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Evolution of sex-biased maternal effects in birds: II. Contrasting sex-specific oocyte clustering in native and recently established populations

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Abstract

In species that produce broods of multiple offspring, parents need to partition resources among simultaneously growing neonates that often differ in growth requirements. In birds, multiple ovarian follicles develop inside the female at the same time, resulting in a trade-off of resources among them and potentially limiting maternal ability for sex-specific allocation. We compared resource acquisition among oocytes in relation to their future sex and ovulation order in two populations of house finches with contrasting sex-biased maternal strategies. In a native Arizona population, where mothers do not bias offspring sex in relation to ovulation order, the male and female oocytes did not show sex-specific trade-offs of resources during growth and there was no evidence for spatial or temporal segregation of male and female oocytes in the ovary. In contrast, in a recently established Montana population where mothers strongly bias offspring sex in relation to ovulation order, we found evidence for both intra-sexual trade-offs among male and female oocytes and sex-specific clustering of oocytes in the ovary. We discuss the importance of sex-specific resource competition among offspring for the evolution of sex-ratio adjustment and sex-specific maternal resource allocation.

Introduction

In species that produce broods of several offspring, the overlap in growth and requirements of neonates strongly affects both parental care and offspring competition (Roff, 1992). In particular, sex differences in growth costs or requirements in viviparous species set the stage for the evolution of sex-biased parental strategies and sibling competition (Drummond *et al.*, 1991; Lessells, 2002; Uller, 2003; Carranza, 2004). For example, differential hormonal requirements of male and female growth and associated hormone leakage among developing neonates lead to a strong effect of brood sex ratio on offspring and maternal life histories (Clark & Galef, 1995; Ryan & Vandenbergh, 2002; Uller, 2006). Moreover, overlapping growth of male and female offspring may limit parental ability to allocate sex-specific resources and hormones to each sex (Ono & Boness, 1996; Oyhenart *et al.*, 1998; Badyaev *et al.*, 2005). A common resolution of this conflict is the evolution of spatial or temporal clustering of neonates of different growth requirements, such as spatial segregation of male and female neonates in the uterus in mammals and sex-bias in ovulation order, hatching asynchrony, and brood sex-ratios in birds and reptiles (Clark & Wilson, 1981; Bortolotti, 1986; Clark & Galef, 1990; Drummond *et al.*, 1991; Burke, 1992; Ricklefs, 1993; Arnold & Griffiths, 2003).

While birds provide some of the best examples of fierce post-hatching sex-specific sibling competition (Mock & Parker, 1997; Drummond, 2001), the maternal opportunities for sex-specific clustering of male and female offspring seem to be limited in birds. Although multiple ovarian eggs develop inside the female at the same time, the sex-determining meiotic division occurs after most of the oocyte growth is complete and this might constrain maternal ability to allocate resources and hormones to a

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specific sex. Nevertheless, sex-specific maternal effects on early offspring growth and sex-specific sibling competition are documented in birds (e.g. Schwabl *et al.*, 1997; Petrie *et al.*, 2001; Badyaev *et al.*, 2002; Blanco *et al.*, 2003a; Strasser & Schwabl, 2004; Groothuis *et al.*, 2005). Two aspects of such sex-biased maternal effects are particularly puzzling. First is the sex-biased maternal allocation of nonsex-specific resources, such as yolk (Weatherhead, 1985; Mead *et al.*, 1987). Especially interesting are the within species fluctuations between male- and female-biased dimorphism in egg size across breeding contexts (Dijkstra *et al.*, 1990; Cordero *et al.*, 2000,2001; Anderson *et al.*, 2003; Magrath *et al.*, 2003), suggesting that the mechanisms of yolk accumulation and sex-determination might be linked. Second is a precise control of sex-biased maternal investment often found within broods; e.g. when sequentially ovulated male and female oocytes differ in nutrients, hormones, and antioxidants (Saino *et al.*, 2002,2003; Blanco *et al.*, 2003b), and when the sex-ratio of sequentially ovulated eggs is strongly biased in opposite directions (Dzus *et al.*, 1996; Komdeur *et al.*, 1997). For example, in 1-year-old females in a Montana population of house finches (*Carpodacus mexicanus*) *ca.* 90% of first-laid eggs are daughters, while *ca.* 80% of the second-laid eggs are sons (Badyaev *et al.*, 2003). Here we examine a novel hypothesis that strong sex-biased maternal effects can be accomplished by pre-ovulation clustering (temporal or spatial) of oocytes that become males or females. We compare yolk partitioning among oocytes within a clutch in relation to their ovulation order and sex in two house finch populations with contrasting maternal strategies: in a recently established Montana population with strongly sex-biased maternal effects and in a native Arizona population with no evidence of sex-biased maternal strategies.

Brief background to avian oogenesis

During the breeding season, the ovary contains a large group of small prerecruitment follicles, few of which are advanced into a hierarchy of rapidly growing pre-ovulatory yolky follicles. Once recruited into the pre-ovulatory pool, the follicles undergo rapid yolk accumulation, form hierarchical arrangement and ovulate sequentially until the clutch is complete (Johnson, 1996,2000). As the follicle grows it develops a follicular stalk with an arterial system that delivers yolk and other resources to the growing ovum. The ovarian blood flow to each follicle, and corresponding yolk precursor delivery, is thought to increase as the follicle grows (e.g. Barber *et al.*, 1991). In most passerines, ovulation occurs daily during egg-laying, typically within an hour of laying the previous egg.

It is commonly observed that the between oocyte ovulation intervals are the most variable when there are fewer simultaneously growing oocytes in the ovary (e.g. for the last-laid eggs in the clutch; Bahr & Johnson, 1984; Etches, 1990; Johnston & Gous, 2003), suggesting that among-follicle inhibiting interactions maintain regular ovulation intervals and egg-laying order within a clutch. One factor that seems to be particularly important is the hormone inhibin, which is secreted at increasingly greater concentrations by the most mature follicle (Lovell *et al.*, 2003). Inhibin's strong negative effects on growth and ovulatory rates of other follicles (Johnson *et al.*, 1993; Wang & Jonhnson, 1993; Chen & Johnson, 1996) have pronounced spatial patterns, such that only follicles in the close proximity or at similar stages of development are most affected (Yang *et al.*, 2001; Johnson *et al.*, 2005). Thus, examination of inhibitory interactions among sequentially ovulated follicles may provide insights into spatial and temporal arrangement of pre-ovulatory oocytes in the ovary.

