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Title: **Sexual dimorphism of egg size in the European blackbird *Turdus merula***

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Abstract

Recently, a number of studies have shown that female birds are able to control the sex of their progeny at the stage of the gamete. There is also some evidence that females adjust their investment in offspring depending on the sex of the embryo during egg formation. Differential maternal investment to the eggs depending on their sex is usually interpreted as an adaptive strategy, by which females can increase competitive abilities of the smaller sex, or preferentially invest towards the sex with the potentially higher fitness returns. Here, we studied variation in egg size in relation to embryo sex and laying order in the European blackbird *Turdus merula*. We found male and female eggs to differ in size, with larger eggs containing male embryos, as well as a significant interaction between embryo sex and laying order. This interaction resulted from the fact that egg size increased with the laying sequence among eggs bearing females but did not change with laying order among eggs bearing males. There was no relationship between offspring sex and the laying sequence within a clutch. We suggest that sexual dimorphism in egg size recorded in the European blackbird may reflect favoritism of the sex which may give higher fitness returns.

Key words: egg volume, hatching asynchrony, laying sequence, maternal effects, sex allocation

Introduction

Sex allocation theory predicts that if a social or ecological circumstance differentially affects the reproductive value of male and female offspring, parents should skew their investment towards the sex with higher fitness returns. Differential sex allocation can be potentially achieved in two ways, by the production of different numbers of sons and daughters, or by unequal allocation of resources to the members of each sex (Trivers and Willard 1973, Charnov 1982). In birds, females are the heterogametic sex, and thus have the opportunity to vary their investment in sons and daughters both during egg production and after hatching by differential provisioning of the offspring. In most bird species, the male is usually the sex with higher variance in reproductive success (Griffith et al. 2002). Thus, according to sex allocation theory mothers may be expected to allocate more resources to male offspring (Charnov 1982). The first evidence showing disparities in size between eggs bearing male and female embryos comes from studies on the White-crowned sparrow *Zonotrichia leucophrys oriantha*, in which authors detected that male offspring hatch from larger eggs (Mead et al. 1987). The same pattern of egg sexual dimorphism where males (the larger sex) come from larger eggs has also been found in other bird species (e.g. Cordero et al. 2000, Cunningham and Russell 2001, Leitner et al. 2006). If male offspring benefit more from increased maternal investment early in life compared to female offspring in terms of lifetime reproductive success, the observed pattern of egg provisioning may be considered as an adaptive favoritism of the sex with potentially higher fitness returns (e.g. Mead et al. 1987, Cordero et al. 2000). Alternatively, it is suggested that females may differentiate investment to eggs to boost the competitive ability of the smaller sex. Two studies on sexually size dimorphic birds, the American kestrel *Falco sparverius* and the Brown songlark *Cinclorhamphus cruralis*, show that eggs containing the smaller, less competitive sex are larger (Anderson et al. 1997, Magrath et al. 2003). Females may also affect performance of sons and daughters by placing

male and female eggs early or late in the laying sequence (e.g. Cichoń et al. 2003, Lezalova et al. 2005). Such differential allocation to sons and daughters according to the laying order might be an important strategy of compensating negative effects of asynchronous hatching (e.g. Rutkowska and Cichoń 2005).

Here, we examined whether eggs containing male and female embryos differ in size and whether sexes are non-randomly ordered in the laying sequence in the European blackbird *Turdus merula*. Sexual dimorphism in egg size might be expected in this species as sexes differ in size (adult males are ca. 5% larger than adult females; Cramp1988, Wysocki 2002, Walasz, unpublished data), and fitness returns resulting from additional maternal investment to sons and daughters may differ, due to the high frequency of extra-pair paternity (in the studied population up to 18% of offspring originate from extra-pair copulation, Rutkowska et al, unpublished data) and possibility of facultative male polygyny (Wysocki 2004). As the European blackbird shows substantial hatching asynchrony (Magrath 1989, Walasz, unpublished data), strategies of allocation of offspring sex to early or late eggs in the laying order might be expected. Thus, we predicted that males would be placed in larger and earlier eggs.

