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Association of Egg Mass and Egg Sex: Gene Expression Analysis from Maternal RNA in the Germinal Disc Region of Layer Hens (*Gallus gallus*)[1]

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ABSTRACT

Female birds have been shown to manipulate offspring sex ratio. However, mechanisms of sex ratio bias are not well understood. Reduced feed availability and change in body condition can affect the mass of eggs in birds that could lead to a skew in sex ratio. We employed feed restriction in laying chickens (*Gallus gallus*) to induce a decrease in body condition and egg mass using 45 chicken hens in treatment and control groups. Feed restriction led to an overall decline of egg mass. In the second period of treatment (Days 9–18) with more severe feed restriction and a steeper decline of egg mass, the sex ratio per hen (proportion of male eggs) had a significant negative association with mean egg mass per hen. Based on this association, two groups of hens were selected from feed restriction group, that is, hens producing male bias with low egg mass and hens producing female bias with high egg mass with overall sex ratios of 0.71 and 0.44 respectively. Genomewide transcriptome analysis on the germinal disks of F1 preovulatory follicles collected at the time of occurrence of meiosis-I was performed. We did not find significantly differentially expressed genes in these two groups of hens. However, gene set enrichment analysis showed that a number of cellular processes related to cell cycle progression, mitotic/meiotic apparatus, and chromosomal movement were enriched in female-biased hens or high mean egg mass as compared with male-biased hens or low mean egg mass. The differentially expressed gene sets may be involved in meiotic drive regulating sex ratio in the chicken.

egg mass, feed restriction, gene expression, laying hens, meiosis, microarrays, primary sex ratio

INTRODUCTION

According to the sex allocation theory, mothers are expected to shift the offspring sex ratio (proportion of male eggs) toward the sex that gives greater fitness returns under given circumstances when one sex is more costly to rear and its fitness depends on available resources [1–3]. Sex ratio biases in relation to environmental or maternal factors have now been demonstrated in a wide variety of taxa, including many bird species [4]. Birds are an interesting case because the female is the heterogametic sex, therefore potentially in charge of determining the sex ratio of their offspring. Indeed, many of the previous studies in various bird species showed that offspring sex ratio varied in relation to food availability [5–8] or female body condition under natural conditions [9–13] or under experimentally controlled conditions [11, 14–17]. Both the food availability [18] and female body condition during egg production have been reported to affect the mass of the egg and of the egg yolk [19], and in several species, these correlate with the offspring sex ratio [20–22].

According to sex allocation theory, in sexually dimorphic species, a sex ratio bias toward the smaller, less costly sex may be expected under conditions of low food availability [3]. The chicken is an interesting study species for studying sex ratio bias in relation to feed availability and decreasing body mass and egg mass for two reasons. First, chickens are sexually dimorphic with females being the smaller, less costly sex [23], having lower reproductive variance than males [24] and lower energetic requirements [25]. Second, improved understanding of mechanisms underlying sex ratio bias may have important implications for commercial poultry farming.

In the present study, our starting hypothesis was that decreasing female body mass and egg mass due to feed restriction would affect the sex of the egg or the primary sex ratio per hen toward female offspring or the females would allocate more resources to the sex with low energetic requirements, that is, females. The sex of the eggs in the present study was determined in unincubated eggs, to best approximate the primary sex ratio, by using a recently developed technique of sexing unincubated chicken eggs [26].

The ovary of reproductively active hens recruit follicles in hierarchical manner (F1–F6), starting from smallest prehierarchical follicles (F6) to biggest preovulatory follicle (F1) [27]. The preovulatory follicle (F1) contains a large amount of yolk as well as a small germinal disk (3.5 mm), visible in the form of a whitish spot on the surface of the follicle [28]. The important functional reproductive processes such as sex-determining meiosis [29], fertilization, and early embryonic development occur in the germinal disk [27, 30]. The germinal disk of the preovulatory follicle in chicken (F1) contains large amounts of RNA (2.1 μg/oocyte) [31]. This RNA serves to regulate the cellular processes of the oocyte as well as earliest development after fertilization. This means that the cellular processes related to meiosis and chromosomal segregation are also regulated by this RNA. Meiosis-I, during which sex of an egg is determined [32–34], occurs about 26–28 h before oviposition [35, 36]. Asymmetric chromosomal segregation during occurrence of meiosis-I (meiotic drive) has been