Once ovulated, an oocyte is captured by the ovary's infundibulum and starts its daylong movement through the oviduct, accumulating albumen, membranes and a shell (Warren & Conrad, 1939). When the oocyte (yolk) size and egg-laying time are known, similarity in the yolk/albumen ratio between sequentially laid eggs might indicate their similarity in the time of ovulation and duration of oviduct passage. For example, when oocytes are ovulated at irregular intervals or simultaneously, the resulting eggs rarely differ in yolk size or egg-laying time, but typically vary strongly in the yolk/albumen ratio (Warren & Conrad, 1939; Meathrel, 1991). Thus, the examination of yolk/albumen ratio (statistically controlling for yolk size) among sequentially ovulated eggs of different sexes can provide insights into the similarity of their ovulation intervals and oviduct passage times, and thus of within-ovary inhibiting interactions. Alternatively, when females vary widely in relative amount and speed of albumen allocation into eggs, the yolk/albumen ratio is less informative of the sequential ovulation intervals.

With this biological background in mind, we tested three predictions of temporal and spatial clustering of follicles with different growth requirements (e.g. future males and females, hereafter male and female follicles) (Fig. 1). Spatial or temporal segregation of male and female follicles within an ovary (Fig. 1a) would enable precise sex-specific allocation of resources, and should produce stronger correlations of yolk partitioning among follicles within a group (that use common pool of resources) compared to follicles from different clusters (that use different pools of resources). Moreover, within clusters the correlation should decrease with the distance between the oocytes (e.g. follicles, which are closer in the hierarchy should have higher correlations; Fig. 1a). Under this scenario, the inhibin-induced coordination of ovulation intervals (see above) is likely to be stronger within each cluster than between clusters, and thus the yolk/albumen ratio in a pair of sequentially laid eggs should be less variable if these eggs came from the same

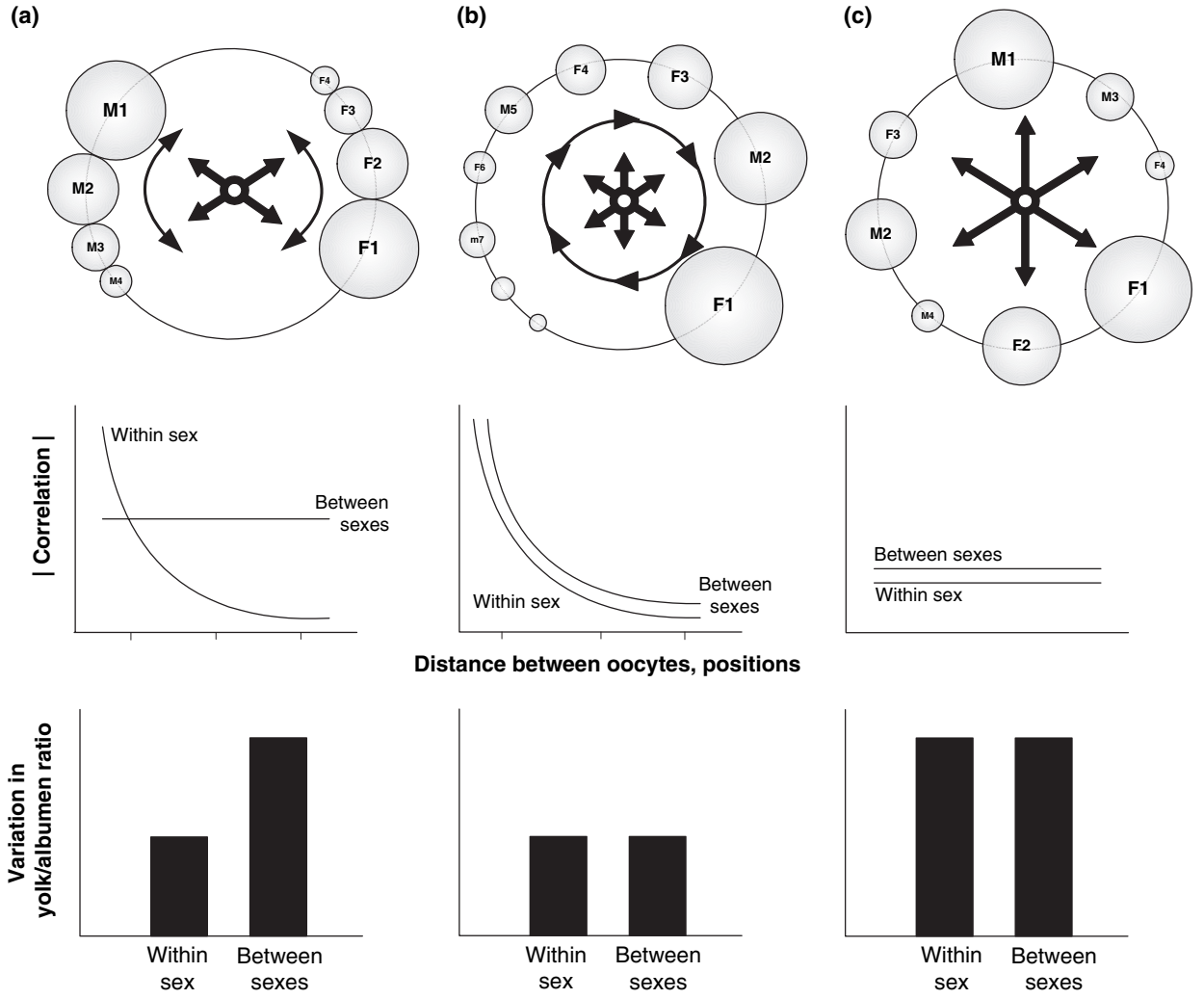

Fig. 1 Conceptual illustration of three hypotheses of spatial and temporal arrangements of classes of follicles with different requirements (e.g. males and females) in the house finch ovary. In all models, yolk precursors are delivered centrally to the ovary through female's vitelline artery (central black circle) and distributed to each follicle (black arrows) in a proportion to its maturation stage. (a) Spatial clustering of follicles with similar requirements, such as those producing males (M) and females (F), and temporal hierarchies *within* each cluster should result in greater within-cluster vs. between-cluster correlation of yolk precursor uptake (middle). Inhibin-induced coordination of ovulation hierarchy (see text for details) is likely to be weaker for *sequential* ovulation from different clusters vs. from the same cluster, thus, yielding higher variation in yolk/albumen ratio in eggs produced from different clusters (bottom). (b) Temporal hierarchy but lack of spatial clustering of male and female follicles will produce similar patterns of correlation in yolk uptake between oocytes within and between sexes (middle) and equally low variation in yolk/albumen ratio in sequentially maturing ova of different sexes (bottom). (c) Lack of temporal and spatial clustering of follicles, including those of different sexes (e.g. oocytes at the similar maturation stage are distributed throughout the ovary) will result in the lack of association between correlation in yolk uptake and ovulation sequence both within and between the clusters of follicles (middle) and equally high variation in yolk/albumen ratio of sequentially ovulated oocytes of the same and the opposite sex (bottom).

cluster compared to weakly coordinated ovulation intervals of eggs from different clusters (Fig. 1a).

