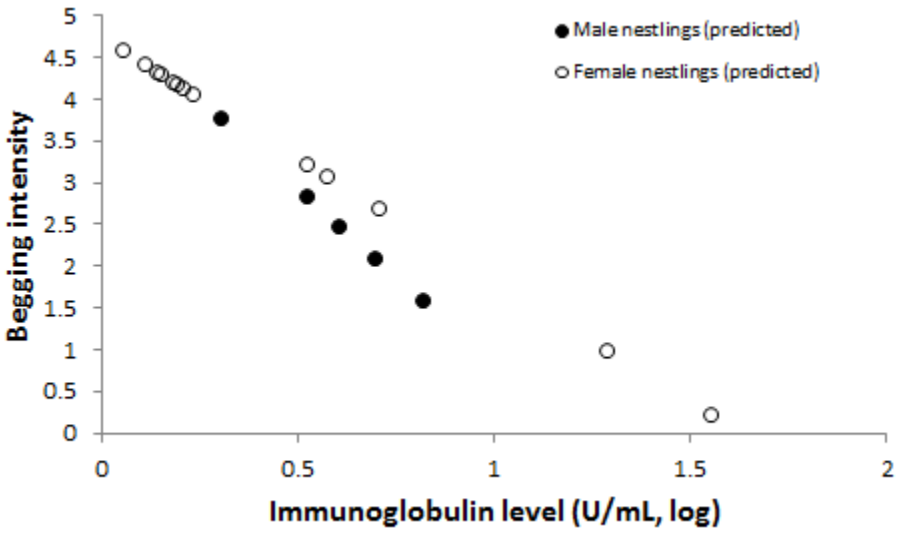

Figure 4-9. Probability of being fed by male parents increased with laying date of the brood.

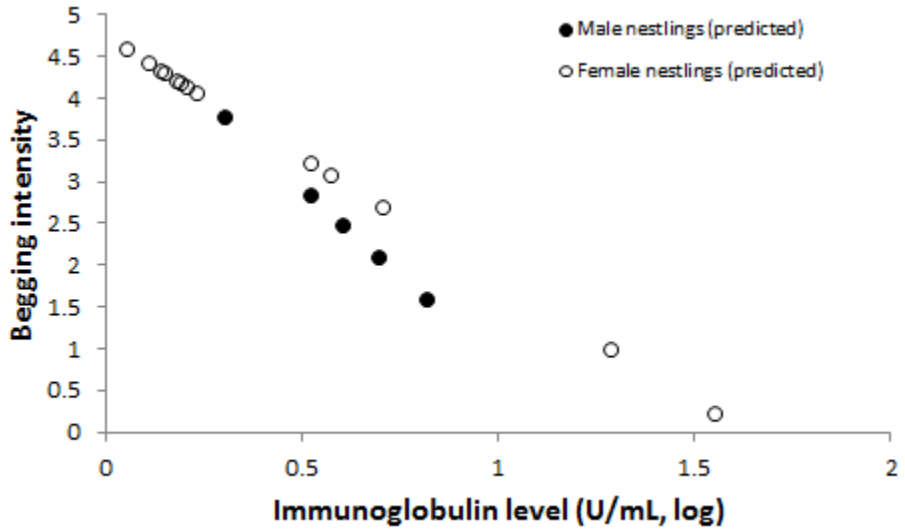

Figure 4-10. Immunoglobulin level at hatching and begging intensity of nestlings

DISCUSSION

We found that mothers delivered generally lower level of maternal antibodies to late hatching nestlings in larger broods. Also, late breeding mothers delivered lower level of antibodies to the nestlings in general. This suggest the total amount of immunoglobulin that mothers deliver to the eggs is limited and condition-dependent, any unequal distribution of maternal immunoglobulin can be regarded as a form of maternal favoritism. Indeed, we found evidence that mothers deliver immunoglobulin to the eggs unequally. Earlier hatching nestlings contained more of maternally derived immunoglobulin in general. This corresponds to the general consensus that earlier laid eggs had better survival prospects and parents are expected to perform more investment towards them.

Considering that the unequal distribution of immune materials on eggs is determined by female parents, we questioned if this favoritism created by females would be maintained during the period of parental care from egg-laying through feeding. If female magpies favor a certain type of nestlings then the females would provide more food to those nestlings that obtained more maternal antibodies. As expected, our results showed that females had a consistent investment strategy by providing more food to the nestlings with more maternal antibodies but it was only for late hatching nestlings. In early hatching nestlings, females preferentially provide more food to nestlings who received less maternal antibodies. This implies that female parents may have different feeding strategies depending on the hatching order of the nestlings; for earlier hatching nestlings, who have higher survival prospects, mothers seem to compensate for low maternal antibodies by providing more feeding to ensure the health and survival of all those nestlings ("egalitarian" strategy, which is known in female parents of many bird species (Stamps 1990, Rosivall et al. 2005, Cameron-MacMillan et al. 2007); on the other hand, for

later hatching nestlings, who have lower survival prospects and thus any further investment can be wasted, they provide feeding according to the investment that they made earlier so that their earlier investment is less likely to be wasted. By having differential feeding strategies based on survival prospects, mothers seem to adjust the amount of current investment depending on the investment that has been already made and thus can optimize the efficiency of their investment.

On the contrary, male parents had a different feeding strategy. Any of the nestling traits, including maternal antibody levels and relative hatching order of the nestlings, did not influence the feeding of the male parents. Instead, laying date solely affected the feeding of the male parents. This means that male parents may not be choosy among the nestlings and their feeding activity may depend on the food availability only. Different food allocation rules between male and female parents suggest that there is a possibility of sexual conflict during the feeding period (e.g. Dickens and Hartley 2007).

Interestingly, a common feature between male and female feeding patterns was that the feeding was not significantly affected by begging intensity of nestlings. In our results, parental feeding seemed to be more influenced by the level of maternally derived immunoglobulin or hatching order than nestling begging behavior. This suggests that parental feeding patterns were performed based on their investment strategies rather than as a simple response to nestlings' begging. This does not mean that nestlings' begging is not important in parental feeding decisions; in a previous study where maternal immunoglobulin level was not accounted for, nestling begging intensity and different aspects of begging display exerted influence on parental feeding with various extents (Lee et al. 2010, 2012). Our results suggest an importance of maternal investment in shaping the feeding decisions of mothers, which has been overlooked in many studies.

In contrast to what can be expected, hunger level of nestlings did not affect begging intensity. Instead, maternal antibody level significantly influenced the begging intensity of nestlings. This is contrary to previous results that manipulated hunger levels affected begging behaviors of magpie nestlings (Redondo and Castro 1992). Our results suggest a possibility that maternal antibodies affect the begging behavior of the nestlings or the tendency to show intense begging displays more strongly than hunger level.

In summary, female magpie parents seemed to maintain a consistent investment strategy throughout the breeding, which has been examined in our study as the transmission of maternal antibodies and food provisioning. Male and female parents showed different feeding strategies and the favoritism towards nestlings between parents may not coincide. For future studies, an experimental paradigm, including cross-fostering of eggs between clutches or experimentally challenging the immune system of embryos or chicks, would allow clearer understanding of multi-faceted parental investment in birds. Particularly, in species with long periods of parental care as magpies, it is likely that investment pattern in earlier stages of breeding may influence investment strategies in the following stages and the dynamics in parental investment strategies would depend on the environmental changes during the breeding as well. Measurements of other possible correlated maternal effects except antibodies (e.g. maternal hormones and antioxidants, see Ardia and Schat 2008) would also help us to understand the maternal investment in more details.

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Chapter 5.

Prolonged brooding by females and behavioral dispute between parents during the feeding period: an evidence for sexual conflict in the Black-billed Magpie (*Pica pica*)?

ABSTRACT

In bi-parental care animals, male and female parents face a situation whether either parent invest more than their mates. Since parents pay the cost of their own investment but benefit from the total investment by both parents, each parent would benefit from larger investment by the other parent. In birds, many female parents brood altricial young and males provide food for the brooding females and the chicks until the chicks can perform their own thermoregulation. As the nestlings reach certain age, female parents switch from brooding to foraging. Here we report prolonged brooding of females in the Black-billed magpie (*Pica pica*). Using small cameras installed in the nests, we recorded brooding and feeding behaviors in 31 nests for three years from hatching to near fledging. We found that, around the 8 – 12th day after hatching, many females sharply decreased the duration of brooding bouts and increased the frequency of foraging trip. Males decreased the feeding to their mates and increased the feeding to their nestlings while increasing total feeding frequency. However, some females still remained in the nests, brooding fully feathered chicks after the 12th day, although the brooding activity does not seem to enhance the survival of nestlings. During this period, males preferred feeding nestlings directly rather than females. Sometimes females refused to move aside to expose nestlings to the provisioning males and they strongly begged to males for food. This 'conflict period' continued for at least several days and the duration varied among the pairs. Within the same pair, the duration of the conflict period was shortened in the consecutive years (up to three years). Furthermore, the duration of the conflict period was shorter for pairs with larger territories. Our results suggest that this unique form of sexual conflict is more likely to exist related with the quality, age

and/or the past experience of the pair and the pairs seemed to adjust the conflict maintaining the pair-bonds over the years.

INTRODUCTION

Parental investment theory predicts that relative amount of investment between the sexes arises raises the issue between the parents for care (Trivers 1972) and this may lead to a conflict between the evolutionary interests of the two sexes, which termed as 'sexual conflict' (Parker 1979). When parents care for offspring, sexual conflict may occur over how much to invest to the offspring at a given moment of time. Female and male parents pay the cost of their own investment separately, but they share the benefit from the total investment made to the offspring. Thus, if one parent can reduce their cost by exploiting the other parent to provide more investment, it would be beneficial for the parent (Lessells 2012). Hence, each sex would reduce their investment as long as the other can provide more investment to the offspring. In species with bi-parental care, there are also sexual conflicts over the relative amount of care during the period of parental care and this may lead to the evolution of parental behavior in both sexes to shift the load towards the other sex (Arnqvist and Rowe 2005).

Since parental care behavior can be affected by signals of their mating partners via hormones or other external stimuli (e.g. Friedman and Lehrman 1968, see Arnqvist and Rowe 2005), both sexes have an opportunity to exploit their partner. For instance, in blue tits (*Parus caeruleus*) females caused synchronous hatching by delaying incubation, which could manipulate males to contribute more to parental care because males provided more efforts to even-aged broods (Slagsvold et al. 1994). This showed that sexual conflict would be a sexually antagonistic adaptation of one parent to manipulate the other to provide more investment for care in monogamous bi-parental animals (Slagsvold et al. 1994, 1995). However, as far as we know, no study presented direct observation of conflict (behavioral dispute) between male and female

parents during the period of parental care regarding the interests of relative parental investment.

Black-billed magpie (*Pica pica*) is a socially monogamous bird. Magpies have relatively a long period of time for parental care and different sex roles in male and female parents (Birkhead 1991). While only female magpies incubate eggs, males provide food to the incubating females in the nests. After hatching, females brood young chicks at early stage and sharply decrease the duration of brooding bouts and increase the frequency of foraging trip (Buitron 1988). In this study, we report the prolonged brooding of females and conflicts between the breeding pairs. Then, we questioned why some females remained in the nests beg food to males and what factors influenced the occurrence of the conflicts, with brood size, laying date, and territory size. Also, we observed whether the probability of having conflicts within the same pairs changed through years while they maintained the pair bond.

MATERIALS AND METHODS

Study population and video-recording

This study was conducted on Seoul National University (SNU) campus (Seoul, Korea). In the campus area, we regularly monitored approximately 50 magpie pairs during the breeding season. From March, we approach magpie nests twice a week and determined laying dates. Magpie incubation is performed by female only during the whole incubation period, 21-22 days after first egg laying (Birkhead 1991, for details see Lee et al. 2013). Around the expected hatching date, we visited the nests and color-marked the nestlings for individual discrimination. Then, we installed small camera (Weatherproof Bullet Cam XB421-W36, Vision Hitech Company)

between 0700 and 1100 daily to observe parental behaviors. In total, 31 nests for three years (7 nests in 2007, 14 nests in 2008, and 10 nests in 2009) were recorded from hatching (day 1 – 3) to around 15th day after hatching. Among the 31 breeding pairs, six pairs were distinguishable by the ring combinations on the legs that we have put at fledging since 1997 in the same study area and by trapping two adult pairs during the winter season in 2008. From the nest locations in our study area, we estimated the distance from the closest nest in order to estimate the territory size of pairs (Birkhead 1991).

Description of prolonged brooding of female and conflict between parents

In our magpie population, we observed that some females remained in the nests even when nestlings were feathered and strongly begged to males for food. The females refused to move aside to expose nestlings to the provisioning males. During this period, males preferred feeding nestlings directly rather than females and sometimes physical conflicts between the begging females and males occurred in the nests. When males ignored begging females in the nests and fed nestlings only with aggressive interactions, we defined such behavior as 'behavioral conflict' between the pairs. We counted how often this behavior was recorded and how long this had lasted once it was observed.