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proposed to be the most likely mechanism of the primary sex ratio bias [24, 32–34, 37–39]. We hypothesized that the RNA from the germinal disk of the preovulatory F1 largest follicle at the time of occurrence of meiosis-I could potentially reflect the physiological and functional mechanisms of meiotic drive and sex ratio bias. None of the previous studies on sex ratio bias in birds investigated the mechanisms of sex ratio bias at the transcriptome level. In the present study, we manipulated the egg mass of laying chickens using feed restriction treatment and tested whether egg mass can predict the sex of eggs at the unincubated stage and also investigated the association of primary sex ratio per hen with mean egg mass per hen. Because a significant negative association was found between egg mass and sex ratio, two groups of hens were selected from the feed restriction group, that is, male-biased hens with low egg mass and female-biased hens with high egg mass. The RNA from the germinal disks of the F1 preovulatory follicles collected at the time of occurrence of meiosis-I from the two groups of selected hens euthanized at the end of the feed restriction was compared in genomewide transcriptome analysis to study the underlying mechanisms of meiotic drive and sex ratio bias.

MATERIALS AND METHODS

Housing Conditions, Inseminations, and Egg Collection

The study was approved by the Animal Experiment Committee of the Animal Sciences Group of Wageningen University and Research Centre, Lelystad (Approval ID 2012002). Ninety hens of a brown layer line 35 wk of age (ISA BV, Hendrix Genetics) were randomly assigned to the control and feed restriction groups with 45 hens per group. Hens were housed individually as of Day −20 (defining the day of start of feed restriction treatment, see below, as Day 0), with visual and audio contact with each other, with a 16L:8D at 25°C. Hens were artificially inseminated twice weekly. Eggs were collected daily from Day −5 (two days after the third insemination) until Day 18 of the experiment and directly stored at 17°C until processing the same day or the next day.

The control group hens had ad libitum access to feed (standard layer diet) during the entire experiment. Hens in the feed restriction group received daily feed rations, each day at 0800, of 80% of ad lib consumption during Days 0–6 and 70% of ad lib on Days 7–18. These rations were calculated for each hen based on its own feed consumption during 4 days prior to starting the treatment. Body mass of hens were measured on Days −15, −12, −10, −4, −1, 2, 4, 6, 9, 11, 13, and 15 between 1500 to 1600.

Collection of Blastodiscs from Preovulatory F1 Follicle

Hens were euthanized at the end of the experiment for collection of the F1 follicle around the time of meiosis-I. For practical reasons, this was done in three batches with 15 hens per day on Days 16–18. On the three days before planned euthanization, the time of egg laying of each hen was recorded to predict the time of meiosis-I, which is reported to occur about 26 to 28 h before oviposition [35, 40]. All hens were euthanized to collect the F1 largest follicle in their respective calculated time window of expected time of meiosis, that is, 2–4 h before expected time of ovulation. Luteinizing hormone surge is known to occur 4–7 h before ovulation in hens [41], which means that the luteinizing hormone surge had occurred and meiosis-I had resumed in all the collected F1 follicles during our experiment. Hens were euthanized by intravenous injection of an overdose (1–1.5 ml/hen) of T61 (Intervet Nederland B.V). The abdomen was quickly opened and the largest follicle (F1) was separated carefully from the ovary by cutting the attaching stalk, and the theca externa was then removed with scissors.