Methods

The study was carried out in an urban blackbird population inhabiting the Dr Jordan Park in Kraków (southern Poland). The European blackbird is a medium-sized, sexually dichromatic, strongly territorial, socially monogamous, and open cup nesting passerine bird (Cramp 1988). The park is located close to the city center and covers 21.3 ha; density of the breeding blackbirds averaged 1.9 pairs per 1 ha (ca. 40 pairs every year). Most males occupy their territories from March and April and some of them stay on their territories throughout the whole year. Females start laying eggs at the end of March and continue into July. In the study

population, females usually lay 3-5 eggs (mean ± SD: 4.3 ± 0.7, N = 99 clutches). As a result of high nest predation, about 95% of females repeat breeding attempts. Most nests are destroyed during egg laying (very often after laying first or second egg) and incubation. All territories were monitored daily and nests were found by following nest-building females and searching bushes and trees throughout the park. Almost all breeding birds in the park were individually marked with an aluminum band and a combination of colour bands. In years 2000-2003 and 2005-2006 laying date of the first egg, clutch size, hatching date and number of nestlings were recorded in most of nests. Additionally, data on laying and hatching order were recorded in a sample of nests. In most cases, data on egg size and sex were obtained from clutches deserted during incubation (N = 45 eggs from 25 clutches). In this case, samples of tissue from embryos were collected. In some nests nestlings hatched successfully (N = 33 hatchlings from 27 clutches), and blood was sampled for further sex determination within 1-2 days after hatching of the first nestling. In the case of 2 clutches, genetic material was collected from both unhatched eggs (N = 2) and alive nestlings (N = 2). Laying order was determined by inspection of nests every day during egg laying. On the day of laying, eggs were numbered with a permanent marker, measured with an electronic calliper (length and width to the nearest 0.1 mm) and weighed with an electronic balance (to the nearest 0.01 g). Starting from the day before expected hatching, nests were visited daily to assign each marked egg to its corresponding hatchling. Nestlings were then marked by nail clipping. In some nests, we were not able to assign hatchlings to the eggs from which they hatched and so they were excluded from the analyses. To calculate egg volume, we used the formula, $V = 0.51 \times LW^2$, where L = egg length and W = egg width, which can be applied to most bird eggs (Hoyt 1979). Egg volume calculated according to this formula is highly correlated with the egg mass ($r = 0.85$, $P < 0.0001$, N = 87). Overall, data on laying order and sex were collected for 82 eggs originating from 54 different females (clutches). 93% of these clutches

were incomplete because of egg infertility (N = 4), unknown hatching order of nestlings (N = 4), partial predation (N = 17) and discovery of the nest after egg-laying initiation (N = 25).

Blood and tissue samples were kept in 0.5 ml of 96% ethanol and stored at room temperature. DNA was extracted using Nucleo Spin Blood kit (Macherey-Nagel) following the manufacturer's protocol. Molecular sexing was performed by PCR amplification of two homologous genes (CHD1-W and CHD1-Z) following the protocol of Griffiths et al. (1998). PCR products were separated by electrophoresis on 3% agarose gels, which were stained with ethidium bromide and visualized under UV trans-illumination. The sex was assessed according to the presence of one band for a male or two bands for a female. This method was positively validated by blindly testing blood samples originating from 10 adult birds of known sex.

To examine variation in egg volume in relation to embryo sex and laying sequence, we used the linear mixed model (PROC MIXED in SAS). In this model, the sex of the embryo was introduced as a categorical variable and laying order as a covariate. To control for possible non-linear effects, the laying order was introduced to the model as a linear and quadratic term and their interactions with sex were also tested. In addition, year as a categorical variable and laying date and clutch size as covariates were introduced to the initial model. To distinguish between within and among clutch effects, clutch sex ratio was entered to the initial model and although non-significant it remained in the final model. This clutch sex ratio was derived only of eggs with known sex and laying order. The level of significance was set at $P < 0.05$ and non-significant terms were systematically removed from the initial model to increase the power of tests. Female identity was entered in each model as a random factor. To analyze the relationship between offspring sex and laying order, we used the generalized linear mixed model using GLIMMIX macro in SAS with logit link function and binomial error variance. To check possible non-linear effects of the position of the egg in the

laying sequence on offspring sex we introduced to the model laying order as a linear and quadratic term. Female identity was introduced to the model as a random factor. Because we did not find any non-linear effects, laying order squared was removed from the final model. All analyses were performed in SAS version 9.1 and denominator degrees of freedom were approximated by the Satterthwaite method.