Statistical analysis

To compare the female brooding time in the nests with conflicts and with no conflicts, we used Welch two sample t-tests (R software 3.0.1). To investigate the factors affecting the probability of having conflicts between the pairs, we used a generalized linear model (SAS 9.3, PROC GENMOD) with main effects and two-way interactions of year, brood size, relative laying

date (rank order divided by the total number of nests), and distance from the closest nest. We implemented paired t-test to compare the duration of conflict periods (days) with the one in previous year within the same pair. Because two pairs were monitored for three years, the two pairs were included in the analysis twice.

RESULTS

We found that, around the 8 – 12th day after hatching, many females sharply decreased the duration of brooding bouts for three years (Figure 5-1). During the feeding period, females reduced the brooding time and increased the frequency of foraging trip while males decreased the feeding to their mates (Figure 5-2). As nestlings aged, males decreased feeding to brooding females and did not feed females after day 11. Meanwhile, females increased feeding trips and reduced brooding time. However, some females still remained in the nests, brooding fully feathered chicks after the 8 - 12th day although the brooding activity does not seem to enhance the survival of nestlings. During this period, males preferred feeding nestlings directly rather than females. Sometimes females refused to move aside to expose nestlings to the provisioning males and they strongly begged to males for food. This 'behavioral conflict' was observed in 20 nests (5 nests in 2007, 10 in 2008, and 5 in 2009) among 31 nests video-recorded. Such conflicts were recorded between 8 – 17th day, and once the conflicts were observed, this behavior occurred 1.70 (± 0.81, SD) times per daily recording (four hours) and lasted for 3.65 (± 2.01, SD) days.

When we compared female brooding time in the nests with conflicts and with no conflicts (Figure 5-3), there were no differences before 12th day after hatching (Welch two sample t-test, $t = 1.4604$, $df = 80.64$, $p = 0.1481$) but there was a significant difference after 12th day ($t = -7.5279$, $df = 65.23$, p

< 0.001). Thus, prolonged female brooding after 12th day was highly related with conflict behaviors between the pairs.

Probability of having conflicts was affected by the distance from the closest nest (Figure 5-4; PROC GENMOD, $\beta = -0.1913$, $p = 0.0004$). This suggests that the conflict was more likely to occur between pairs with smaller territories. Within the same six pairs for three years (2007-2009), the duration of the conflict period was shortened in the consecutive years (Figure 5-5; Paired t-test, $df = 7$, $t = 2.392$, $p = 0.048$).

When we investigated the breeding results of the nest, the number of hatchlings and fledglings did not differ between the nests with conflicts and no conflicts (Figure 5-6, Welch two sample t-tests, $t = 0.1656$, $df = 18.675$, $p = 0.8702$ for hatchlings; $t = -0.8372$, $df = 20.582$, $p = 0.4121$ for fledglings). Body condition index of fledglings in the two groups were not significantly different each other (Figure 5-7; Welch two sample t-tests, $t = 0.733$, $df = 37.357$, $p = 0.4681$).

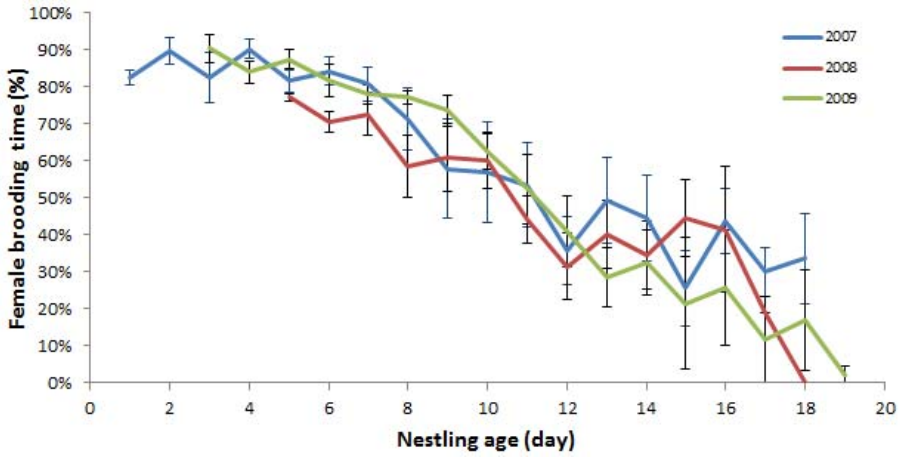

Figure 5-1. Nestling age (day) and female brooding time (%) for three years (2007 - 2009). Females sharply reduced brooding time after day 5 – 6 and it highly varied between day 12 – 18 among the nests.

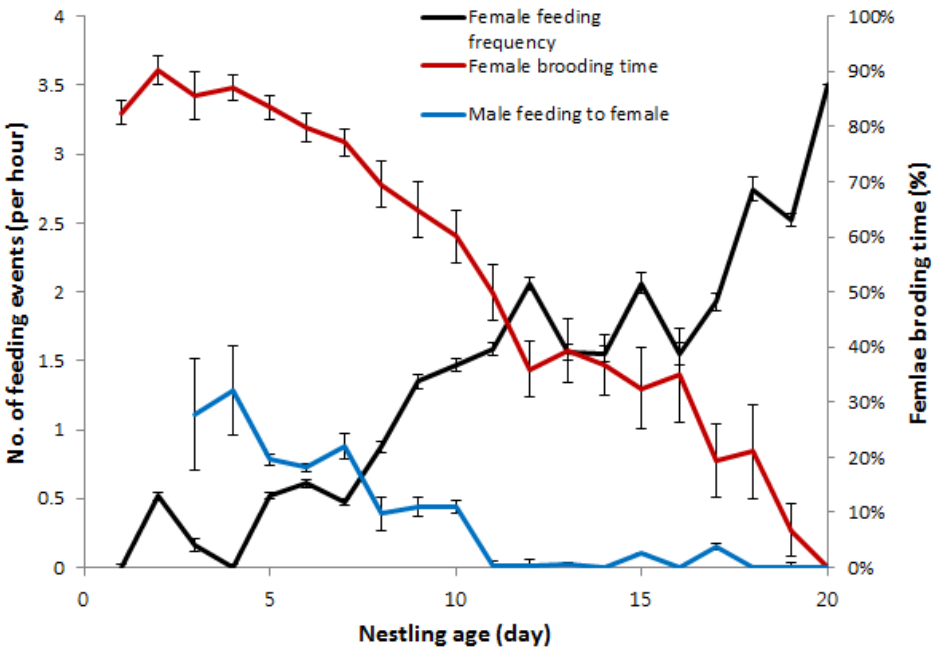

Figure 5-2. Nestling age (day), female brooding time (%), female feeding to nestlings and male feeding to female in the nest (no. of events per hour). As nestlings aged, female reduced brooding time and increased feeding trips while male reduced feeding to female in the nest.

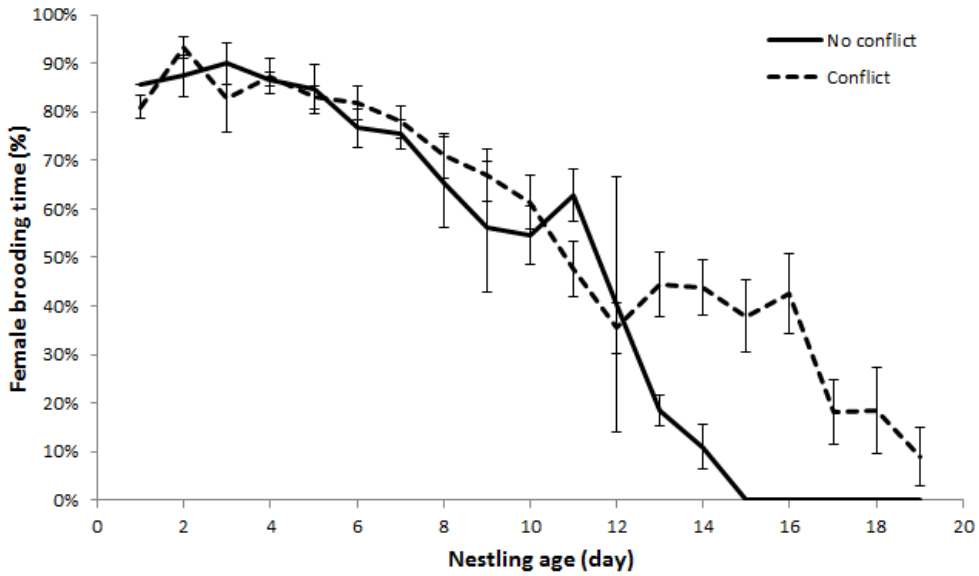

Figure 5-3. Nestling age and female brooding time (%) when conflicts were observed between pairs during the feeding period (solid line) and when no conflicts were observed (dashed line). In the nests with conflicts, females remained in the nests even after day 15.

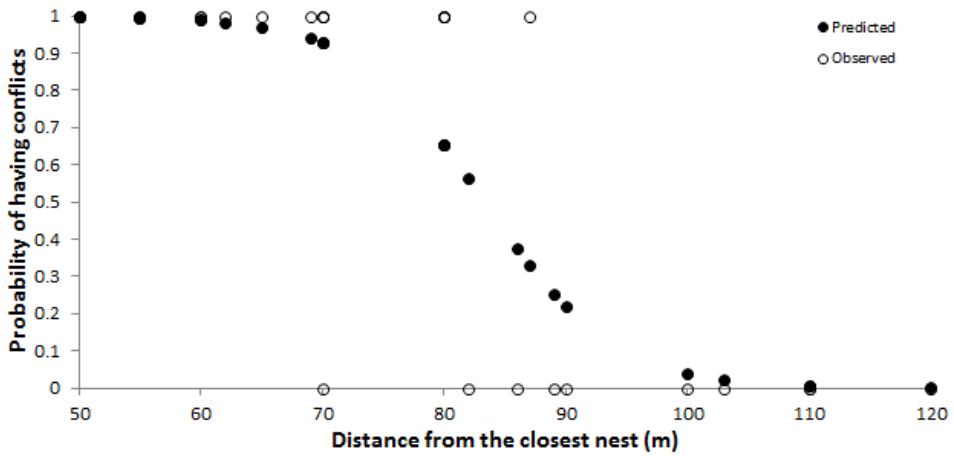

Figure 5-4. Distance from the closest nest affected probability of having conflicts between pairs (Proc genmod, $\beta = -0.1913 \pm 0.0791$, $p = 0.0004$). Pairs in a large territory are less likely to have conflicts during the feeding period.

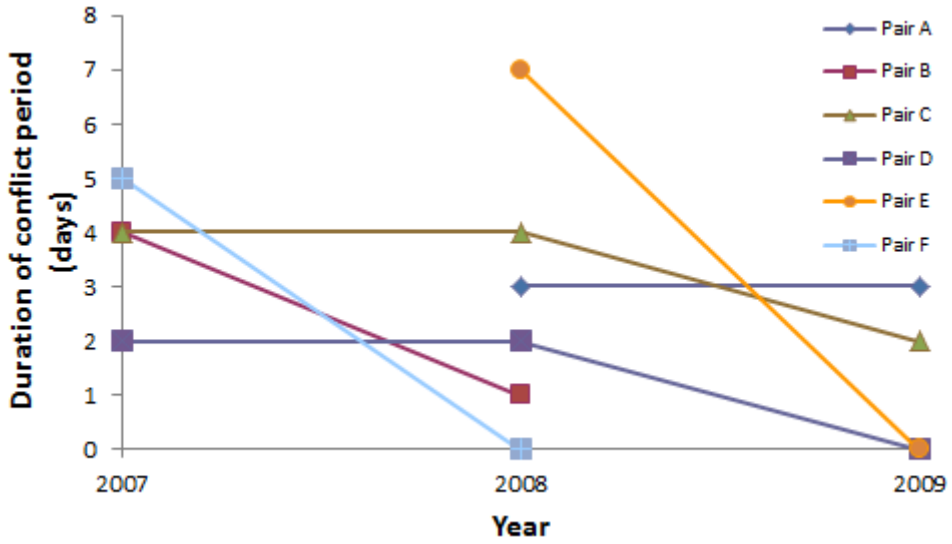

Figure 5-5. Duration of conflict period (days) in six breeding pairs for three year (2007 – 2009). The pairs reduced the conflict period thorough years while maintaining the pair bonding.