The germinal disk region was isolated from the F1 follicle as described previously [30, 42]. In brief, a small piece of filter paper with a round 6 mm diameter hole was placed over the germinal disk region to support the follicle membrane. The follicle membrane was cut on one side of the paper, allowing insertion of artery forceps to clamp the paper plus germinal disk region. Then the follicle membrane was cut along all sides of the paper square, and the paper square together with the adhering yolk membrane section, including the germinal disk region, was lifted from the follicle and transferred to a Petri dish with PBS. Adhering yolk was removed with a gentle stream of PBS from a pipette tip and while being gently stroked with a flexible horsehair fixed onto a small stick. By manipulation with forceps and horsehair, the theca interna plus overlying filter paper was removed. The material around the edges of the whitish area of the germinal disk was cut away, and the germinal disk area (including oolemma and perivitelline layer and possibly some granulosa cells) was then transferred to clean RNase-free Eppendorf tubes, frozen in liquid nitrogen, and stored at −80°C until the purification of the RNA.

Blastodisc Isolation and Sexing Using PCR

The laying rates in the control and the feed restriction groups were 95% and 87%, respectively. Blastodiscs were isolated from all eggs (943 in the control and 900 in the feed restriction group from Days −5 to 18) using a technique similar to that described by Chapman et al. [43]. Isolated clean blastodiscs free of yolk were stained with Hoechst 33342 for determining fertility as described previously [26]. A total of 1696 eggs were fertile (878 in control and 818 in feed restriction groups) and 147 were infertile (65 in control and 82 in feed restriction groups), and the fertility rate in the control and feed restriction groups were 93.1% and 90.8%, respectively. The fertile blastodiscs were suspended in 20 μl of PBS and stored frozen at −20°C until use for nucleic acid extraction after a maximum storage of 3 wk. The sex of the blastodiscs was determined by PCR amplification of the *CHD1* gene exactly as described [26].

Sample Sizes, Statistical Analysis, and Selection of Hens for Microarrays

Statistical analysis was performed using software R2.12.2 with the lme4 and nlme package applying a generalized linear mixed modeling approach. All the tests were two tailed with significance delimited by $\alpha = 0.05$. Hens were used as random factor to account for the fact that they contributed with more than one egg to the data sets.

Two hens in the feed restriction group gave very few ($\leq$4) fertile eggs during the experiment. In the control group, one hen died at the beginning of the experiment and another hen did not produce eggs during the experiment. These hens were excluded, so data from 43 hens of each group are used for statistical analysis. For practical reasons, egg mass was not determined on Days 0 and 18. In addition, the egg mass of 41 eggs (on various days and from both groups) had inadvertently not been recorded. Egg sex and egg sex ratio data as well as laying rate and fertility rate were analyzed by logistic regression with binomial errors using logit link function. In the feed restriction group, for analyzing egg sex of hens while under the influence of feed restriction, the eggs laid between Days 2 and 18 were considered because these would have had undergone completion of meiosis-I during the treatment period (between Days 0 and 18). Likewise, for the period of more severe feed restriction (70% of ad lib), the eggs laid between Days 9 and 18 were considered.

The effect of treatment on egg sex was tested in a model with interaction of treatment and day (as the effect of treatment may increase over time), and backward stepwise regression was then performed. The effect of treatment on hen body mass was tested by comparing hen body mass at Days −1 and 15, using a paired *t*-test. Details of testing the effect of declining body condition on sex ratio are described together with the respective results in the results section.

For the feed restriction and the control group, regression of egg mass on the day of treatment was performed for individual hens. In the feed restriction group, the slope of the egg mass over time was negative for the majority of the hens (38 out of 43), and all the hens were included for analyzing the effect of egg mass on egg sex or egg sex ratio, considering the eggs of Days 2–18 or Days 9–18, respectively. In the control group, the slope of the egg mass over time was negative for 18 of the 43 hens and was positive for the other 25 hens (see Fig. 1 for egg mass, i.e., mean of hens per group, over time). The effect of egg mass on egg sex in the control group was analyzed using either all hens or using the two subpopulations of hens with negative and positive regression, respectively, considering eggs of Days −5 to 18.