Results

We found that within a clutch, eggs containing male and female embryos to differ in size (Table 1). Male eggs were significantly larger (least sq mean ± SE; egg volume: 6988.7 ± 112.9 mm^3) in comparison to female eggs (least sq mean ± SE; egg volume: 6842.6 ± 110.9 mm^3). There was also a significant interaction between embryo sex and laying order (Table 1, Figure 1). Separate analysis performed for each sex showed that egg volume significantly increased with the laying sequence among eggs bearing female embryos ($F_{1, 25.4} = 8.46$, $P = 0.007$; Figure 1). The effect of laying order was not significant among eggs bearing male embryos ($F_{1, 23.4} = 1.44$, $P = 0.24$; Figure 1). We also found a non-linear effect of position of the egg in the laying sequence on egg size (Table 1). This results from the fact that initial eggs were larger than middle eggs and final eggs were the largest within a clutch. There were no between clutch effects on egg volume (Table 1), thus the observed within-clutch effects were independent from differences in sex ratio. The sex ratio in the studied sample of eggs was not significantly different from parity (49% males; Binomial test, $P = 0.90$). Offspring sex did not vary with the position of the egg in the laying sequence analyzed both as a linear (laying order: $F_{1, 80} = 1.62$, $P = 0.21$) and quadratic term (laying order: $F_{1, 79} = 0.26$, $P = 0.61$; laying order laying order: $F_{1, 79} = 0.08$, $P = 0.78$).

Discussion

In this study, we report within-clutch differences in eggs size between sexes but do not find evidence that offspring sex is differently allocated in early and late laid eggs in the European blackbird. Sexual egg size dimorphism has been reported in at least six passerine species (Mead et al. 1987, Cordero et al. 2000, 2001, Arnold and Griffiths 2003, Magrath et al. 2003, Leitner et al. 2006). There are also similar findings from non-passerine species (Anderson et al. 1997, Petrie et al. 2001; see also Cunningham and Russell 2001, Blanco et al. 2003, Müller et al. 2005). However, the observed patterns of egg sexual size dimorphism are inconsistent and multiple hypotheses have been suggested to explain the possible adaptive value of disparities in egg size between male and female eggs. Among passerines, female offspring of the Spotless starling *Sturnus unicolor* and the Brown songlark hatched from larger eggs (Cordero et al. 2001, Magrath et al. 2003). This pattern of egg provisioning was interpreted as an adaptive strategy of support for the more vulnerable smaller sex (Cordero et al. 2001, Magrath et al. 2003). In contrast, in the White-crowned sparrow, the House sparrow *Passer domesticus* and the captive canaries *Serinus canaria*, males were found in larger eggs, despite them being larger than females (Mead et al. 1987, Cordero et al. 2000, Arnold and Griffiths 2003, Leitner et al. 2006). Mead et al. (1987) and Cordero et al. (2000) explained their results in light of the sex allocation theory, arguing that better provisioning of eggs bearing male embryos may be a adaptive strategy of increased investment to the sex with the potential to give higher fitness returns. In fact, males of those species exhibit higher variation in reproductive success compared to females resulting from extra-pair copulation, very common in these species. Therefore, the increased maternal investment towards male offspring might be expected. Support for this hypothesis comes from studies on mammals, which indicate that the breeding success of a male is related to maternal investment at early stages of life, however, only mothers in good condition can produce more successful males (e.g. Clutton-Brock et al. 1984, 1986, Sheldon and West 2004). Thus, extra investment in eggs containing

male embryos may be considered as an adaptive strategy by which females could increase reproductive success of their sons (e.g. Mead et al. 1987, Cordero et al. 2000). Such scenario may also contribute to explain sexual egg size dimorphism in the European blackbird, in which extra-pair paternity is common and generate a substantial variation in male reproductive success (Creighton 2000, Rutkowska et al, unpublished data). However, rigorous test of the adaptive role of sex-specific variation in egg size is missing. The observed pattern of sexual dimorphism in egg size may alternatively be explained without invoking adaptive explanations. For instance, if the same maternal hormones regulate oocyte growth and simultaneously affect sex-determining chromosome segregation, sexual egg size dimorphism may arise as a not-necessarily adaptive consequence of this hormonal mechanism (e.g. Young & Badyaev 2004).