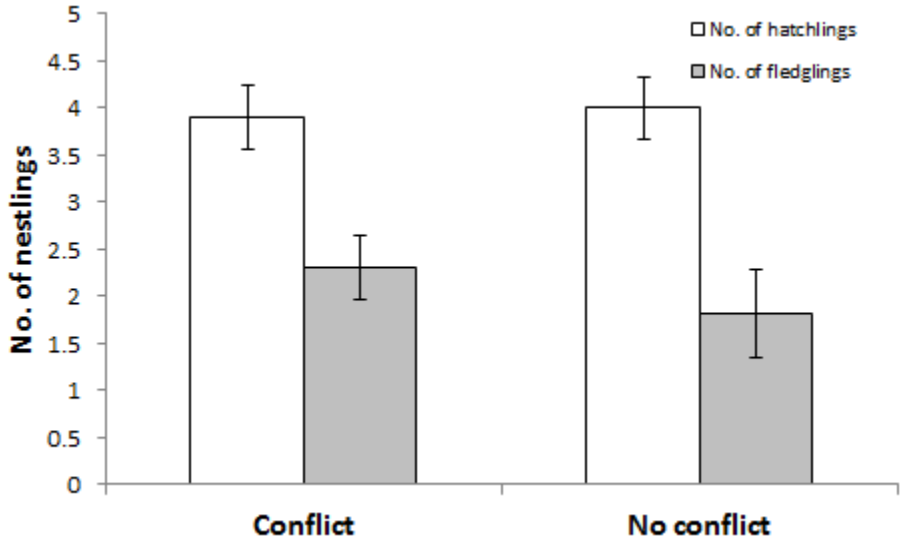

Figure 5-6. The number of hatchings and fledglings in the nests with conflicts (n = 20) and with no conflicts (n = 11).

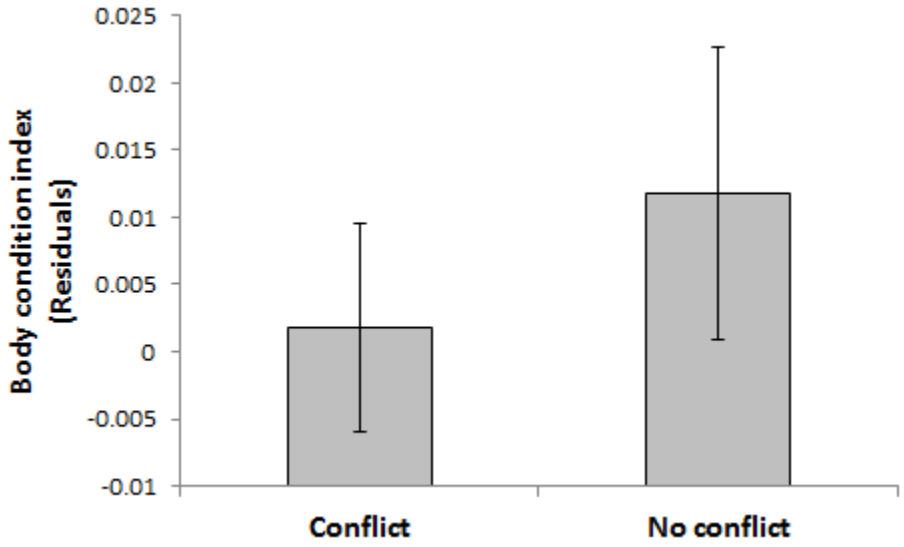

Figure 5-7. Body in the nests with conflicts (n = 20) and with no conflicts (n = 11).

DISCUSSION

In this study, we report a conflict behavior between the pairs and discuss whether the breeding pairs resolve the conflict in a bi-parental monogamous bird. We found that some females still remained in the nests, brooding fully feathered chicks after the 8 - 12th day when thermoregulation of their nestlings did not seem to be necessary. Then, why do the females remain in the nests and beg food to males? One possibility is that the prolonged brooding is necessary for protection of nestlings against nest predators. It would be beneficial if females remained in the nests responding to small nest predators (Buitron 1988). However, nest predators may not play an important role in prolonged brooding of females at least in our population since we could not observe any nest predations from five year video-recordings during the feeding period (74 nests; 8 in 2007, 16 in 2008, 19 in 2009, 16 in 2010, and15 in 2011). Therefore the brooding activity does not seem to particularly enhance the survival of nestlings. Another explanation is that females remained in the nests to acquire food and more care for nestlings from males. In magpies, males and females specialize their role in parental care (Buitron 1988). Only females incubate eggs and males feed the incubating females. After hatching, females switch the role from incubation to foraging. However, if females could acquire food from their partners without self-feeding trips and burden to males for feeding nestlings, this may bring benefits to the females and males should take more works to compensate the reduced feeding of females. This would be in accordance with sexual conflict (Parker et al. 1972, see Lessells 2012). When we compared the nests with and without conflicts, the presence of prolonged brooding of females was highly related with the presence of conflict behavior. Thus, we think that the prolonged brooding is the main reason for the conflict between the parents.

Considering that this behavior is temporarily observed when females switch their role from brooding to foraging, such conflict does not seem to be a manipulating behavior of one sex to the other sex to provide more investment (Slagvold et al. 1994). Instead, this could be an extension of deceptive behavior (Whiten and Byrne 1991) by concealing current information of nestlings to induce more contributions from males temporarily. In fact, during this period, females refused to move aside to expose nestlings to the provisioning males and they strongly begged to males for food. This may be due to complex feeding patterns of magpie parents. In early stage of feeding, males deliver food to females and females allocate a part of food from males to the nestlings. Simultaneously, males participated in the food allocation by themselves. Therefore, males were often deceived by females hiding nestlings even when nestlings were not necessary for female brooding.

Then, what factors affect the conflict behavior? Our results suggest that the pairs occupying small territories are more likely to have such conflicts. Also, the pairs seemed to reduce the conflict period through years. Currently, we do not have data for age or body conditions of adult birds to extend our findings. If we assume that social status is related with the age and the low rank young breeders have small territories in magpies (Birkhead 1986, 1991), we guess that naïve breeders have more conflicts and manage the conflict for years while maintaining the pairbonds.

Sexual conflict may lead to a reduction of total amount of parental care for offspring by antagonistic negotiation between the pairs ('negotiation hypothesis', Parker 1985, McNamara et al. 1999, 2003). In our results, the conflict did not significantly influence the breeding success, such as the number of fledglings and body condition of the fledglings. Since conflict behaviors were not observed frequently (0.44 times (± 0.38, SD) per hour) and lasted for 3.65 (± 2.01, SD) days between the 8th – 17th day), this does not seem to severely suffer offspring despite the conflict.

To summarize, we observed conflict between the pairs during the feeding period, possibility due to the prolonged brooding of females. Our results suggest that this unique form of sexual conflict exists in magpies during the feeding period, and this may be related to their tendency of repeated breeding with the same partner over the years and the degree of conflict seems to be shaped by the quality, age and/or the past experience of the pair. Future studies with adult information and pair-bonding history, we could be close to understanding of why such conflict occurs and how the parents adjust the problems in a fine scale.

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Supplementary information

Breeding information and recording dates of the magpie nests from 2007 to 2009.

Year	Nest ID	Brood size	Hatching date	Recorded period (nestling age)
2007	131	3	30-Apr	4–17
2007	251	4	4-May	0–13
2007	5662	4	18-Apr	4–17
2007	97	4	1-Apr	4–17
2007	29	5	9-Apr	1–8
2007	45	7	18-Apr	8–15
2007	24	2	10-Apr	12–14
2008	3	5	23-Apr	5–9
2008	32	2	27-Apr	7–11
2008	73	6	20-Apr	10–16
2008	74	4	8-May	7–10
2008	109	5	3-Apr	6–15
2008	151	3	1-Apr	8–17
2008	314	5	10-Apr	10–15
2008	138	6	11-Apr	9–15
2008	66	4	4-May	7–11
2008	G6	3	7-Apr	10–16
2008	16103	3	24-Apr	6–8
2008	1109	5	3-Apr	9–11
2008	MR1	5	9-Apr	11–13
2008	17	5	2-May	6–9
2009	13	2	25-Apr	5–20
2009	27	2	29-Mar	2–10
2009	84	4	12-Apr	3–11
2009	310	6	14-Apr	9–14
2009	1670	3	8-May	6–16
2009	1683	4	24-Apr	6–18
2009	2511	4	18-Apr	6–14
2009	58B	6	16-May	10–19
2009	71150	4	28-Mar	4–19
2009	1773	6	8-Apr	15–18

Chapter 6

Maternal antibodies and parental provisioning in the Black-billed magpie (*Pica pica*): an experimental study

ABSTRACT

Maternal antibodies are important for nestlings' immunity since newly hatched nestlings are dependent on the maternally derived antibodies. By differentially allocating maternal antibodies to certain nestlings, mothers can aid the health or survival of those nestlings. However, it is currently not clear if the parents exhibit consistent investment patterns throughout the period of parental care. In this study, we questioned whether nestlings that received more maternal antibodies were also more provisioned by the parents. To disentangle any effects from other parental factors, we cross-fostered nestlings at hatching randomly with respect to hatching order and investigated the correlation between parental feeding, nestling begging and the effect of maternally derived antibodies on these two. Early hatching nestlings and nestlings from smaller broods contained higher level of maternally derived antibodies. We found that parents preferentially fed nestlings who received more antibodies from genetic mothers, regardless of hatching order and nestling sex. Probably because of this biased feeding, nestlings which had more maternal antibodies were also more likely to survive until fledging. Our results suggest that parents can recognize nestlings which received more maternal antibodies, probably through the begging behavior of the nestlings, and allocate more feeding effort to ensure the survival of those nestlings.

INTRODUCTION

Theory predicts that parental investment adjusts their current expenditure to expected future benefits of parental care and this is in relation to past investment (Trivers 1972, Maynard Smith 1977, see Clutton-Brock 1991). Since past investment reduces parents' capacy for prospective investment, the benefits of future expenditure are related with the past investment of parents (See Maynard Smith 1977). Several empricial studies suppoted that current expenditure is correlated with the past expenditure in parental investment of birds (e.g. Weatherhead 1982, Winkler et al. 1991). For instance, Winkler et al. (1991) found that tree swallow parents decided their investment rules, whether or not to abandon a nestling attempt, based on their past reproductive effort.

In birds, maternally antibodies (mainly Immunoglobulin; Tella et al. 2002) are transmitted to the offspring at egg-laying. Young nestlings largely rely on maternal antibodies for improving immunity and the anitbodies could help protection against infectious diseases (see Grindstaff et al. 2003). Empricial studies supported that maternal antibodies also have long-term effects on offspring immune system (Reid et al. 2006, Grindstaff 2008). However, several recent studies found no effects of maternal antibodies on immunity of short-term (King et al. 2010) or long-term at the adult stage (Addison et al. 2010). It is still unclear whether the maternal antibodies boost nestling immunity and consequently increase nestling survival. Moreover, most studies on maternal effects highlighted on maternal materials and nestling growth or survival. However, we do not know if the maternal materials directly help nesltings' immunity and if the biased maternal distribution indirectly induces biased parental provisioning at feeding stage. For mother, production and transmission of maternal antibodies are costly and condition dependent (Philaja et al. 2006, Boulinier and Staszewski 2008). If

the past investment for biased maternal materials is related with future investment, parents would put more investment to the nestlings with more maternal materials by biased feeding. Therefore, we predicted that offspring which was invested more than others (with more maternal antibodies) at an early stage of investment would be favored (with more feeding) at a later stage.

Here, we investigated the effect of the maternal antibodies on parental food provisioning and nestlign survival in the Black-billed Magpie (*Pica pica*). Brood reduction is common in this species, mostly due to starvation (Birkhead 1991). In a previous study in magpies, young hatchlings were dependent on maternal antibodies and began to produce their own antibodies at near 8-10 days after hatching (Philaja et al. 2006). Also, a nestling with more maternal antibodies had a higher value of antibodies, which were synthesized mainly from their food acquisition. The positive relationship between the maternal antibodies at hatching and the antibodies at near fledging suggests that parental feeding may play an important role to mediate the correlation. Hence, we manipulated nestlings' maternal antibody levels by cross-fostering at hatching and examined how parents allocated food resources to their nestlings during the feeding period in relation to the maternal antibodies. Furthermore, we estimated if the maternal antibodies affected nestlings' survival until fledging.

MATERIALS AND METHODS

Experimental procedure

In our study area, Seoul National University (SNU) campus, more than 50 breeding magpies were monitored in 2010 and 2011 during the breeding season. We regularly visit the nests from nest building and

determined laying and hatching dates. At hatching dates, we sampled 10 – 15 microliters of blood from the nestlings from medial metatarsal vein. Blood samples were centrifuged to separate blood cells and plasma, which then were used for molecular sexing and maternal antibody measurement (see below) respectively. Molecular sexing was conducted on the same or the next day using P2-P8 primers (Griffiths et al. 1998). After two to three days, we selected pairs or triplets of nests with the same hatching date and we exchanged 2-3 nestlings among these pairs or triplets to change the sex composition of the brood as an experimental procedure for other experiment that investigates the effect of brood sex ratio on the mortality of the nestlings (see Chapter 6). As the cross-fostering was conducted in a blind manner except for the sex, the hatching order of the nestlings was randomly changed (correlation between the original hatching order and newly assigned hatching order; $r = 0.248$, $p = 0.105$). Brood size of the nests was not altered. When we cross-fostered the nestlings, we installed small video cameras (Weatherproof Bullet Cam XB421-W36, Vision Hitech Company, 8cm (L) × 2cm (r)) into the nests to record the feeding behaviors of parents and begging of nestlings (for details of video-recordings, see Lee et al. 2012). We individually marked the nestlings with non-toxic nail polish (Kiss nail products, NY, USA) on their beak and head to distinguish them in the video-recordings. Behavior of 26 hatchlings (7 nests) in 2010 and 23 hatchlings (8 nests) in 2011 were included in the data analysis.