Because it was found that hens differed in sex ratio and mean egg mass per hen, with a significant negative association between these two variables (see Fig. 2), we selected 16 hens ranging from high sex ratio and low mean egg mass per hen to lower sex ratio and higher mean egg mass per hen to study gene expression in the F1 follicle (isolated at the last day of treatment) by microarray analysis. Microarrays were done on the individual hen level, that is, we used one microarray for each of these 16 hens, allowing to study associations of gene expression values with sex ratio/mean egg mass per hen. We did not select hens on the basis of sex ratio alone, but also on the basis of mean egg mass per hen, because the two variables are confounded anyway and especially because the estimate for the binomial quantity sex ratio (or fraction of male eggs) is less reliable when considering the relatively small number of eggs of a single hen. It was not always possible to select hens with the most extreme estimates for sex ratio/mean egg mass because we also considered three other criteria. One is that the five (out of 43) hens that did not show a decline in egg mass in response to feed restriction were not considered. Second, the (few) hens with relatively low laying or fertility rates were not given

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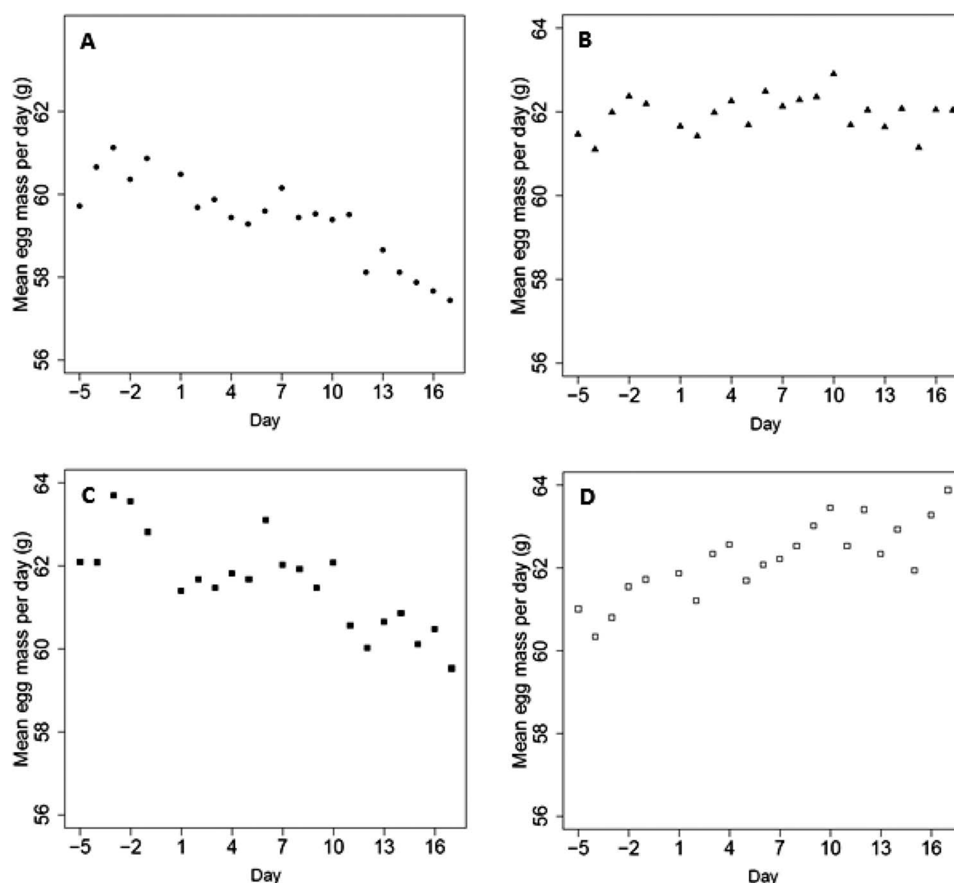

FIG. 1. Mean egg mass per day (mean per group) as a function of day number. **A**) Feed restriction group (43 hens). **B**) Control group (43 hens). In the control group, subpopulations were defined of hens showing a decrease (**C**, 18 hens) or increase (**D**, 25 hens) of egg mass over time.