Alternatively, when male and female follicles are not clustered, and ovulated in strict hierarchical sequence (Fig. 1b), correlations of yolk deposition should be similar between male and female follicles and vary only with distance between oocytes. Yolk/albumen ratio should be similar between sequentially ovulated oocytes of the same and the opposite sexes (Fig. 1b). Under this scenario, sex-specific allocation is difficult; oocytes that are closer in ovarian hierarchy are expected to share (passively or actively) resources, such as hormones, with each other. Finally, oocytes of different sex and developmental stages can be distributed throughout the ovary without particular clustering or strict ovulation hierarchy (Fig. 1c). Under this scenario, sex-specific allocation is

likely accomplished by follicle-specific allocation of resources, no correlation in yolk uptake is expected between sequentially ovulated oocytes, and weak within-ovary coordination of ovulation intervals should produce equally variable yolk/albumen ratio between sequentially ovulated eggs of the same and the opposite sex (Fig. 1c).

Materials and methods

Field methods

We studied house finches at two long-term study sites: at the northernmost edge of the species' range in northwestern Montana, where house finches started breeding in late 1970s and at the southern edge of their native range in southwestern Arizona. The Montana and Arizona study sites have been maintained since 1995 and 2000, respectively. At both sites all resident adults were trapped and marked with a unique combination of one aluminium and three coloured plastic rings, and pairing associations and nest-initiation behaviour were closely followed for all birds (Badyaev *et al.*, 2003; Oh & Badyaev, in review). At the time of nest building, thermocouples (iButton-TMEX, Dallas Semiconductor) were installed at each nest to monitor the onset of incubation. All females laid one egg per day between 630 and 1000 until the clutch was complete and eggs were numbered sequentially on the day of laying (Badyaev *et al.*, 2002,2005). To calibrate the method of egg measurement used in this study (see below), 66 freshly laid eggs from 17 nests were collected in 2002–2003 and yolk and albumen separated and weighed as described in Young & Badyaev (2004). All other eggs used in this study were photographed within a 24 h of laying and allowed to develop.

Egg and yolk measurements

To measure egg and yolk sizes, freshly laid eggs were photographed on a specially designed photo stand, with 5 megapixel digital 35 mm camera mounted in a standard position and connected by a cord with the dedicated point-source tube flash attached 5 mm directly under the egg placement plate. The egg placement plate had a round opening (∅ 6 mm), marks delineating the longest ellipsoid axis of an egg, and a ruler. The photo stand was placed to block ambient light, and operated by remote controller. Point-source flash was set to a constant output and operated by the camera. The setup produced high-resolution digital images of an entire yolk, egg size, eggshell and all membranes. Egg, yolk and albumen areas as well as the reference ruler (1 mm) of each image were measured with the SIGMASCAN PRO 5.0 image analysis software (SPSS Inc., Chicago, IL, USA). Two hundred and eighty-one eggs from 63 nests in Montana population and 104 eggs from 26 nests in Arizona population were repositioned on the photo stand and photographed twice to obtain repositioning error and subsequently measured twice, with the interval of at least 1 week, to estimate measurement error. We obtained complete data on egg, albumen and yolk size, egg-laying order and the sex of resulting nestlings from 378 eggs (84 nests) from the Montana population and 304 eggs (64 nests) in Arizona population. To verify yolk and egg measurements used in this study, we compared area-based measurements and weights of 66 freshly collected eggs from 17 broods. Area measurements accurately represented size of both egg ($F_{1,85} = 187.22$, $P < 0.0001$, linear regression $b = 0.86 \pm 0.60$ (SE), $t = 13.68$, $P < 0.0001$; standardized regression coefficient $b_{ST} = 0.83$) and yolk sizes ($F_{1,86} = 156.45$, $P < 0.0001$, linear regression coefficient $b = 2.03 \pm 0.16$, $t = 12.51$, $P < 0.0001$; standardized regression coefficient $b_{ST} = 0.80$).

Sex determination

Sex of nestlings and embryos was determined molecularly by amplification of an intron of the CHD1 genes on the sex chromosomes (Griffiths *et al.*, 1998). In eggs that were allowed to develop, genomic DNA was obtained from nestling tissues – either from a 15 μL blood sample of 7–8-day-old nestlings or from a down follicle of 2–3-day-old nestlings (Badyaev *et al.*, 2001). In a sample of eggs used for the method calibration, early embryo cells of eggs that were incubated for 18–20 h were separated from the surrounding tissues of the whole yolk under 12 times magnification. DNA extraction, PCR protocol and verification of molecular sexing of these samples are described in Young & Badyaev (2004).

Statistical analysis

Clutches with less than four or more than five eggs were excluded from this study. We used the general linear models with nest identity as a random effect and ovulation order as categorical variable to analyse the among-clutch trends in egg, albumen and yolk variation (Table 1). Sex-ratio bias was tested with the binomial test. Sex differences in egg composition *within* ovulation orders were compared with nonparametric two-tailed Kruskal–Wallis tests, multiple comparisons were conducted with Waller-Duncan *K*-ratio *t*-tests. All area measurements had normal distribution and were log-transformed before the analyses of variance.