From the plasma that were separated from the blood cells, the amount of immunoglobulin Y (IgY, an avian prototype of IgG) proteins were measured using ELISA method (Enzyme-linked immunosorbent assay) with anti-chicken immunoglobulin antibody (for details, see Philaja et al. 2006).

Statistical procedure

To elucidate what factors influenced the level of maternal antibodies and begging intensity, we used a general linear mixed models (SAS software 9.3, PROC MIXED) where nest identity was treated as the block of analysis. To determine the factors affecting the probability of being fed by parents, we used a generalized linear mixed model (SAS software 9.3, PROC GENMOD) and nestling identity was treated as the nested factor (nestlings within each nest). From the initial model that contained main effects and two-way interactions among the explanatory variables, we searched minimal model by stepwise backward elimination (Crawly 1993) based on p-value (criterion for deletion is $p \geq 0.05$). In the analysis where the response variable was the level of maternal antibodies, we considered the factors that describe the condition of the brood where a focal nestling was born ("original brood"); thus, sex ratio, brood size, relative laying date of the original brood, and relative hatching order (in the original brood) and sex of the focal nestling were included as explanatory variables. In the analysis where the binary response of the parental feeding (fed or not fed) was considered, we included the factors that describe the condition of the brood that was experimentally altered ("manipulated brood"); thus; sex ratio, brood size, relative laying date of the manipulated brood, and relative hatching order (in the manipulated brood), immunoglobulin level (maternal antibodies) and begging intensity (see below) of the focal nestling were included as explanatory variables. For begging intensity as the response variable, we used the same sets of explanatory variables as for the analysis of parental feeding except we added hunger level (see below) and excluded begging intensity. In all analyses, year was included as additional explanatory variable to control for, if any, the year difference.

Relative laying date was calculated from dividing the relative rank of laying date by the total number of nests. Relative hatching order was from

dividing the relative rank of hatching order by the number of hatchlings. Hatching order was determined based on the tarsus length.
Begging intensity (1 – 5, from the weakest to the strongest) was measure from the posture of nestling in the recordings (see Redondo and Castro 1992).
Hunger level (1 – 4, from the least hungry to the most hungry) was categorized as the time interval (minutes) since the last food acquisition from the parents (based on time passed without parental feeding; level 1: less than 8 minutes, level 2: between 8 and 16 minutes, level 3: between 16 and 30 minutes, and level 4: more than 30 minutes).

Results

Maternal immunoglobulin level

At hatching, the level of maternally derived immunoglobulin was affected by an interaction of brood size and hatching order (Table 6-1). Early hatching core broods (nestling of low hatching orders) contained higher level of maternally derived immunoglobulin than late hatching marginal broods (nestlings of high hatching orders), and the difference between core and marginal broods was more pronounced in larger broods (Figure 6-1).

Factors affecting the probability of being fed by either parent

Among the explanatory variables, only immunoglobulin level and begging intensity remained in the minimal model (Table 6-2). The nestlings who received more immunoglobulin from the mothers at hatching had more probability to be fed by the parents (Figure 6-2). Also, begging intensity of nestlings increased the chances to be fed (Figure 6-3).

Factors affecting nestling mortality

In the minimal model to elucidate the factors affecting nestling mortality, we found that immunoglobulin level at hatching, laying date and an interaction between feeding frequency and year significantly influenced the mortality (Table 6-3). Nestlings who had higher level of maternal immunoglobulin (Figure 6-4) or nestlings that were produced by late breeding parents (Figure 6-5) had higher chances to survive until fledging. Feeding frequency had different effects on mortality between 2010 and 2011; nestlings that were fed more often in general survived better in 2010, but in 2011, many nestlings died in spite of parental feeding (Figure 6-6).

Factors affecting the begging intensity of nestlings

In the minimal model for begging intensity of nestlings (Table 6-4), hunger level increased the intensity. Interaction of immunoglobulin and sex was included in the model. Nestlings with more immunoglobulin reduced the begging intensity and this pattern was obvious in male nestlings (β = -0.9190 for male and -0.0015 for female). Interactions between brood size and sex ratio, brood size and hatching order also remained in the minimal model (Table 6-4). These interactions simply reflect nestlings in more competitive environment begged more intensely. Additionally, brood size, sex ratio and immunoglobulin had sex-specific effect on the begging intensity of the nestlings.

Table 6-1. Minimal model for maternal immunoglobulin levels at hatching.

Effect	df	F value	P value	β (±SE)
Brood size	1	3.83	0.0587	-0.2409 (0.1232)
Hatching order	1	7.57	0.0094	-2.2999 (0.8358)
Brood size × Hatching order	1	8.14	0.0073	0.5117 (0.1794)

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, original sex ratio and sex of nestlings.

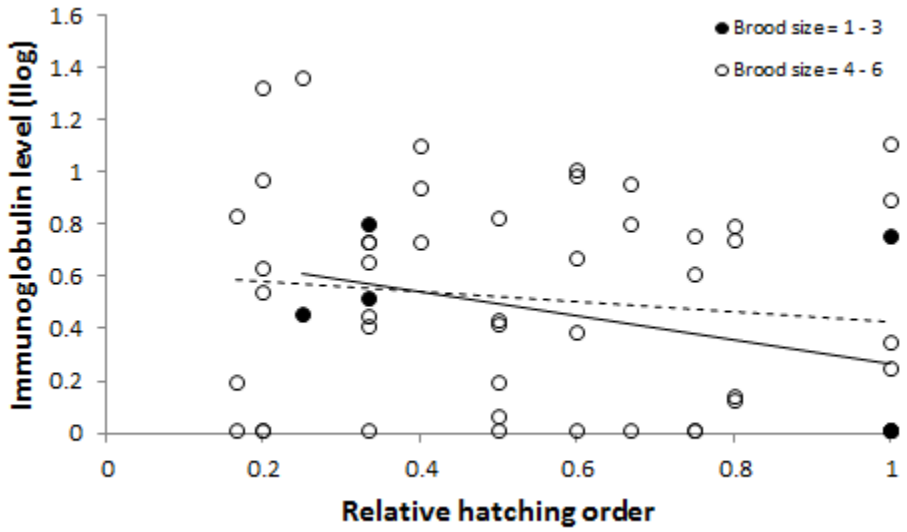

Figure 6-1. Immunoglobulin level (log) of hatchlings decreased by relative hatching order of nestlings ($\beta = 0.5117$, $p = 0.0073$). Fitted lines were drawn by linear regression, the solid line ($y = -0.4666x + 0.7312$) is when brood size is from one to three and the dashed line ($y = -0.2018.x + 0.6255$) is when brood size is from four to six.

Table 6-2. Minimal model for feeding (Y/N) by parents in relation to manipulated sex ratio.

Effect	df	χ^2	P value	β (±SE)
Immunoglobulin level	1	6.38	0.0116	0.6165 (0.1658)
Begging intensity	1	8.86	0.0029	0.4056 (0.0975)

The initial model included main effects and two-way interactions of year, brood size, immunoglobulin level at hatching, sex of nestlings, manipulated sex ratio, relative laying date, relative hatching order, and begging intensity.

Table 6-3. Minimal model for the mortality of nestlings in relation to manipulated sex ratio.

Effect	df	χ^2	P value	β (±SE)
Year	1	6.07	0.0137	4.8453 (1.9110) for 2010
Immunoglobulin level	1	7.08	0.0078	-3.2192 (1.0055)
Laying date	1	4.28	0.0386	-4.085 (1.7770)
Feeding frequency	1	3.2	0.0736	0.7639 (0.3632)
Feeding frequency × Year	1	10.23	0.0014	-3.5594 (1.1051) for 2010

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, feeding frequency, manipulated sex ratio, sex of nestlings and immunoglobulin level at hatching.

Table 6-4. Minimal model for begging intensity of nestlings in relation to manipulated sex ratio.

Effect	df	F value	P value	β (±SE)
Hunger level	1	3.98	0.0464	0.047 (0.0235)
Brood size	1	35.09	<.0001	-0.5543 (0.1388)
Manipulated sex ratio	1	106.71	<.0001	-7.7461 (0.7588)
Immunoglobulin level	1	5.13	0.0302	-0.9190 (0.2667)
Sex	1	20.23	<.0001	1.4485 (0.3220) for female
Hatching order	1	20.87	<.0001	-2.8908 (0.6327)
Brood size × Manipulated sex ratio	1	92.02	<.0001	1.8139 (0.1891)
Brood size × Sex	1	10.03	0.0033	-0.2809 (0.0887) for female
Brood size × Hatching order	1	25.06	<.0001	0.6610 (0.1320)
Manipulated sex ratio × Immunoglobulin level	1	16.38	0.0003	1.745 (0.4312)
Manipulated sex ratio × Sex	1	11.13	0.0021	-1.7048 (0.5111) for female
Immunoglobulin level × Sex	1	29.08	<.0001	0.9175 (0.1702) for female

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, manipulated sex ratio, sex of nestlings, immunoglobulin level at hatching, and hunger level of nestlings (from 1 to 4; measured by the time interval between the parental feeding events from the weakest to the strongest).

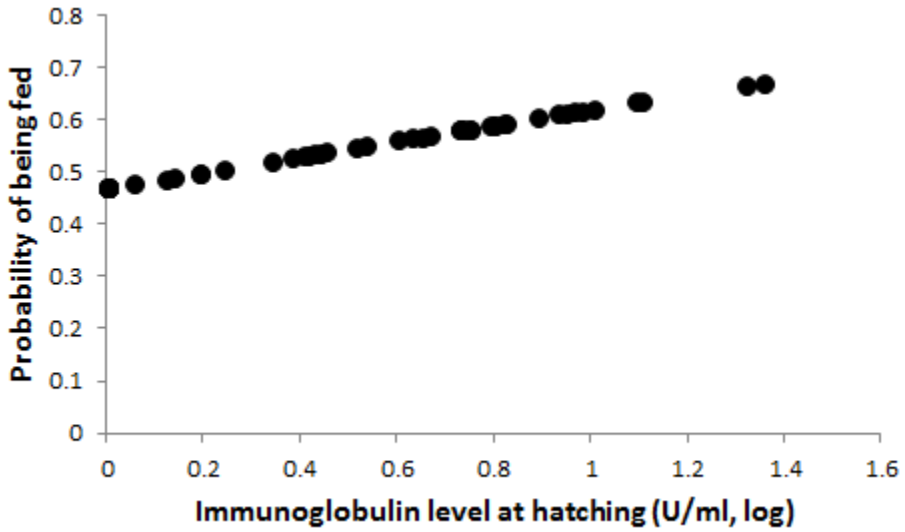

Figure 6-2. Probability of being fed by parents increased by maternal immunoglobulin levels (log) in male nestlings ($\beta = 3.0502$, $p = 0.0285$). Predicted values were obtained from a generalized linear mixed model.

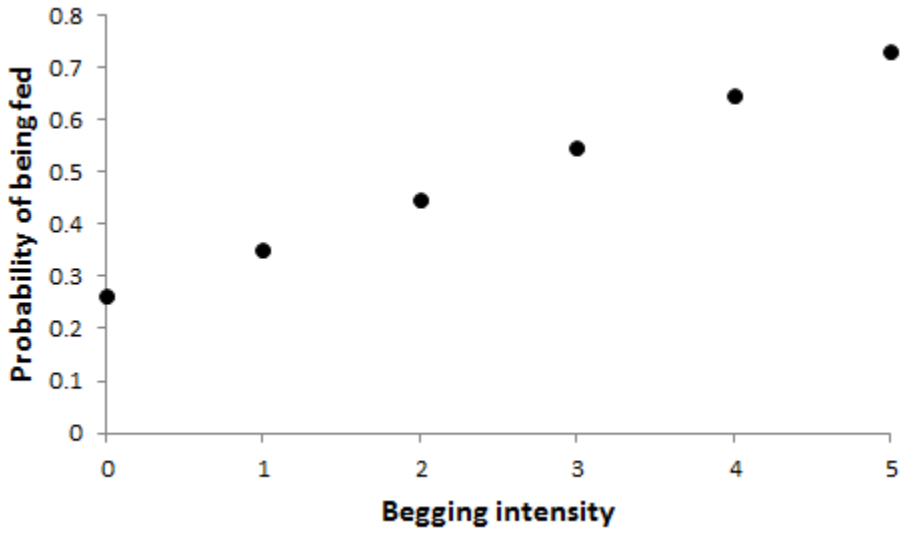

Figure 6-3. Probability of being fed by parents increased by begging intensity of nestlings (from 1 to 5, by posture of the weakest to the strongest). Predicted values were obtained from a generalized linear mixed model.