priority because estimates for the sex ratio or mean egg mass per hen would be less reliable, and third, only hens with good quality of the RNA isolated from the germinal disk were selected. To compare gene expression levels of individual genes in two distinct groups of hens, we grouped the data into two subgroups of eight hens with relatively high sex ratio/low mean egg mass per hen and eight hens with relatively low sex ratio/high mean egg mass per hen. The overall sex ratio and egg mass (mean ± SD) in all the hens of both groups of high sex ratio/low mean egg mass and low sex ratio/high mean egg mass were 0.71/54.63 ± 3.25 and 0.44/61.61 ± 3.25, respectively. Selected hens are depicted by solid symbols in Figure 2. Laying rates were 95% and 92% (not significantly different, $P = 0.38$), respectively, and fertility rates were 100% and 96% (not significantly different, $P = 0.32$) for the high sex ratio/low mean egg mass subgroup and the low sex ratio/high mean egg mass subgroup, respectively. Note that in the gene set expression analysis (GSEA, explained below) and in the analyses of associations with sex ratio/mean egg mass, the expression data of the 16 hens were used individually.

RNA Purification and Microarray Analysis

RNA extraction. RNA from the blastodiscs of F1 follicles from the selected hens was purified using master pure RNA purification kit from the Epicenter (MCR85102) and following the protocol as described by the manufacturer. All 16 purified RNA samples were checked for integrity on the Agilent Bioanalyzer according to Agilent Technologies Protocol. The amount of RNA isolated from the germinal disk region per hen ranged from 1.5–5 μg.

Labeling, hybridization, scanning, and feature extraction. Labeling was done as recommended by Agilent Technologies using One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling. The input was 10 ng of total RNA, and 600 ng of labeled cRNA was used on the eight pack array.

Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling protocol from Agilent in the hybridization oven (G2545A hybridization oven; Agilent Technologies). The hybridization temperature was 65°C with rotation speed 10 rpm for 17 h. After 17 h, the arrays were washed as described in the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling protocol from Agilent.

The arrays were scanned using the DNA microarray scanner with Surescan high-resolution technology from Agilent Technologies. Agilent Scan Control was used with resolution of 2 μm, 16 bits, and photomultiplier tube of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for one color gene expression.

Data loading and statistical and functional analysis. Array data has been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database (accession number GSE59041). The files generated by the feature extraction software were loaded in GeneSpring GX 12 for quality control. On the basis of aberrant values for the quality control probes of one microarray, used to analyze the transcriptome of one female sex ratio bias hen, the data of this hen were taken out. The normalization and transforming of the data was performed within R 3.0.0 [44] using the LIMMA package. First, a principle component analysis was performed to explore the data. Second, using data of all 15 hens (both groups) together, we performed functional annotation clustering analyses with Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 [45, 46]). With DAVID Functional Annotation Clustering, enrichment is calculated for genes of interest across multiple databases and ranked accordingly to their enrichment score (ES). An ES above 1 suggests that this process/these processes are dominant. Thus, processes were identified that were enriched in highly expressed genes (gene expression >10 in each hen sample). Third, we performed a statistical analysis (t-test) using Benjamini-Hochberg correction for multiple testing to identify differentially expressed genes (false discovery rate < 0.05) between the two groups (hens with male sex ratio bias/low mean egg mass and female sex ratio bias/high mean egg mass, respectively). Finally, a GSEA [47] was performed to identify whether there were differences at the process level between the two groups. To represent the major biological processes, we only used the KEGG and GO database in the GSEA. In the GSEA, human gene annotation was used because in the DAVID functional annotation clustering, many more enriched processes were found when the human gene database was used instead of the chicken database (see Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). Furthermore, we assumed that most processes are generic