Correlational structure of similarity among follicles in yolk uptake (Online Figs S1, S2) was converted to distances in canonical discriminant analysis (PROC CANDISC in SAS 9.2). The cluster analysis of similarity in correlational structure between follicles of different ovulation order and sex was then conducted by Ward's method using pseudo-F and pseudo-t^2 statistics to estimate the number of statistically distinct clusters. Pseudo-t^2 statistics suggested four clusters for Montana

Table 1 Egg, albumen and yolk sizes in relation to sex and ovulation order in recently established Montana population and a native Arizona population of the house finches. Shown are *F*-values from general linear models that include nest identity as a random effect

	Population			
	Montana, $n = 378$ eggs, 84 nests		Arizona $n = 304$ eggs, 64 nests	
Effect	*F*	*P*	*F*	*P*
Egg size				
Sex	7.34	0.01	6.65	0.01
Ovulation order	2.86	0.05	0.59	0.67
Sex × ovulation order	2.55	0.04	3.36	0.01
Albumen amount				
Sex	4.21	0.04	0.36	0.55
Ovulation order	3.11	0.02	4.07	0.01
Sex × ovulation order	2.76	0.03	3.07	0.04
Yolk size				
Sex	0.05	0.82	4.14	0.04
Ovulation order	1.71	0.15	3.51	0.01
Sex × ovulation order	3.18	0.02	2.93	0.02

d.f. are as follows: sex (d.f. = 1), ovulation order (d.f. = 4), sex × ovulation order (d.f. = 4).

and three clusters for Arizona. Pseudo-*F* statistics suggested six distinct clusters in Montana and four clusters in Arizona. Most conservative measures – four clusters for Montana and three clusters for Arizona – were selected for the analyses. The per cent of variance due to sex of the preceding follicle in the analyses of yolk/albumen ratios was calculated from the total phenotypic variance.

Results

Sex ratio, egg, albumen and yolk sizes in relation to ovulation order

Populations differed in ovulation order of male and female eggs ($Z = 3.93$, $P < 0.01$; Fig. 2). In Montana, the first-, second- and the last-laid eggs were strongly sex-biased (all $\chi^2_1 > 5.17$, $P < 0.05$; Fig. 2a). In Arizona, there was no sex bias in any egg-laying position (first: $\chi^2_1 = 0.01$, $P = 0.90$; second: $\chi^2_1 = 0.42$, $P = 0.51$; third: $\chi^2_1 = 0.00$, $P = 0.99$; fourth: $\chi^2_1 = 0.81$, $P = 0.37$), except for the strongly female-biased last-laid egg ($\chi^2_1 = 4.55$, $P < 0.05$; Fig. 2b). In Montana, female eggs were larger than male eggs (Kruskal–Wallis $\chi^2_1 = 4.69$, $P < 0.05$), which was due to larger amount of albumen in female eggs ($\chi^2_1 = 3.73$, $P = 0.05$), yolk sizes did not differ between the sexes ($\chi^2_1 = 0.00$, $P = 0.99$; Table 1; Fig. 3a). Both albumen and overall egg size differed between the sexes and ovulation orders, and the sex difference in yolk size, as well as contribution of yolk and albumen to overall egg size varied with ovulation order (Table 1; Fig. 3a). For example, while male and female penultimate (fourth) eggs did not differ in size ($\chi^2_1 = 0.72$, $P = 0.39$), they consisted of distinct proportions of yolk and albumen: albumen amount was greater ($\chi^2_1 = 4.67$, $P < 0.05$), but yolk was smaller ($\chi^2_1 = 3.82$, $P = 0.05$; Fig. 3a) in female eggs compared to male eggs. Overall, in Arizona, male and female eggs did not differ in size ($\chi^2_1 = 1.91$, $P = 0.160$, albumen ($\chi^2_1 = 0.13$, $P = 0.70$) or yolk amount ($\chi^2_1 = 0.81$, $P = 0.37$), however the relative contribution of yolk and albumen to egg size varied strongly with ovulation order and sex of an egg (Table 1 and Fig. 3b). Despite the opposite bias in the

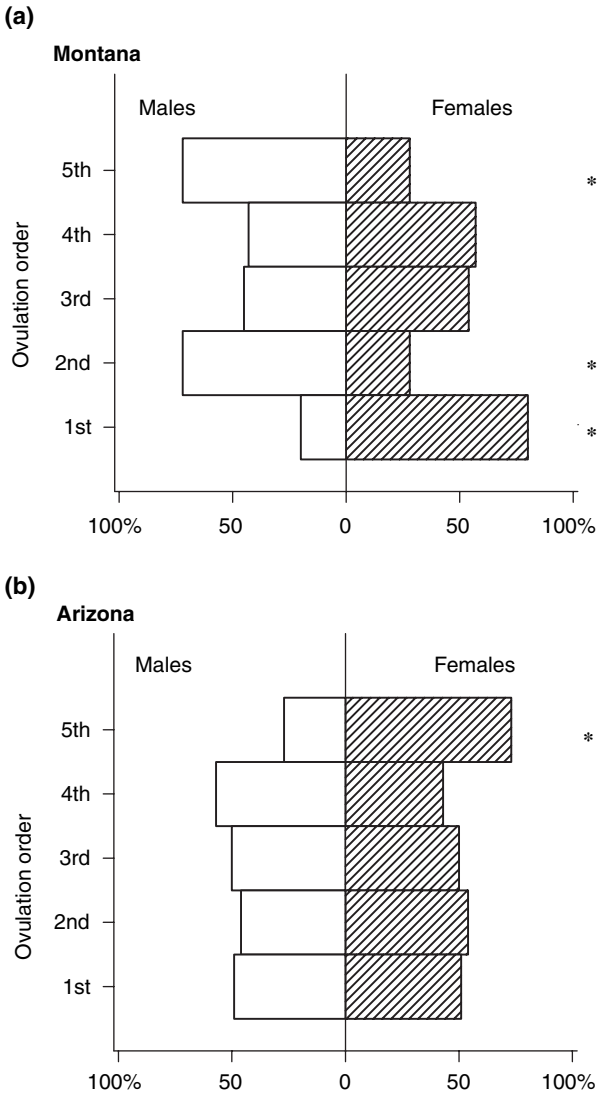

Fig. 2 Sex-ratio in relation to ovulation order in nests of house finches in (a) recently established Montana population, and (b) native Arizona population. Asterisks indicate significant deviation from the equal sex ratio for a given ovulation order.