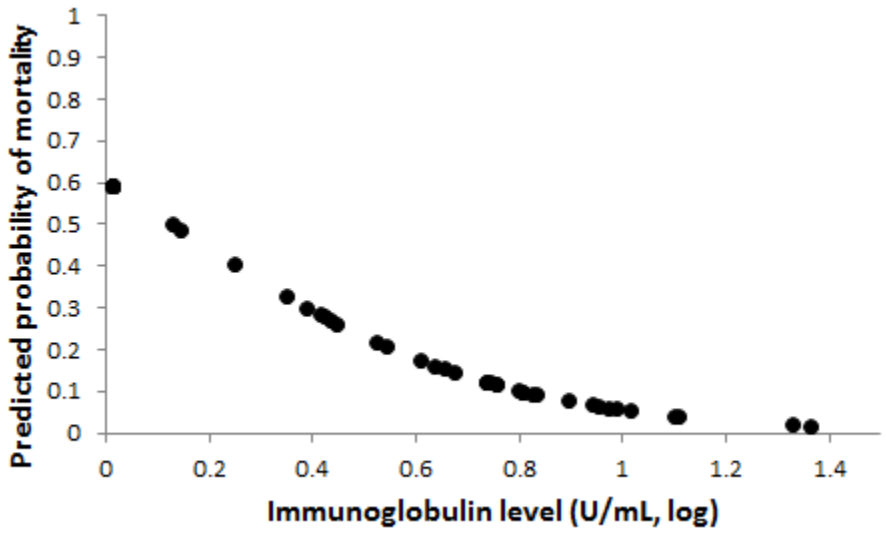

Figure 6-4. Immunoglobulin level at hatching and the probability of nestling mortality. Predicted values were obtained from a generalized linear mixed model.

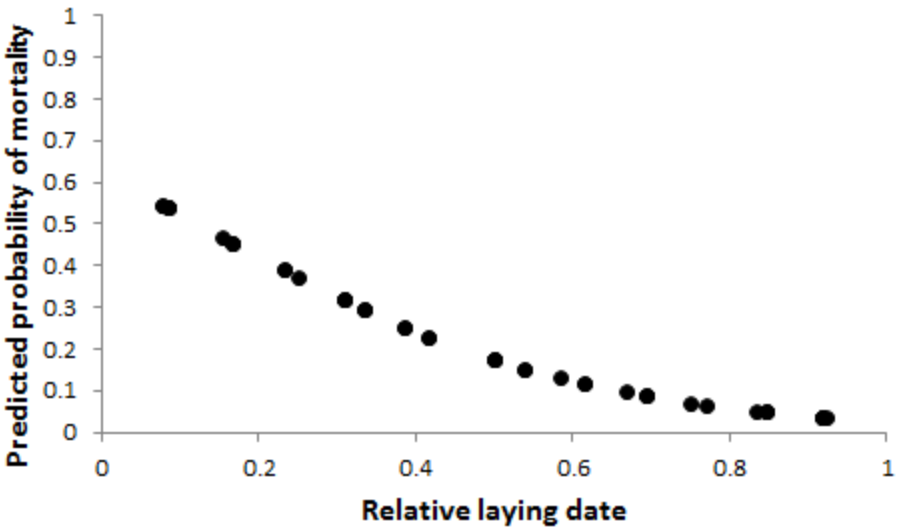

Figure 6-5. Relative laying date and the probability of nestling mortality. Predicted values were obtained from a generalized linear mixed model.

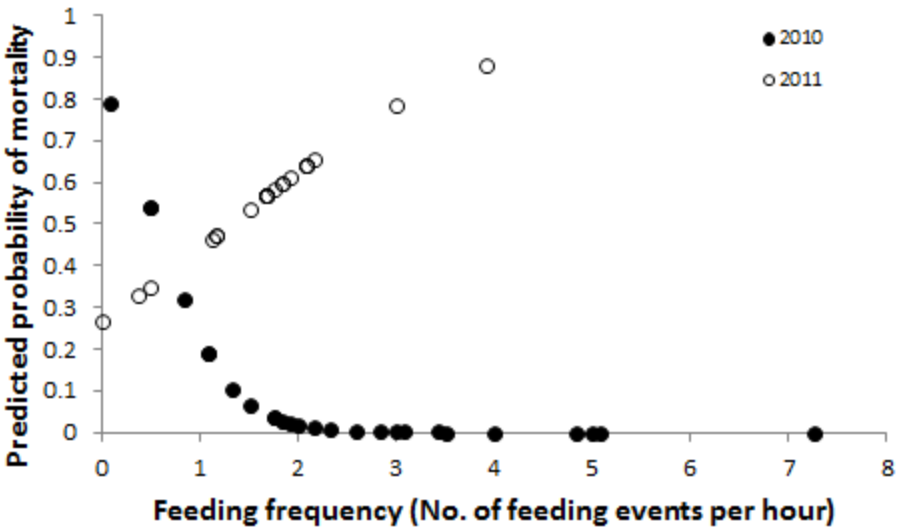

Figure 6-6. Feeding frequency and the probability of nestling mortality in 2010 and 2011. Predicted values were obtained from a generalized linear mixed model.

Discussion

Previous studies suggest hatching order as a factor that mediates the effect of maternal antibodies on parental feeding (e.g. Saino et al. 2001, Hargitai et al. 2006). In accordance with these findings, magpie mothers in our study population delivered more antibodies to early hatching nestlings. However, as our study involved experimental alteration of nestling composition and rearrangement of hatching order of nestlings, we did not detect any effect of hatching order on parental feeding. Instead, the level of maternal antibodies strongly influenced the feeding decision of the parents. Our results suggest that, although maternal antibodies may influence parental feeding via hatching order in natural conditions, maternal antibodies in the nestlings per se exert a strong effect on feeding decision of parents regardless of survival prospects of the nestlings that may be indicated by the hatching order in the brood.

Our results imply that parents can recognize those nestlings that received maternal antibodies and preferentially deliver more provisioning effort towards them. As discussed above, they could not have used hatching order as the indicator for the level of maternal antibodies, because, due to our experimental procedure, hatching order have been altered and feeding decision of parents did not depend on hatching order. If transmitted maternal antibodies were expressed to the nestlings' phenotypes either directly or indirectly, parents could distinguish and favor the nestlings among others. We think that the indicator for the level of maternal antibodies should be related to the begging display of the nestlings. In our results, we found only two factors for parental feeding decisions; maternal antibodies and begging intensity of the nestlings. However, begging intensity of the nestling was also influenced by the amount of maternal antibodies. Although there were confounding effects by other factors such as brood size and brood sex ratio,

and although the effect was not straightforward, maternal antibodies seemed to exert influence on begging intensity of nestlings. Thus, our result on maternal antibodies and begging intensity influencing parental feeding activities suggest that the effect of maternal antibodies should be very strong, as its effect still remained significant even after some portion of the variance is removed through begging intensity. Similar results were obtained in other species (Saino and Møller 2002).

It is also plausible that the amount of maternal antibody is reflected in some aspects of begging display that are not examined in our study. It is known that maternal antibody itself could create phenotypic changes as signals (egg color, Morales et al. 2006; for review see Boulinier and Staszewski 2008). Other correlated maternal effects of hormones or antioxidants (carotenoids, Blount et al. 2003) could indirectly function as potential signals to the parents (e.g. beak color and carotenoids, Navarro et al. 2010).

Maternal antibody level increased the survival of nestlings. This implies that maternal antibodies could help nestlings' immune function for survival and growth against pathogens (reviewed in Grindstaff et al. 2003). As expected, parental feeding sharply decreased the nestling mortality in 2010 and the nestlings with more than twice an hour were not likely to die before fledging. On the other hand, feeding increased the mortality in 2011. Considering that nestling mortality was mostly due to starvation in our magpie population (Lee et al. 2010, 2012), we could hardly explain this pattern but we suspect that this could have been related to unknown environmental reasons such as diseases. In fact, the nestling mortality nearly doubled in 2011 in comparison to 2010 (62% of hatchlings died in 2011 whereas 34% in 2010). Nestlings from early breeding parents were more likely to die before fledging. Although early breeders typically produced more

hatchlings, nestlings in early breeding nests might be in a worse condition possibly due to low food availability and cold weather (Lee et al. 2010).

In summary, we found that parental feeding in magpies is directed to those nestlings which had higher level of maternal antibodies. Furthermore, maternal antibodies increased the survival of the nestlings, even after the effect of parental feeding frequency was accounted for. Our results suggest that parents can recognize those nestlings that received maternal antibodies and preferentially deliver more provisioning effort towards them. This supports Trivers's investment theory (1972) that the amount of current parental investment is related to the amount of investment that has already been made in the past.

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Chapter 7.

Parental provisioning strategy and sex-specific mortality in relation to original sex ratio in the Black-billed magpie (*Pica pica*): an experimental study

ABSTRACT

Theory predicts that parents should differentiate their investment into male and female offspring to maximize their fitness. Recent studies on birds have focused on whether and how parental adjustment is made for primary brood sex ratio. Currently it is not clear whether parental adjustment is achieved for secondary sex ratio and how sex-biased parental food provisioning contributes to that. Here, we studied parental provisioning in the Black-billed Magpie (*Pica pica*). We manipulated initial brood sex ratio shortly after hatching by cross-fostering and video-recorded parental feeding behavior. Our results showed that parents who initially created male-biased broods provided more food to female nestlings and parents who created female-biased broods provided more to male nestlings. The sex-specific mortality of nestlings was affected by the original sex ratio that the parents created. Neither manipulated sex ratio nor the difference between the manipulated sex ratio and the original sex ratio affected parental feeding or nestling mortality. Our results suggest that magpie parents actively shape brood sex ratio by differential feeding to adjust the sex ratio bias which was initially induced at the early stage of parental investment.

INTRODUCTION

Theory predicts that natural selection should favor parents to invest differentially to male and female offspring and thus to adjust offspring sex ratio in a way to maximize the parents' fitness (Fisher 1930, Trivers and Willard 1973, Charnov 1982). Fisher's equal allocation theory (1930) presented that male and female ratio is adjusted by selection because frequency-dependent selection stablizes the offspring sex ratio to near an equal state. Conversely, Fisher's theory means that parents would bias the sex ratio unless the resource investment is equal in the two sexes. Trivers and Willard (1973) hypothesized that parents would bias the sex ratio of offspring to increase their fitness returns in different environmental conditions. Since Triver and Willard, many empirical studies supported the hypothesis that parents adjust their relative allocation to males and females (see Hardy 2002 and West 2009).

In birds, sex ratio adjustment by parents can be achieved by controlling offspring's sex at birth (West and Sheldon 2002, Cassey et al. 2006) or inducing differential mortality of embryos (Krackow 1995, Palmer 2000) or nestlings (Wiebe and Bortolotti 1992, Torres and Drummond 1999, Lee et al. 2010). If parents can regulate initial brood sex ratio, offspring sex ratio would be adjusted at an early stage (West and Sheldon 2002). On the other hand, if parents are not able to adjust initial brood sex ratio, for instance by random segregation of sex chromosomes (Charnov 1982), or if they are under the pressure of unpredictable environment throughout the nestling rearing period (Lee et al. 2010), offspring sex ratio would be adjusted at a later stage. However, it is still unclear whether parents induce adaptive sex ratio adjustment by affecting sex-specific nestling mortality during the period of parental care.

In sexually size-dimorphic bird species, two mechanisms for differential nestling mortality were suggested: 'larger-sex vulnerability' and 'size dominance'. Larger sex vulnerability predicts that the larger nestlings show higher mortality and lower growth rate because they consume more resources than smaller ones, especially when amount of food is not sufficient to be distributed to all nestlings. Size dominance predicts that the nestlings of the larger sex dominate the smaller sex by physically competing with smaller nestlings (e.g. golden eagles, Bortolotti 1986, reviewed in Komdeur 2012) or by demanding more food to the parents (Lee and Moss 1986). Size dominance was reported in species where direct sibling competition is observed (great tits, Kölliker et al. 1999; Alpine swifts, Bize et al. 2005). On the other hand, several comparative studies revealed that the degree of size dimorphism influences the degree of mortality in larger sex nestlings in birds (Weatherhead and Teather 1991, Benito and González-Solís 2007), supporting the prevalence of the larger-sex vulnerability mechanism among birds.

We chose the Black-billed Magpie (*Pica pica*) for testing differential investment to control brood sex ratio owing to their sexual size dimorphism. Black-billed magpies are sexually size-dimorphic birds (males are 10% larger than females [Husby 1992]). Lee et al. (2010) suggested a possibility that parental favoritism is involved for this pattern of mortality. In the follow-up of their study, it was suggested that parental feeding may play an important role in nestling survival (Lee et al. 2012). In our magpie population, high nestling mortality has been observed: average nestling mortality was 46.45% in 2009 (1.89 ± 1.48 of nestlings died among 4.08 ± 1.51 of hatchlings, mostly due to starvation from video-recordings, Lee, unpublished data; mean ± SD). In our study area, nest predation was not observed during the nestling period (Lee, personal observation). Therefore, we expected that biased feeding by the parents would be one of the main reasons for the mortality of

magpie nestlings. However, the direct link between the brood sex ratio and the parental provisioning effort has never been investigated.