In the current study we found a significant interaction between sex and laying order resulting from the fact that egg size increased with laying order among female but not male eggs. An increase in egg size is often explained in terms of compensatory mechanism of negative effect of hatching asynchrony (e.g. Clark and Wilson 1981). Such compensation could be expected in the blackbird, as eggs hatch asynchronously in this species (Magrath 1989, Walasz, unpublished data). Interestingly, such compensation would occur only among eggs bearing daughters. Since eggs bearing daughters are smaller, they may pay larger cost of hatching asynchronously and the increase in egg size in late laid eggs may offset negative effects of hatching asynchrony. Such sex-specific effect has already been reported in asynchronously hatching zebra finches *Taeniopygia guttata*, in which egg mass and egg laying sequence were significant predictors of differential survival of male and female offspring (Rutkowska and Cichoń 2005).

Zusammenfassung

Sexueller Dimorphismus der Eigröße in Amseln *Turdus merula*

In letzter Zeit haben mehrere Studien zeigen können, dass weibliche Vögel in der Lage sind, das Geschlecht ihrer Nachkommen während des Keimzellenstadiums zu kontrollieren. Ferner gibt es Hinweise darauf, dass Weibchen ihre Investitionen in ihre Nachkommen von dem Geschlecht des Embryos während der Eiformation abhängig machen können. Die unterschiedliche mütterliche Investition in Eier in Abhängigkeit des Geschlechts wird normalerweise als adaptive Strategie interpretiert, wobei Weibchen die Wettbewerbsfähigkeiten des kleineren Geschlechts verbessern können, oder vorzugsweise in das Geschlecht mit dem potenziell höheren Fitnessgewinn investieren. In der vorliegenden Arbeit haben wir Variation in Eigröße relativ zum Geschlecht der Embryos und Legereihenfolge in Amseln *Turdus merula* untersucht. Männliche und weibliche Eier waren unterschiedlich groß — größere Eier enthielten männliche Embryos. Wir fanden auch eine signifikante Interaktion zwischen dem Geschlecht von Embryos und der Legereihenfolge. Diese Interaktion entstand durch eine Zunahme von Eigröße mit Legereihenfolge innerhalb von Eiern die weibliche Embryos enthielten; die Eigröße von männlichen Embryonen veränderte sich dagegen nicht mit der Legereihenfolge. Wir fanden keinen Zusammenhang zwischen dem Geschlecht der Nachkommen und der Legereihenfolge innerhalb einzelner Gelege. Wir schlagen vor, dass der sexuelle Dimorphismus der Eigröße bei Amseln möglicherweise die Begünstigung des Geschlechts mit dem höheren Fitnessgewinnversprechens reflektiert.

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Table 1.

Final model	Rejected terms	Estimate	SE	d.f.	F	P
Sex		-720.03	281.67	1, 37.3	6.53	0.015
Laying order		-390.81	202.28	1, 36.7	2.47	0.124
Laying order laying order		73.95	30.57	1, 35.4	5.85	0.021
Sex laying order		187.51	78.52	1, 42.8	5.70	0.021
Clutch sex ratio		398.13	245.61	1, 74	2.63	0.109
	Laying date	6.90	3.73	1,45.5	3.43	0.071
	Sex laying order laying order	-59.61	67.10	1, 38.3	0.79	0.380
	Year			5, 47.2	0.70	0.627
	Clutch size	41.69	168.11	1, 45.4	0.06	0.805

Figure 1.