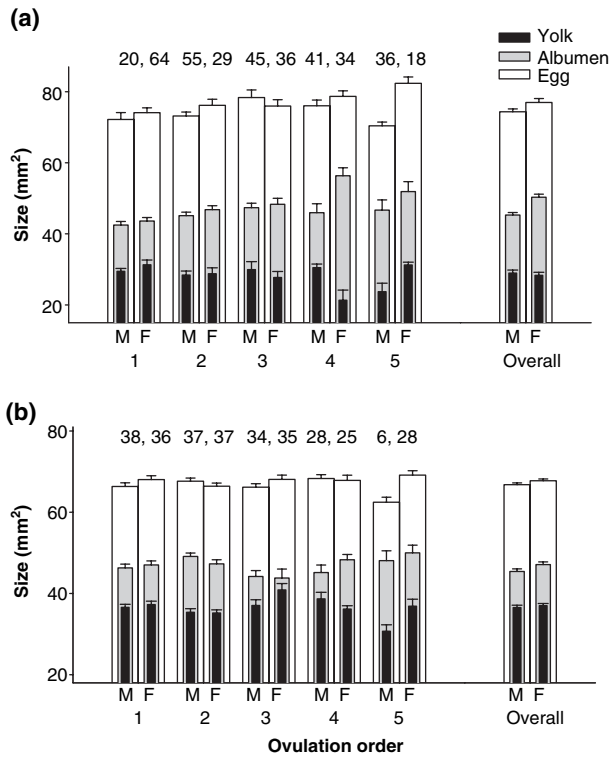

Fig. 3 Descriptive statistics (mean ± SEM) of yolk, albumen and egg sizes in relation to ovulation order and sex in (a) Montana and (b) Arizona populations. Numbers above bars are sample sizes for male (M) and female (F) eggs. See text for tests.

sex-ratio of the last-laid egg between the populations (Fig. 2), in both populations the last-laid egg was larger when female than when male (Montana: $\chi^2_1 = 9.78$, $P = 0.001$; Arizona: $\chi^2_1 = 8.30$, $P < 0.05$), and the difference was produced by a larger yolk of last-laid female eggs (Montana: yolk $\chi^2_1 = 3.28$, $P = 0.050$, albumen: $\chi^2_1 = 1.80$, $P = 0.18$; Arizona: yolk $\chi^2_1 = 3.70$, $P = 0.05$, albumen: $\chi^2_1 = 0.21$, $P = 0.60$; Fig. 3).

Similarity of oocyte yolk uptake in relation to sex and ovulation sequence

In Montana, in pairs of same-sex follicles in adjacent positions, yolk uptake was strongly negatively correlated (Online Fig. S1, above diagonal), whereas adjacent follicles of the opposite sex showed either positive or no correlation (Online Fig. S1, below diagonal). In the follicle pairs of the same sex, the strength of correlation gradually decreased with distance between the follicles (Fig. 4a; correlation between follicles separated by three and more positions is different from follicles adjacent to each other in both male-male and female-female pairs: Waller Duncan K-ratio $t = 3.05$, $P < 0.001$). In pairs of follicles where male follicles ovulated after female follicles (FM pairs; Fig. 4b), the strength of correlation did not vary with the distance between the follicles, whereas in pairs where male follicles ovulated before female follicles (MF pairs), the follicles adjacent to each other differed from pairs of follicles separated by three or more positions, $t = 3.52$, $P < 0.01$; Fig. 4b). In Arizona, the strength of correlation did not vary with the distance or follicle sex in the pairs of the same sex follicles (Fig. 4c), whereas in follicle pairs of the opposite sex, those separated by three and more positions had greater correlations in yolk uptake than more closely positioned follicles (Fig. 4d; $t = 3.31$, $P < 0.01$).

Cluster analysis of the correlational matrix of yolk size similarity based on Ward's minimum distance revealed four significantly different clusters of follicles for Montana and three clusters for Arizona (Fig. 5; cluster structure is different between the populations: Wilk's $\lambda = 0.88$, $P < 0.01$). In Montana, the clusters widely segregated male and female oocytes that ovulate early in the sequence (i.e. clusters F1-F2-F3-F4 and M1-M2-M3, Mean square distance = 0.31) and a both-sexes cluster of late ovulating oocytes (i.e. M4-F5; distance = 1.2; Fig. 5a). In Arizona, there was no sex-specific segregation of follicles (Fig. 5b). The first, poorly differentiated, cluster contained a mixture of early ovulating male and female follicles, and was significantly distinct only from the cluster of late ovulating follicles (M4-F4; distance = 0.6; Fig. 5b). In both populations, the last ovulating male follicle was highly distinct in yolk size variation from all other follicles (M5 distances > 1.6; Fig. 5).

Duration of oogenesis stages in relation to sex and ovulation sequence

Similarity between two sequentially ovulating oocytes in yolk/albumen ratio (controlled for yolk size) depended strongly on whether the two oocytes were the same sex (Fig. 6 and Table 2). In Montana, with the exception of the second-laid eggs, male oocytes that ovulated after another male oocyte were less variable (i.e. more similar) in inferred ovulation intervals compared to male oocytes ovulating after a female oocyte (Fig. 6a and Table 2). In females, only in the second and the last-laid egg positions, oocytes that ovulated after another female oocyte had more similar inferred ovulation intervals compared to oocytes preceded by a male oocyte (Fig. 6b and Table 2). In Arizona, in males the ovulation interval between sequentially ovulating oocytes was less variable than in Montana, and did not covary with the sex of the preceding oocyte (Fig. 6c), while in female oocytes, those ovulating after a female oocyte had more variable ovulation intervals in the last-laid eggs (Fig. 6d and Table 2).

Discussion

When male and female offspring differ in costs of growth, viviparity imposes a strong constraint on maternal ability