In this study, we manipulated brood sex ratio and related it with the response of the parents in their provisioning effort. We examined whether parents direct their feeding effort towards a certain sex of nestlings in a brood in response to the manipulated sex ratio. We questioned (1) whether parents with sex-biased broods show biased feeding towards a certain sex and, if it is yes, (2) whether parents' biased feeding is a response to the original sex ratio or the manipulated sex ratio. We also examined the effects of sex-specific effect of brood sex ratio on nestling begging behavior.

Recent studies on brood sex ratio adjustment in birds have focused on offspring sex ratio at egg-laying (Palmer 2000, Hardy 2002, West and Sheldon 2002). It is still not clear whether parental control operates brood brood sex ratio by inducing sex-specific mortality as an adaptive strategy for sex ratio adjustment. To our knowledge, this is the first attempt to investigate the effect of feeding effort on the adjustment of brood sex ratio. Through this study, we experimentally tested if sex-specific mortality is achieved by parental biased feeding, and if yes, how this contributes to brood sex ratio adjustment.

MATERIALS AND METHODS

Brood sex ratio manipulation

In Seoul National University (SNU, Seoul, Republic of Korea) campus area, we monitored approximately 40 breeding pairs of magpies (see Lee et al. 2010, 2012 for details). In 2010 and 2011, we regularly visited the magpie nests more than twice a week to determine laying dates and hatching

dates. At near hatching, we approached the nests and marked hatchlings individually using non-toxic nail polish (Kiss Products Co., USA) on their beak and head and sampled blood for molecular sexing (Griffiths et al. 1998). Once the sexes were identified, within 2 or 3 days after hatching, the nestlings were randomly exchanged among two or three broods which hatched at the same date. Two or three nestlings in each brood (approximately half of a brood) were exchanged. Within nests, nestlings were randomly chosen to reduce the bias due to the difference of hatching order in a brood. Hence, the relative hatching order of a nestling was randomly rearranged by cross-fostering in a newly manipulated brood. In total, 190 nestlings (86 in 17 nests in 2010 and 104 in 22 nests in 2011) were cross-fostered for the experiment. We maintained the original brood size when exchanging the nestlings.

Video recording

When the nestlings were exchanged, small video cameras (Weatherproof Bullet Cam XB421-W36, Vision Hitech Company, 8cm (L) × 2cm (r)) were installed to record the feeding behaviors of parents and begging of nestlings. The recordings for 3 days (day 10 – 12, daily 0700–1100)) were used for the analysis. We categorized the begging intensity (from 1 to 5; see Redondo and Castro 1992) of the focal nestling and measured begging and feeding frequency. Totally, 53 nestlings (29 in 7 nests and 23 in 7 nests in 2011) from 14 nests were successfully video-recorded and used for the analysis.

Statistical analysis

We used generalized linear mixed models to find the minimal model to explain the significant variables affecting nestling mortality, feeding

frequency, and begging intensity (SAS software 9.3). Model selections were conducted by stepwise backward elimination (Crawley 1993) from the initial full model of the main effects and two-way interactions (based on p-value < 0.05). To estimate what factors affected the nestling mortality, we used generalized linear mixed models (PROC GENMOD) with the logit link function and individual nest ID was set as a random effect. Response variable was mortality (death or survival at fledging) and explanatory variables included brood sex ratio (original sex ratio or manipulated sex ratio or the difference of brood sex ratio between the manipulated sex ratio and original sex ratio), sex of nestlings, relative hatching order (dividing rank of hatching order by brood size), relative laying date (dividing rank of laying date by the number of nests), feeding frequency (number of feeding events per hour), brood size and year. To elucidate the factors for feeding frequency, we used generalized linear mixed models (PROC MIXED) and nest ID was a random effect. Response variable was feeding frequency and explanatory variables were brood sex ratio (either original sex ratio or manipulated sex ratio or the difference of brood sex ratio (delta brood sex ratio) between the manipulated sex ratio and original sex ratio), sex of nestlings, relative hatching order, relative laying date, begging frequency (number of begging events per hour), brood size and year. For begging intensity of nestlings, we used a generalized linear mixed model (PROC MIXED) and nestling ID within each nest ID was treated as a nested random effect. Explanatory variables were manipulated sex ratio, sex of nestlings, relative hatching order, relative laying date, hunger level of nestlings, brood size and year. Begging intensity was categorized from 1 to 5 (from the weakest to the strongest) by the begging posture of nestlings (see Redondo and Castro 1992). Hunger level of nestlings was defined as the time interval (minutes) after their last food acquisition from parents and categorized into four levels by the four quartile time interval values between the parental feeding events (based on time passed without

parental feeding; level 1: less than 8 minutes, level 2: between 8 and 16 minutes, level 3: between 16 and 30 minutes, and level 4: more than 30 minutes).

RESULTS

In total of 190 hatchlings in 39 nests (86 in 17 nests in 2010 and 104 in 22 nests in 2011), 95 individuals in 22 nests (31 in 14 nests in 2010 and 64 in 18 nests in 2011) died before fledging. Nestling mortality occurred in 82.05% of nests (82.35% in 2010 and 81.82% in 2011). At hatching, there were 35 male nestlings and 51 female nestlings in 2010 and 43 and 61 in 2011, respectively. Among the nestlings, 20 male nestlings and 35 female nestlings in 2010 and 19 and 21 in 2011 survived at near fledging (57.14% for male and 68.63% for female in 2010 and 44.19% and 34.43% in 2011; survival rate).

Nestling mortality

The minimal model for the mortality of nestling in relation to original sex ratio, which was initially created by parents before manipulation, showed that there was a significant interaction between original sex ratio and sex of nestlings (Table 7-1). The predicted values and observed nestling mortality in three groups (female-biased brood, equal, and male-biased) indicated that female nestlings had higher mortality in female-biased broods and males had higher mortality in male-biased broods (Figure 7-1). We also found the effects of laying date and feeding frequency on nestling mortality (Table 7-1), which suggests that nestlings in early breeding nests died more than the ones in late nests and less fed nestlings were more likely to die. Year, relative hatching order and brood size were removed during the process of model reduction.

In relation to manipulated sex ratio, the minimal model for the mortality of nestlings had an interaction between year and feeding frequency and an interaction between hatching order and feeding frequency (Table 7-2). Feeding frequency increased the mortality in 2010 and more sharply increased the mortality in 2011, and late-hatched marginal broods had higher mortality with a differential degree of effects by feeding frequency. Overall, parental feeding frequency seemed important for nestling survival. However, manipulated sex ratio, sex of nestlings and laying date did not significantly relate with nestling mortality in the minimal model.

With delta brood sex ratio (manipulated sex ratio – original sex ratio) in the initial model, interactions of feeding frequency with year, hatching order, and laying date remained in the minimal model (Table 7-3). Feeding frequency was differentially affecting the mortality; it reduced the mortality in 2010 and increased the mortality in 2011. Also, nestlings in late breeding nests and early-hatched core broods had lower mortality, and the interactions of feeding frequency with hatching order and laying date had significant effects on the mortality. This implies that feeding frequency was strongly affecting nestling mortality. Similarly with the results of manipulated sex ratio, brood sex ratio and sex of nestlings were deleted in course of the model reduction.

Feeding frequency

From the initial model with original sex ratio, we found that there was a sex-specific effect on parental feeding (Table 7-4). The pattern of sex-specific feeding was similar to the results of sex-specific mortality (Figure 7-2). Female nestlings in female-biased broods had less feeding from parents and male nestlings in male-biased broods acquired less feeding. We also found that begging frequency of nestlings affected feeding frequency (Table 7-4, Figure 7-3).

In the minimal model with manipulated sex ratio, manipulated sex ratio was removed. Instead, hatching order and interactions of begging frequency with sex and year were included (Table 7-5). Core hatchlings (early hatched offspring) had more feeding frequency and begging frequency positively affected feeding frequency despite the different estimates with sex and year (Table 7-5).

The minimal model with delta sex ratio (manipulated sex ratio – original sex ratio) had an interaction of begging frequency and year (Table 7-6). Begging frequency affected feeding frequency and the estimate value in 2011 was slightly higher than the one in 2010 (Table 7-6). Delta sex ratio, sex of nestlings, hatching order and laying date were deleted in the model reduction.

Begging intensity

For the begging intensity of nestlings, hunger level and interactions of manipulated sex ratio and sex, brood size and hatching order, and brood size and manipulated sex ratio remained in the minimal model (Table 7-7). As expected, hungry nestlings begged for food intensively to their parents. Marginal broods (late hatched nestlings) had lower begging intensity. Manipulated sex ratio affected begging intensity in both male and female nestlings and the intensity decreased as the sex ratio was male-biased. Also, hunger level increased begging intensity.

Table 7-1. Minimal model for the mortality of nestlings in relation to original sex ratio.

Effect	df	χ^2	P value	β (±SE)
Original sex ratio	1	0.55	0.4578	3.1266 (4.9222)
Sex	1	8.35	0.0038	6.7726 (2.9009) for female
Laying date	1	4.72	0.0298	-3.175 (1.6990)
Feeding frequency	1	4.76	0.0291	-0.8974 (0.4036)
Original sex ratio × Sex	1	8.42	0.0037	-14.234 (5.8500)

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, feeding frequency, original sex ratio and sex of nestlings.

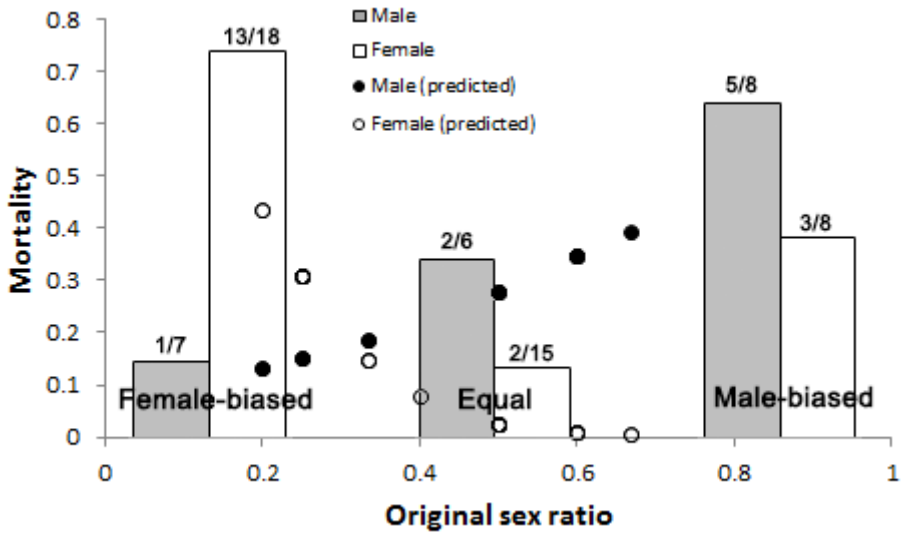

Figure 7-1. Original sex ratio and nestling mortality. Sex-specific mortality was detected in relation to original sex ratio by parents. Predicted values were acquired from a generalized linear mixed model. 63 nestlings in 16 nests were used for the analysis.

Table 7-2. Minimal model for the mortality of nestlings in relation to manipulated sex ratio.

Effect	df	χ^2	P value	β (±SE)
Year	1	1.67	0.1968	1.8661 (1.3647) for 2010
Hatching order	1	5.77	0.0163	7.2808 (3.1181)
Feeding frequency	1	2.03	0.1546	2.4957 (1.1190)
Year × Feeding frequency	1	7.68	0.0056	-2.3912 (0.6605) for 2010
Hatching order × Feeding frequency	1	5.69	0.0171	3.8596 (1.5518)

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, feeding frequency, manipulated sex ratio and sex of nestlings.

Table 7-3. Minimal model for the mortality of nestlings in relation to Δ sex ratio (manipulated sex ratio – original sex ratio).

Effect	df	χ^2	P value	β (±SE)
Year	1	3.31	0.0689	5.5904 (3.2769) for 2010
Hatching order	1	6.88	0.0087	9.6987 (3.5627)
Laying date	1	5.86	0.0155	-12.4925 (5.8326)
Feeding frequency	1	0.41	0.522	1.1947 (1.3741)
Year × Feeding frequency	1	7.57	0.0059	-4.4657 (1.7803) for 2010
Hatching order × Feeding frequency	1	6.81	0.0091	-4.7915 (1.8245)
Laying date × Feeding frequency	1	4.95	0.0261	5.0034 (2.6710)

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, feeding frequency, manipulated sex ratio and sex of nestlings

Table 7-4. Minimal model for feeding frequency by parents in relation to original sex ratio.