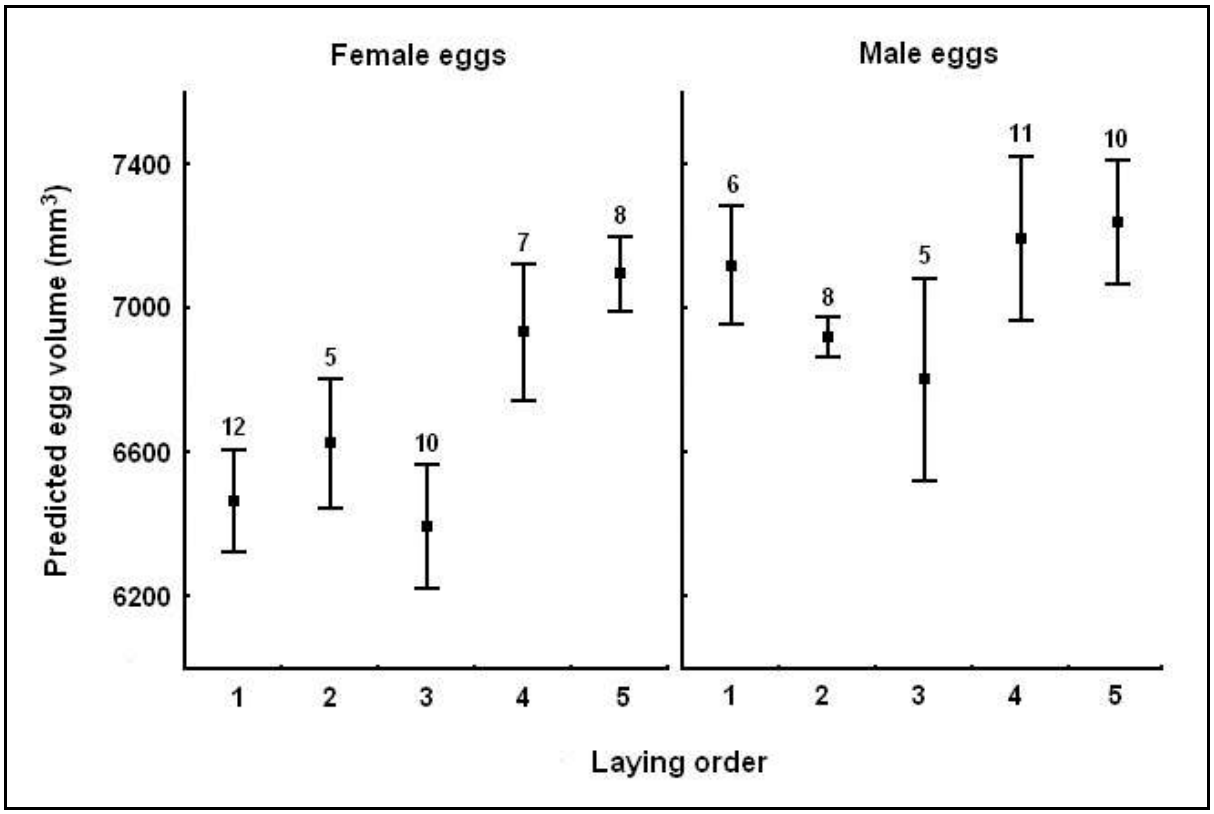
Female eggs
Male eggs
Predicted egg volume (mm³)
7400
7000
6600
6200
12
5
10
7
8
6
8
5
11
10
1
2
3
4
5
1
2
3
4
5
Laying order

Table 1. Results of the Linear Mixed Model (PROC MIXED in SAS) analyzing variation in egg volume in relation to embryo sex and year as categorical variables, laying order, clutch sex ratio, clutch size and laying date as covariates. Laying order was entered to the model both as a linear and quadratic term and all possible interaction between sex and laying order were tested. Non-significant terms were systematically removed from the initial model. In table F and P values, degrees of freedoms (d.f.) and estimates with SEs presents values for the final model, whereas in the case of rejected terms on the time when they were dropped from the model. Clutch sex ratio was entered to the initial model and remained in the final model to control for between clutch effects. Female identity was included as a random factor ($Z = 3.75$, $P < 0.0001$; the final model).

Figure 1. Predicted egg volume (least square means ± SEs) in relation to embryo sex and laying order in the European blackbird. The respective sample sizes are indicated above bars.