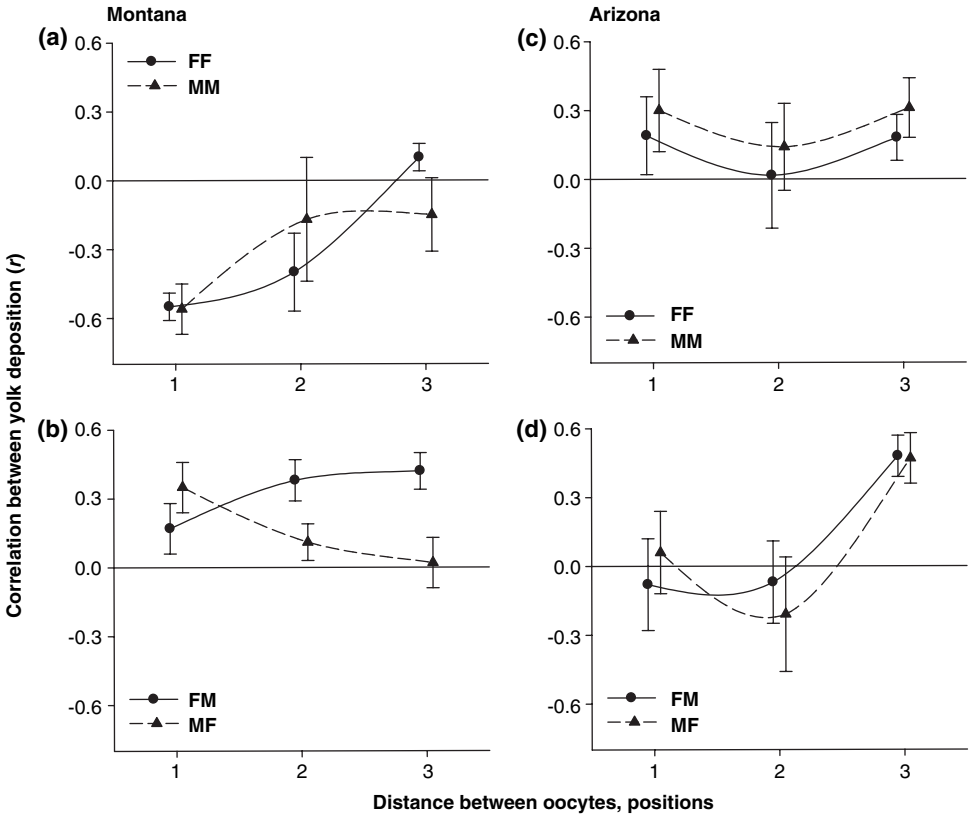

Fig. 4 Correlations between yolk sizes of simultaneously growing oocytes in relation to their sex and sequence of ovulation. *X*-axis shows numbers of ovulation positions between oocytes involved in correlations (e.g. second and third ovulating oocytes have a 'distance' of one position, first and fourth ovulating oocytes have a 'distance' of three positions, correlations between first and fifth follicles are included in the three-positions group). Fitted lines are shown to facilitate comparisons with predictions of Fig. 1 and are for illustration only. (a) Correlation between oocytes of the same sex (—●—, solid lines – females; – –▲– –, dashed lines – males) in Montana and (c) Arizona populations. (b) Correlation between oocytes of the opposite sex (—●—, solid lines – female preceding males, e.g. F1M2; – –▲– –, dashed lines – males preceding females e.g. M1F2) in Montana and (d) Arizona population. See text for details.

to meet sex-specific demands of offspring thus setting a stage for the evolution of sex-specific sibling competition (Taylor, 1981; Richter, 1983; Slagsvold, 1990; Krackow, 1995; Uller, 2006). In many animals, spatial or temporal segregation of eggs or embryos of different sexes not only enables sex-specific parental resource allocation, but also reduces the detrimental effects of passive exposure to sex-specific growth substances (such as hormones) between male and female neonates (Ryan & Vandenbergh, 2002). In birds, such parental strategies include modification of the order in which offspring of different sexes are produced within a clutch and biases in brood sex-ratio; often precise sex-biased maternal strategies (Dijkstra *et al.*, 1990; Komdeur *et al.*, 1997; Cordero *et al.*, 2001; Krebs *et al.*, 2002; reviewed in Komdeur & Pen, 2002; Pike & Petrie, 2003) point to close interdependencies among maternal resource allocation, resource acquisition by growing follicles, and ova's sex-determination. Yet, these interactions are poorly understood in birds.

We examined evidence for sex-specific clustering of developing follicles in two populations of house finches – a 36-year-old population at the northern limit of the species' range (Montana) where strong sex-specific modification of oocyte growth and ovulation are favoured by natural selection (Badyaev *et al.*, 2002, 2005) and the native southern population (Arizona) where finches show no evidence of sex-specific maternal effects on ovulation sequence (Fig. 2). Our tests of three predictions of yolk uptake similarity and ovulation intervals among follicles of different sex and ovulation order (Fig. 1), provided evidence for sex-specific oocyte clustering in the population with strongly sex-biased maternal effects (i.e. prediction A in Fig. 1), but no evidence for such clustering in the population with no sex-biased egg-laying order (i.e. prediction C in Fig. 1). First, in Montana, but not in the Arizona population we found negative trade-offs in yolk uptake between follicles of the same sex; these trade-offs were decreasing with the distance between the follicles, whereas follicles of the

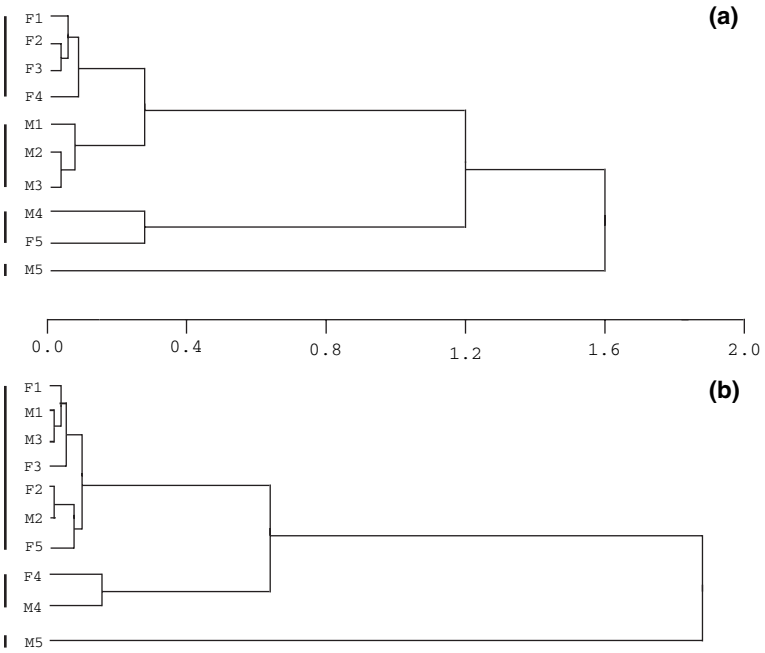

Fig. 5 Clusters of yolk uptake similarity among oocytes (*X*-axis – Ward's minimum distance) in relation to oocyte sex and ovulation order in (a) Montana and (b) Arizona populations. Vertical bars show four significantly different clusters in (a) and three clusters in (b).