Effect	df	F value	P value	β (±SE)
Begging frequency	1	149.81	<.0001	0.3954 (0.0323)
Original sex ratio	1	1.3	0.2765	-2.7722 (1.1424)
Sex	1	8.54	0.017	-1.9721 (0.6749) for female
Original sex ratio × Sex	1	7.63	0.0091	3.8775 (1.4040) for female

The initial model included main effects and two-way interactions of year, brood size, sex of nestlings, original sex ratio, relative laying date, relative hatching order and begging frequency.

Table 7-5. Minimal model for feeding frequency by parents in relation to manipulated sex ratio.

Effect	df	F value	P value	β (±SE)
Begging frequency	1	180.12	<.0001	0.8080 (0.0858)
Hatching order	1	8.43	0.0065	-0.9962 (0.3432)
Sex	1	4.28	0.0684	0.7170 (0.3464) for female
Year	1	7.65	0.0171	0.9737 (0.3520) for 2010
Begging frequency × Sex	1	9.31	0.0045	-0.2147 (0.0704)
Begging frequency × Year	1	11.88	0.0016	-0.2825 (0.0820) for 2010

The initial model included main effects and two-way interactions of year, brood size, sex of nestlings, manipulated sex ratio, relative laying date, relative hatching order and begging frequency.

Table 7-6. Minimal model for feeding frequency by parents in relation to Δ sex ratio (manipulated sex ratio – original sex ratio).

Effect	df	F value	P value	β (±SE)
Begging frequency	1	138.01	<.0001	0.6166 (0.0765)
Year	1	6.19	0.0285	0.9212 (0.3702) for 2010
Begging frequency × Year	1	9	0.0049	-0.2508 (0.0836) for 2010

The initial model included main effects and two-way interactions of year, brood size, sex of nestlings, Δ sex ratio (manipulated sex ratio – original sex ratio), relative laying date, relative hatching order and begging frequency.

Table 7-7. Minimal model for begging intensity of nestlings in relation to manipulated sex ratio.

Effect	df	F value	P value	β (±SE)
Hunger level	1	5.45	0.0197	0.0526 (0.0225)
Brood size	1	136.55	<.0001	-1.0475 (0.0896)
Manipulated sex ratio	1	312.05	<.0001	-10.2216 (0.6671)
Sex	1	41.12	<.0001	1.1993 (0.187) for female
Hatching order	1	37.93	<.0001	-3.3743 (0.5479)
Brood size × Manipulated sex ratio	1	432.93	<.0001	2.6995 (0.1297)
Brood size × Hatching order	1	44.35	<.0001	0.7714 (0.1158)
Manipulated sex ratio × Sex	1	54.89	<.0001	-2.7747 (0.3745) for female

The initial model included main effects and two-way interactions of year, brood size, relative laying date, relative hatching order, manipulated sex ratio, sex of nestlings, and hunger level of nestlings (from 1 to 4; measured by the time interval between the parental feeding events from the weakest to the strongest).

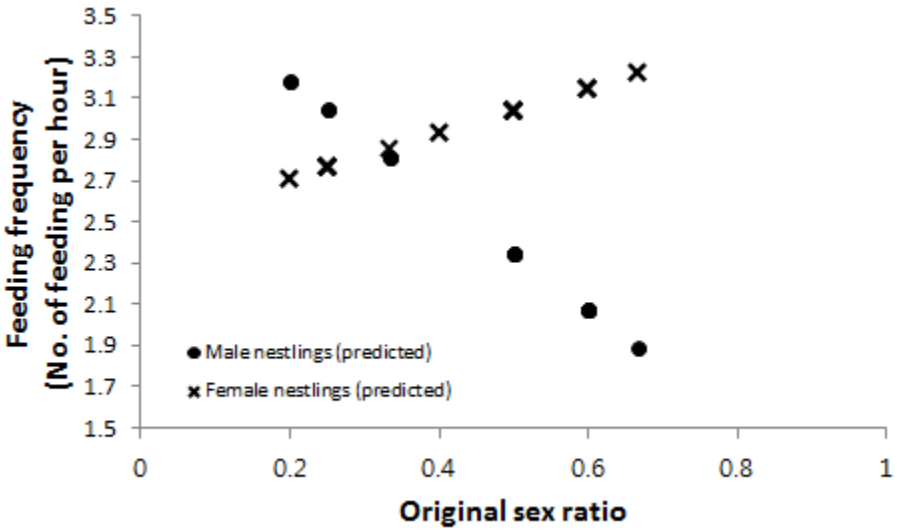

Figure 7-2. Original sex ratio and feeding frequency by parents. Predicted values were acquired from a generalized linear mixed model. In total, 63 nestlings in 16 nests were used for the analysis to observe the feeding events for three days (0700-1100, at day 10-12).

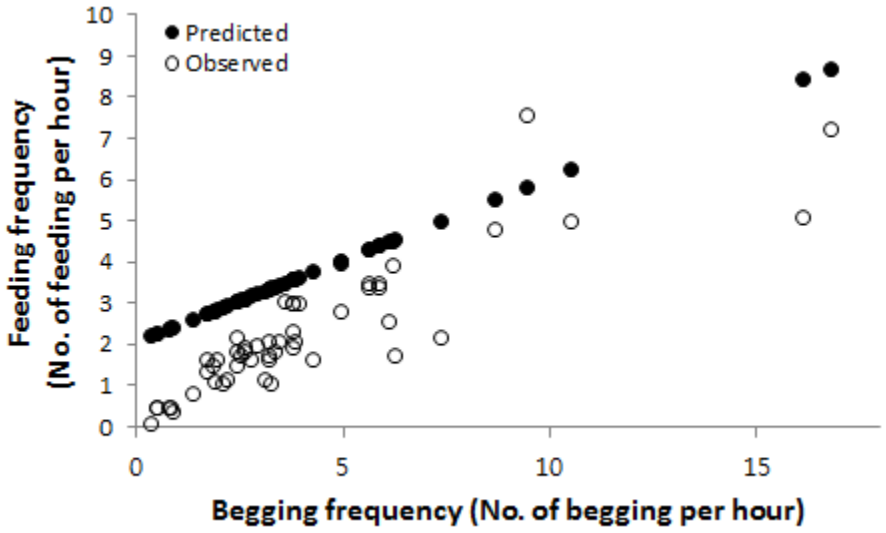

Figure 7-3. Begging frequency and feeding frequency in relation to offspring sex ratio. Frequency was the number of events per hour and the predicted values were obtained from a generalized linear mixed model. In total, 63 nestling in 16 nests were used for the analysis to observe the feeding events for three days (0700-1100, at day 10-12).

DISCUSSION

In this study, we found that nestling mortality was differentially related with original sex ratio and sex of nestlings. The sex-specific mortality was affected by the original sex ratio that was initially created by parents. Instead, neither manipulated sex ratio nor the difference between the manipulated sex ratio and the original sex ratio affected nestling mortality. A sex in the sex-biased broods was more likely to die than the other sex and this sex ratio related mortality was affected by original sex ratio, not by manipulated sex ratio. In addition to that, the pattern of nestling mortality was quite similar with the result from feeding frequency by parents. Male nestlings in female-biased broods had more food from parents and less mortality at fledging, and female nestlings in male-biased broods had more food and less mortality. This suggests that parents actively control brood sex ratio by differential feeding to adjust the sex ratio bias which was initially induced by parents.

Our results support Fisher's equal allocation theory (1930). Considering that male fledglings are larger than female fledglings (approximately 7% in our population, Lee et al. 2010), the costs are not same between male and female production. At population level, original sex ratio of male and female hatchlings was slightly skewed to 4.1:5.9 in both 2010 and 2011 and sex ratio of fledglings were 3.6:6.4 in 2010 and 4.8:5.2 2011. In addition, figure 7-1 showed that the predicted mortality of male and female was met at around 0.33 of original sex ratio and males were more likely to die than females at 0.41 of original sex ratio of hatchlings. This implies that selection favors parents to select to bias the offspring sex ratio and cheaper sex is advantaged when the cost differs. In our magpie population, the sex ratio was biased to females, the cheaper sex, while the sex-specific mortality was adjusted in relation to original sex ratio.

In the view of nestlings, our findings of parental feeding on sex-specific mortality do not exclude the possibilities of nestling competition. Begging intensity of nestlings in relation to manipulated sex ratio was also correlated with manipulated sex ratio and sex of nestlings. Under manipulated situations, feeding was biased to the early-hatched core offspring and the nestlings with more begging trials. This means that offspring sex may influence nestling begging behavior as well as parental feeding (Lessells 2002). Because begging frequency was mainly affecting the feeding frequency in manipulated sex compositions, the sibling competition and the corresponding parental feeding could indirectly contribute to the sex-specific nestling mortality although the effects were not included in the models.

The two hypotheses in size-dimorphic birds could not relate with our results because the hypotheses explain, under the manipulated nestling composition, how sibling competition contributes to the biased mortality ('size dominance') and how larger sex nestlings suffer when food resources are scarce ('larger sex vulnerability'). In our results, the sex-specific mortality was not influenced neither by the manipulated sex composition of nestlings nor by the difference of sex ratio (delta sex ratio) between the manipulated sex ratio and the original sex ratio. Therefore, our results do not support the previous hypotheses studied in size-dimorphic birds.

In manipulated situations we made, the pattern of nestling mortality was affected by feeding frequency. However, nestlings which fed more by parents were more likely to die in 2010 and 2011. It is difficult to interpret this mortality pattern since starvation was the main cause of nestling death in our magpie population. We think that this might be related with manipulating the original sex ratio and original hatching order of nestlings. If we destroyed the original plan of the parents by disturbing the natural breeding ecology, the resulted pattern would not reflect the effects of manipulated situations.

The results of an observational study in the same population were contrary to what we found from the experiment (Lee et al. 2010). Lee et al. found that females had higher mortality in male-biased broods and males had higher mortality in female-biased broods. When we compared the nestling mortality between our study and the earlier study, our magpies were in worse conditions (50% of nesting mortality in 82% of nests, our magpies in 2010 and 2011; 28% of nestling mortality in 51% of nests, magpies in Lee et al.'s study in 2000 and 2004). The increased nestling mortality in the population, possibly related with unknown environmental changes, might cause a parental strategy to adjust offspring sex ratio.

In summary, our results suggest that sex-specific mortality of magpie nestlings were affected by the original sex ratio which was initially created by the parents and the sex-specific mortality was adjusted by biased parental provisioning. To our knowledge, this is the first study to provide evidence for parental active sex ratio adjustment by feeding to induce the sex-specific mortality. For future research, it would be necessary to study the proximate mechanism to create primary sex ratio. It is still unclear if the parents induce primary sex ratio adjustment at egg-laying or hatching and reinforce the adjustment during the later stages. Other parental investment, such maternally derived hormones or antibodies, would help us to understand how parents deliver such nutrients at early stages that are necessary for nestling survival.

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Chapter 8.

General Discussion

General Discussion

In this thesis, I studied parental care in the Black-billed Magpie (*Pica pica*). I investigated incubation effects on eggshell microbes using PCR and pyrosequencing analysis and parental investment strategies using immunoessays and video-recordings. First, I showed that incubation selectively affected microbial growth on eggshells (Chapter 2 and 3). Antibiotic-producing bacteria greatly multiplied on eggs whereas pathogenic bacteria decreased. Here I suggest that incubation reduces the risk of pathogenic infection by harboring beneficial microbes. Second, I found that maternal investment is consistently maintained during egg-laying and feeding (Chapter 4 and 6). At feeding stage, females favored nestlings with more maternal antibodies that they injected at egg-laying. On the other hand, males' feeding rules were not affected by maternal antibodies. Instead, males were more likely to provide food to the nestlings in the late-breeding nests. Third, I reported behavioral conflict between male and female parents (Chapter 5). When females remained the nests and begged food to their mates during the feeding period, males and females had physical interactions. Here, I defined 'behavioral conflict' as a behavioral disputes between male and female parents when males ignored begging females and fed nestlings only. Fourth, I found that parents actively adjusted offspring sex ratio by biased feeding strategies (Chapter 7). Parents induced sex-specific mortality by biased feeding in relation to original sex ratio as they initially created. Male nestlings in male-biased broods had higher mortality than female nestlings and female nestlings in female-biased broods showed higher mortality. The feeding pattern of the parents was in accordance with the sex-specific mortality: Male nestlings in male-biased broods had less chance to be fed by parents and female nestlings in female-biased broods had less chance to be fed.