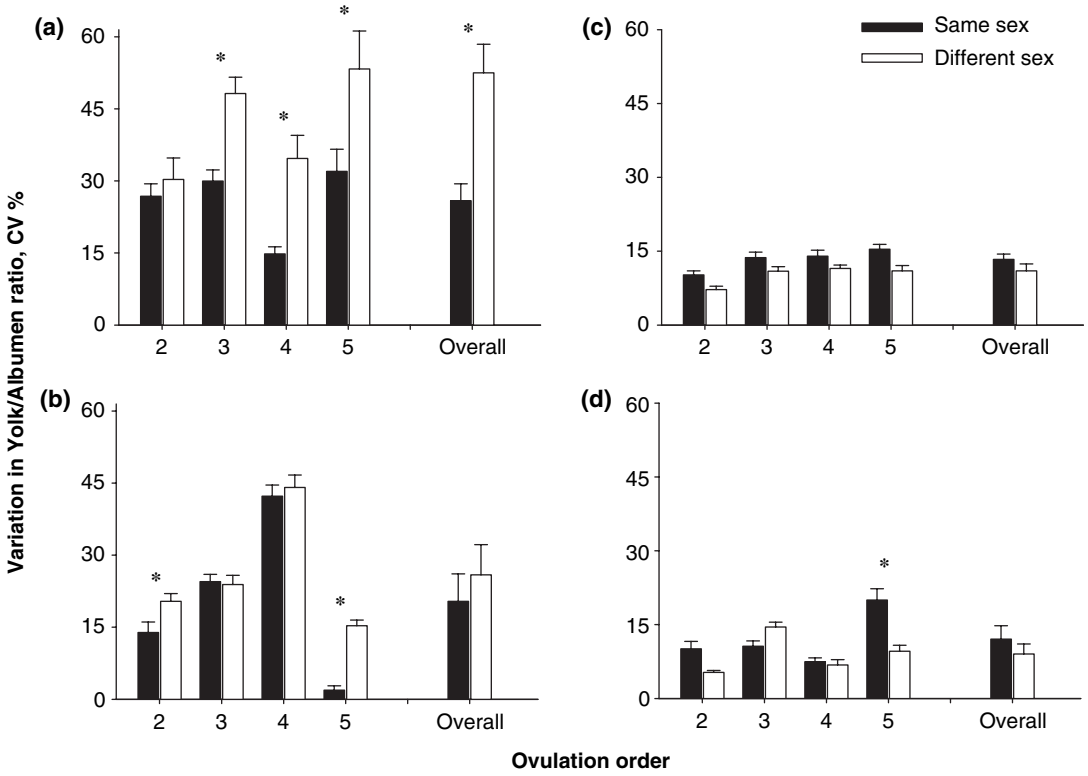

Fig. 6 Variation in yolk/albumen ratio as a measure of ovulation intervals consistency in relation to ovulation order and the sex of the immediately preceding egg. Shown is variation in residual egg's yolk/albumen ratio (controlled for yolk size and representing duration of oviduct passage in CV, %) in (a) male eggs and (b) female eggs in Montana population, (c) male eggs and (d) female eggs in Arizona population. *$P < 0.05$ for the Kruskal–Wallis test of significant differences between the effect of the sexes of preceding egg. For other tests see Table 2.

Table 2 Contribution of the sex of the immediately preceding follicle to the yolk/albumen ratio (a measure the ovulation time and oviduct passage) in Montana and Arizona house finches. Shown are *F*-values for the sex of the preceding follicle (same vs. the opposite, d.f. = 1) from the general linear models that include nest identity (Montana: $n = 84$ nests, Arizona: $n = 64$) and yolk size as covariates (sample sizes for each ovulation position are in Fig. 3). VAR (%) is the amount of phenotypic variance attributable to the sex of preceding follicle.

	Population					
	Montana			Arizona		
Ovulation sequence	*F*	*P*	VAR	*F*	*P*	VAR
Males						
2	1.31	0.26	2.7	2.94	0.10	13.9
3	5.34	0.03	12.1	2.01	0.12	13.0
4	7.91	0.01	23.4	1.83	0.20	11.8
5	6.02	0.02	17.8	3.63	0.06	18.5
Females						
2	6.46	0.02	24.8	3.78	0.06	21.7
3	13.91	0.01	48.2	1.31	0.27	11.5
4	0.23	0.63	11.5	0.03	0.87	0
5	10.08	0.01	34.2	12.10	0.01	44.5

opposite sex showed positive or no correlation (Fig. 4). In Montana, stronger trade-offs in yolk uptake among follicles of the same sex and ovulation order produced distinct clusters of male and female follicles (Fig. 5a). In Arizona, only early and late ovulating oocytes were distinct in their yolk uptake, and there were no evidence for sex-specific segregation of oocytes (Fig. 5b). Second, in Montana, but not in Arizona finches, the inferred among-follicle inhibiting interactions, which maintain ovulation hierarchy, were stronger among sequentially ovulating follicles of the same sex than among follicles of the opposite sex (Fig. 6), providing further, albeit indirect, evidence for spatial or temporal sex-specific clustering in the population with strongly sex biased egg-laying order. Finally, the relative contribution of yolk and albumen to the overall egg size depended on the sex of the previously ovulated eggs and the follicle position in the ovulation order.

We discuss these findings in relation to their three important implications. First, the documented variable and sex-specific trade-offs in yolk uptake provide insights into the evolution of sex-biased maternal resource allocation in birds. Second, the sex-specific clustering of follicles in temporal and spatial ovarian hierarchy enables inference about the timing and mechanisms of sex-determination in birds, especially in species that show consistently sex-biased ovulation sequence. Third, the finding that egg size variation – a commonly measured trait in birds – is attributed to different components depending on follicle juxtaposition in relation to other follicles in the hierarchy is highly relevant to studies of maternal effects and life histories in birds.

Negative trade-offs in yolk uptake within adjacent follicles of the same sex, such as observed in Montana finches (Fig. 4; Online Fig. S1) can be produced by either partitioning of a common pool of yolk precursors between the follicles that share access to the yolk supply source (e.g. spatial proximity of follicular stalks), or by adjacent follicles' similarity in their yolk uptake requirements (i.e. similar efficiency in yolk uptake), or by inhibiting effects that the follicles exert on each other. Diminishing strength of trade-offs with the distance between the follicles in the ovarian hierarchy and the strongly opposite within- and between-sex correlations (Online Fig. S1), suggest that the trade-offs are due to spatial or temporal proximity of the same sex follicles within an ovary. This conclusion is corroborated by the cluster analysis of correlational structure of yolk uptake within each population; in Montana, follicles form distinct sex-specific clusters, whereas in Arizona, there is no evidence of sex-biased clustering. These results are also in concordance with the highly sex-biased egg-laying order in Montana population and the absence of such bias in Arizona population (Fig. 2, Badyaev *et al.*, 2003).

At the same time, positive correlations in yolk uptake between the groups of follicles of the different sex or between widely separated follicles in the ovary might reflect general organism-wide allocation into egg-production (de Jong, 1993). For example, in Arizona finches, with no evidence of distinct spatial or temporal ovarian hierarchy between the sexes, the positive correlations in yolk uptake may reflect larger variation in allocation between follicles at different growth stages (Online Fig. S2) compared to lesser variation in the relatively constant or demand-driven supply of yolk to the entire ovary (Van Noordwijk & de Jong, 1986; Salvante & Williams, 2002; Worley *et al.*, 2003). Resource acquisition by individual ovum often negatively varies with the temporal overlap in growth among ovarian follicles (Surai *et al.*, 1998; Challenger *et al.*, 2001). In the previous study of Montana finches, we found that oocytes that become males, do not show the extensive overlap with other follicles, which could otherwise be expected from their position in the ovarian hierarchy, because of their earlier and faster growth but delayed ovulation (Young & Badyaev, 2004). Such temporal segregation of growing oocytes enables precise and sex-specific allocation of resources to oocytes destined to become male and females and limits passive exposure to these sex-specific products by the opposite sex (Badyaev *et al.*, 2005). Here we extend these findings by showing that yolk uptake negatively correlates in the adjacent male follicles, revealing a substantial sex-specific overlap in follicle growth (Fig. 4). An important next step would be to extend the predictions tested in this study (Fig. 1) to detailed measurements of oocyte growth (Young & Badyaev, 2004).

Three alternative scenarios of sex-specific temporal or spatial arrangement of pre-ovulatory follicles (Fig. 1) also

provide testable predictions for the timing and the mechanisms of sex determination in females that bias sex ratio in relation to egg-laying order. For example, spatial or temporal clustering of male and female follicles within an ovary (Fig. 1a), such as found in this study, would enable their differential exposure to maternal hormones. For example, a group of follicles that will eventually become males can be recruited earlier than other follicles, be exposed to and accumulate different amounts of steroids, thereby affecting both their sex-determination and ability to accumulate sex-specific resources (Badyaev *et al.*, 2005; Williams *et al.*, 2005). During egg-laying, sex-biased ovulation sequence can then be accomplished by recruiting follicles from different clusters within an ovary (similar scenarios were proposed for mammals and reptiles, Fukuda *et al.*, 2000; Lovern & Passek, 2002). Alternatively, when follicles that become males or females are not segregated but form a single ovulation hierarchy (Fig. 1b), precise sex-determination might be accomplished by an exposure of oocytes to an influx of maternal hormones immediately prior to ovulation, during the time of sex-determining meiotic division (see Badyaev *et al.*, 2005 for discussion). Finally, when oocytes of different sex and maturation stage are distributed throughout the ovary (Fig. 1c), sex-determination is likely accomplished by follicle-specific mechanisms and processes unrelated to the presence of other follicles (Johnson, 1996).

A link between sex of the oocytes and sex-specific patterns of resource deposition has been documented before (e.g. Legge *et al.*, 2001; Magrath *et al.*, 2003; see also Kraak & de Looze, 1993). For example, in zebra finches (*Taeniopygia guttata*), androgen concentration in yolk increased with ovulation order in male eggs, but decreased in female eggs within the same clutches (Rutstein *et al.*, 2005), which may be explained by either sex-specific follicle segregation before ovulation or by follicle-specific absorption or synthesis of steroids. In American Kestrels (*Falco sparverius*), the size of male, but not female eggs depended on the sex-ratio of the entire clutch (male eggs were larger in male-biased clutches; Anderson *et al.*, 1997; see also Blanco *et al.*, 2003b); a pattern most easily produced by a stronger interaction within vs. among groups of same sex follicles (Fig. 1a). Similarly, in a recent study, Müller *et al.* (2005) found evidence for significant interdependency of sex-bias and egg-mass gradient within a clutch; an egg-laying gradient of yolk deposition covaried with the sex of the initial egg. Interestingly, in the present study, the only egg that showed biased sex ratio in the Arizona population – the last-laid egg – also showed significant sex-bias in yolk and egg sizes (Figs 2b and 3b).

Our results corroborate previous findings that variation in egg size and variation in yolk size are partially independent and most variation in egg size is due to changes in the amount of albumen and not yolk size (Warren & Scott, 1935; Warham, 1983; Finkler *et al.*, 1998). We extend these observations by showing that the relative contribution of albumen and yolk – that provide distinct classes of nutrients to an embryo – depends on the timing and intervals of ovulation, sex of the preceding oocyte (Fig. 6 and Table 2), as well as on female's hormonal profile (Badyaev *et al.*, 2005; see also Zakaria, 1999). Thus, within-clutch fluctuations in egg size may often reflect female constraints and adaptations to laying eggs of different sexes (such as fluctuations in ovulation intervals), rather than egg-specific or sex-specific maternal investment (see also Nager *et al.*, 1999; Krist *et al.*, 2004), explaining the high repeatability of egg size for each female in different breeding contexts (Williams, 1994).

Our results suggest that, in a recently established Montana population, differences in growth patterns, recruitment and ovulation sequence, and hormonal exposure and accumulation between oocytes that become males and females (this study, Young & Badyaev, 2004; Badyaev, 2005) might enable sex-specific resource allocation and sex-biased ovulation even under the constraints imposed by offspring growth overlap. At the same time, the absence of these patterns in the native population – where there is no evidence of sex-biased maternal effects in relation to ovulation order – suggests that the sex-specific oocyte growth and clustering in a recently established population may represent rapidly evolving maternal adaptations for breeding at the northern limit of this species' expanding range.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 Partial correlations of yolk size between oocytes in relation to their ovulation order and sex in Montana.

Fig. S2 Partial correlations of yolk size between oocytes in relation to their ovulation order and sex in Arizona. *ND* indicates lack of data.

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