Effect of incubation on eggshell microbes in a temperate area

In previous studies on tropical eggs, researchers revealed that parental incubation inhibits microbial growth on eggshells (Cook et al. 2005, Shawkey et al. 2009, D'alba et al. 2010). Here I tested the inhibitory hypothesis in a temperate area to see if it is applicable to the birds which live in relatively cool and dry regions. Interestingly, I found that various bacterial taxa existed on magpie eggshells which were even more diverse than the thrasher eggs in tropics (Shawkey et al. 2009). Also, I found that incubation affects microorganisms on eggshell selectively. From pyrosequecing and real-time PCR analyses, I found that incubation increased the growth of total bacteria. While total bacteria increased, pathogenic species, such as *Escherichia coli* and *Pseudomonas*, were suppressed by incubation. In the results, two out of 10 most abundant genera showed significant changes in abundance in almost all incubated eggs: *Pseudomonas* decreased and *Bacillus* increased. *Pseudomonas* includes opportunistic human pathogens. On the other hand, the majority of *Bacillus* are harmless or antibiotics. Although it needs further verification, some of the sequences that were obtained seemed to match those of antibiotics producing *Bacillus* (*B. sutilis, B. pumilus,* and *B. licheniformis*). Thus, the results suggest that incubation decreases potentially pathogenic bacteria by promoting the growth of non-pathogenic or antibiotics-producing bacteria.

Based on my results, I suggested the causal relationships among incubation effort, hatching success, and microorganisms on eggshell surfaces in a wild avian field study (Figure 3-1 in Chapter 3). I found that hatching success was higher as females spend more time for incubation. By detecting and quantifying total bacteria and several expected microbial species on eggshells, I showed that the microbes affected hatching success and incubation would increase hatching success by suppressing the microbial

growth. The results support that the incubation effort increases hatching success, possibly by suppressing the pathogenic microbial growth since pathogens reduce hatching success. Considering that avian gut flora has a large number of *Escherichia coli* and it can be transmitted to the egg surface through cloaca, I suspect that maternal gut would be one of the main sources for microbes among the possible microbial sources on eggshells, such as maternal cloaca, nest materials, parental body, and airbones.

In humid tropic areas (e.g. Cook et al. 2005ab, Shawkey 2009), drying mechanism (D'Alba 2010, Ruiz-de-Castañeda et al. 2011) would be an important function to suppress the growth of pathogenic microbes on eggs. However, the drying mechanism does not fully explain my results that incubation selectively affected the microbial growth. Such selective response would be well understood with antibiotic activities of beneficial bacteria and competition among the taxa.

Maternal investment strategy thorough egg-laying to feeding stage

Here, I found that female parents delivered higher levels of maternal antibodies to early-hatched core nestlings. This is evidence that females unequally distributed maternal materials among offspring. Also, I showed that female parents' feeding was affected by the amount of maternal antibodies that they delivered. The results from maternal antibody and feeding provisioning suggest that parents maintain a consistent investment strategy through the period of parental care. Nestlings with more maternal antibodies at hatching were favored after hatching by biased parental feeding and the nestlings which had more maternal antibodies were more likely to survive at fledging. This supports Trivers's investment theory (1972) that parental investment is related to the past investment. According to his theory, parents are expected to invest more to the offspring that they cost more in the past

investment. In magpies, the consistent investment strategy by females was only for late hatching nestlings. In early hatching nestlings, females preferentially provide more food to nestlings who received less maternal antibodies. This implies that female parents may have different feeding strategies depending on the hatching order of the nestlings; for earlier hatching nestlings, who have higher survival prospects, mothers seem to compensate for low maternal antibodies by providing more feeding to ensure the health and survival (Stamps 1990, Rosivall et al. 2005, Cameron-MacMillan et al. 2007); on the other hand, for later hatching nestlings, who have lower survival prospects and thus any further investment can be wasted, they provide feeding according to the investment that they made earlier so that their earlier investment is less likely to be wasted. By having differential feeding strategies based on survival prospects, mothers seem to adjust the amount of current investment depending on the investment that has been already made and thus can optimize the efficiency of their investment.

Parental feeding and nestlings' begging with maternal antibodies

Parental feeding is often accompanied by begging displays (Johnstone and Godfray 2001). As Trivers (1974) mentioned, selection favors that offspring demand more resources than that parents provide. This evolutionary conflict leads to the exaggerated begging displays to manipulate parents to bring more food (Mock and Parker 1997). Then, why do parents allow themselves to be manipulated as nestlings do? According to 'signal of need' hypothesis (Godfray 1991), parents respond to the begging displays of nestlings since it reflects the state of the nestlings. In this thesis, maternal feeding was biased to the nestlings with more antibodies at hatching. Even though I blindly manipulated the hatching orders, mothers responded to the nestlings of higher levels of maternal antibodies. This means that parents, at

least females, could distinguish and favor the nestlings among others. In my results, nestlings' begging intensity was affected by the maternal antibodies. Thus, I think that the maternal antibodies should be related to the begging postures. First, the nestlings' phenotypes could be either directly or indirectly expressed the parents so that they could distinguish and favor the nestlings among others. Second, the amount of maternal antibody could be mediated in some aspects of begging display. It is known that maternal antibody itself could create phenotypic changes as signals (e.g. egg color, Morales et al. 2006; for review see Boulinier and Staszewski 2008). Third, other correlated maternal effects of hormones or antioxidants (carotenoids, Blount et al. 2002) could indirectly function as potential signals to the parents (e.g. beak color and carotenoids, Navarro et al. 2010).

Maternal feeding rules vs. paternal feeding rules

Female parents' feeding strategy was highly related with the amount of maternal antibodies at hatching. On the other hand, Male parents had a different feeding strategy. Any of the nestling traits, including maternal antibody levels and relative hatching order of the nestlings, did not influence the feeding of the male parents. Instead, laying date solely affected the feeding of the male parents. This means that male parents may not be choosy among the nestlings and their feeding activity may depend on the food availability only. Different food allocation rules between male and female parents suggest that there is a possibility of sexual conflict during the feeding period (e.g. Dickens and Hartley 2007).

Interestingly, a common feature between male and female feeding patterns was that the feeding was not significantly affected by begging intensity of nestlings (Chapter 4). In the results, parental feeding seemed to be more influenced by the level of maternally derived immunoglobulin or

hatching order than nestling begging behavior. This suggests that parental feeding patterns were performed based on their investment strategies rather than as a simple response to nestlings' begging. This does not mean that nestlings' begging is not important in parental feeding decisions; in previous studies where maternal immunoglobulin level was not accounted for, nestling begging intensity and different aspects of begging display exerted influence on magpie parental feeding with various extents (Lee et al. 2010, 2012).

Behavioral conflict between male and female parents during the feeding period

I report conflict behaviors between the pairs and discuss whether the breeding pairs resolve the conflict in a bi-parental monogamous bird. I found that some females still remained in the nests, brooding fully feathered chicks after the 8 - 12th day when thermoregulation of their nestlings did not seem to be necessary. I observed behavioral disputes between the pairs during the feeding period, possibility due to the prolonged brooding of females. Here, I defined such behaviors as 'behavioral conflict' when males ignored begging females and fed nestlings only in the nests. The behavioral conflict was often accompanied with physical interaction between male and female parents. Male parents moved the females sitting on the nestlings with the beak and tried to feed nestlings only with avoiding the begging females. Behavioral conflict were more likely to happen in the pairs with small territories and they seemed to adjust the conflict while maintain the pair-bonding. The results suggest that this unique form of sexual conflict exists in magpies during the feeding period, and this may be related to their tendency of repeated breeding with the same partner over the years and the degree of conflict seems to be shaped by the quality, age and/or the past experience of the pair.

Brood sex ratio adjustment by parental feeding

In parental adjustment of brood sex ratio, I found that nestling mortality was differentially related with original sex ratio and sex of nestlings. The sex-specific mortality was affected by the original sex ratio that was initially created by parents. Male nestlings in female-biased broods had more food from parents and less mortality at fledging, and female nestlings in male-biased broods had more food and less mortality. This suggests that parents actively control brood sex ratio by differential feeding to adjust the sex ratio bias which was initially induced by parents. Overall, the results support Fisher's equal allocation theory (1930) that male and female ratio is adjusted by selection because frequency-dependent selection stablizes the offspring sex ratio to near an equal state. However, since the costs of male and female were different in magpies, the sex ratio was biased to females (the cheaper sex) while the sex-specific mortality was adjusted in relation to original sex ratio.

Suggestions for future studies

For future studies, I suggest that the microbial studies will benefit from comparison between geographic variation and characteristics of incubation effects on eggshell microbial activities. In maternal antibodies and parental investment studies, it would be good to conduct an experimental manipulation of hatching asynchrony (e.g. inducing hatching synchrony by placing early laid eggs in temporary cold storage to delay hatching) and separate the effects of hatching order. These experimental studies with hatching orders will elaborate the effect of maternal antibodies as signals to the parents. Experimentally manipulated immune systems of embryos or chicks would be worth trying to test the effects of regulated maternal antibodies of hatchlings on parental behaviors and nestlings' growth. For

instance, researchers can manipulate nestlings' immune stat by injecting antibodies. In sexual conflict issues, it still remains unclear why behavioral conflicts occurred between the evolutionary interests and how the parents adjust the problems. I expect that breeders' qualities and pair-bonding history would provide evidence for the reason why such conflict occurs in magpies. In offspring sex ratio adjustment, it would be necessary to study the proximate mechanism to create primary sex ratio. It is unclear if the parents induce primary sex ratio adjustment at egg-laying or hatching and reinforce the adjustment during the later stages. Other parental investment, such maternally derived hormones or antibodies, would help us to understand how parents deliver such nutrients at early stages that are necessary for nestling survival.

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Abstract in Korean (국문초록)

본 연구는 까치의 포란과 먹이분배를 포함한 부모양육 행동을 다루고 있다. 첫 번째로, 까치의 알 표면에 있는 미생물에 포란이 어떤 영향을 미치는지 알기 위해서 실시간유전자증폭(Real-time PCR) 방법과 파이로시퀀싱 (Pyrosequencing)방법을 이용하였다. 그 결과, 이전 연구들과는 다르게 포란이 이루어진 알의 표면에서 전체 박테리아의 양이 크게 증가하는 것을 확인하였다. 특히 병원성 미생물은 줄어든 반면, 항생물질을 분비하는 것으로 알려진 미생물은 증가하였다. 이 결과는 부모의 포란 행동이 알 표면 미생물에 선택적인 영향을 끼쳤다는 것을 시사한다. 이것은 미생물 분류군에 따라 포란에 서로 다르게 영향을 받은 결과일 것으로 여겨진다. 포란으로 인해 알 표면의 습기가 낮아지는 정도에 따라 다르게 반응한 결과이거나 항생물질을 만들어내는 미생물이 증가하면서 그 미생물들이 분비한 항생물질의 작용에 따른 결과일 가능성이 있다. 두 번째로, 어미가 알을 낳고 새끼를 키울 때 일정한 투자전략에 따라 새끼에게 자원을 분배하는지 확인하였다. 알을 낳을 때 어미는 '모성효과(Maternal effect)' 와 관련하여 알에 영양 물질을 전달하는데, 이때 그 물질을 알에 따라 불균등하게 주입할 수 있다. 따라서 더 많은 모성물질을 받은 알이 부화 후에도 어미로부터 더 많은 먹이를 받게 되는지 연구하였다. 그 결과, 예측했던 것과 마찬가지로 어미는 산란 단에서 전달한 모성항체의 양에 따라 먹이분배 전략이 다르게 나타났다. 따라서 어미는 산란과 먹이분배에 있어서 일정한 투자전략을 가지고 새끼를 양육하는 것으로 보였다. 암컷과 달리 수컷은 먹이를 분배하는데 있어서 암컷이 얼마나 모성항체를 전달했는지에 영향을

받지 않았다. 대신 번식 시기에 따라 먹이분배 전략을 다르게 나타내는 것으로 여겨진다. 세 번째로, 암컷과 수컷이 새끼에게 먹이를 주는 시기에 관찰된 부부간의 '갈등행동(Behavioral conflict)'을 보고하였다. 암컷이 먹이 활동을 하지 않고 둥지에 머무는 시간이 긴 번식 쌍들에서 이러한 갈등행동이 많이 나타나는 것으로 보였으며, 특히 영역의 크기가 작은 경우에 갈등이 발생할 확률이 높은 것으로 나타났다. 그리고 해가 지날수록 갈등이 지속되는 기간이 줄어드는 것으로 보아, 부부가 번식을 지속해나가면서 이러한 갈등을 조정해나가는 것으로 추측하였다. 다섯 번째로, 부모가 자녀의 성비에 따라 먹이를 주는 전략이 달라지고 이로 인해 새끼 사망률에 영향을 미친다는 것을 확인하였다. 실험적으로 자녀의 성비를 바꿔준 결과, 조작된 자녀의 성비가 아닌 처음 부모가 낳은 자녀의 성비에 반응하는 것으로 나타났다. 수컷을 많이 낳은 둥지에서는 암컷에게 먹이를 주는 경향이 높았으며 이로 인해 수컷의 사망률이 높았다. 반대로 암컷을 많이 낳은 둥지에서는 수컷에게 먹이를 주는 경향이 높았고 암컷의 사망률이 높게 나왔다. 이러한 결과를 토대로 살펴볼 때, 부모는 차별적인 먹이 공급을 통해 자녀의 성비를 조정하는 것으로 여겨진다.

주요어: 양육행동, 미생물, 자녀 성비, 갈등행동, 까치, *Pica pica*
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