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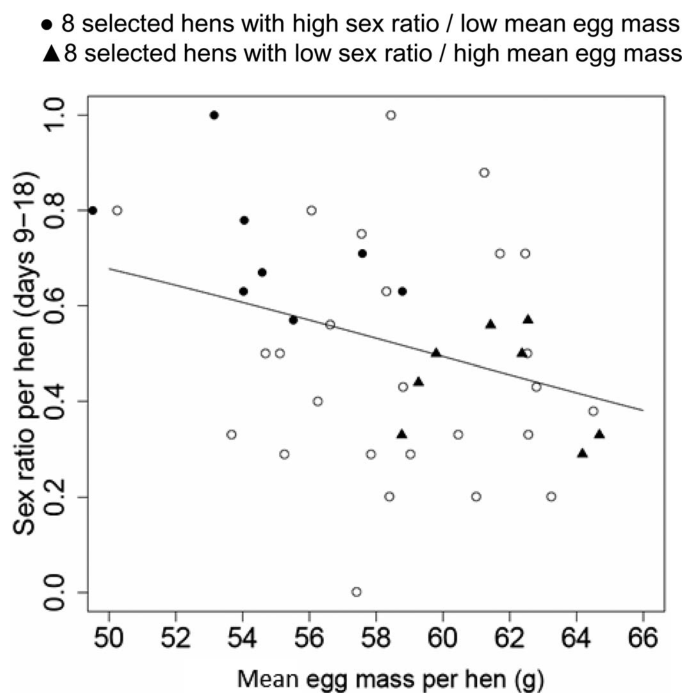

FIG. 2. Association of sex ratio per hen with mean egg mass per hen. Graphical representation of the association of sex ratio (proportion of male eggs) per hen with mean egg mass per hen for the eggs of the last 10 days of treatment, Days 9–18 (statistics given in Table 1), showing hens selected for the two groups to be compared in microarray transcriptome analysis, that is, male-biased hens with low egg mass (solid circles) and female-biased hens with high egg mass (solid triangles).

when comparing chicken and human, including cell cycle, spindle, as well as (cellular) metabolic processes.

RESULTS

Effect of Treatment on Egg Sex, Hen Body Mass, and Egg Mass

The interaction of treatment (feed restriction) times treatment day number was almost significant for predicting the sex of egg (eggs of Days 2–18, no. of eggs = 1162, variance of hen as random factor <0.0001, estimate = −0.04, $z = -1.87$, $P = 0.06$). After removing day and interaction of treatment times day from the model, the main factor treatment was not significant (Days 2–18, no. of eggs = 1162, variance of hen as random factor <0.0001, estimate = 0.11, $z = 0.96$, $P = 0.33$). In the control group (no feed restriction) 18 (out of 43) hens showed an unexpected decline of egg mass. If the data from these hens were excluded from the analysis, the main effect of treatment on sex ratio was significant (eggs of Days 2–18, no. of eggs = 900, variance of hen as random factor <0.0001, estimate = 0.27, z = 2.00, $P = 0.04$).

On average, feed restriction resulted in a significant decrease of body mass of hens over time (from day −1 to day 15) (77 ± 48 g, mean ± sd) (df = 84, $t = 2.54$, $P = 0.01$), while mean body mass of hens from the control group did not change significantly (2 ± 46 g). In the feed restriction group, treatment day number (Days 1–17) had a significant negative effect on egg mass (no. of eggs = 621, residual for hen as random factor = 2.80, estimate = −0.17, $t = -7.35$, $P < 0.0001$) (see Fig. 1). In the control group during the same period of Days 1–17, the mean egg mass per day did not decline over time, that is, the effect of treatment day number on egg mass was not significant (no. of eggs = 665, residual for hen as random factor = 1.93, estimate = 0.006, $t = 0.43$, $P = 0.66$) (see Fig. 1).

Association of Hen Body Mass, Laying Rate, and Fertility Rate with Egg Sex Ratio per Hen

The interaction of treatment times the slope of hen body mass over time between two consecutive days on which body mass was measured (every second or third day between Days 2 and 15) did not significantly predict the sex ratio of eggs laid in the corresponding period (no. of observations 588, variance of the hen as random factor <0.0001, estimate = 0.005, $z = 0.64$, $P = 0.51$) or the sex ratio of eggs that had meiosis in the corresponding period (no. of observations 582, variance random factor <0.0001, estimate = 0.002, $z = 0.31$, $P = 0.75$). In a model without interaction, the main factors were not significant either.

In the feed restriction group, the laying rate and fertility rate per hen were not associated with egg sex ratio per hen (considering eggs of Days 2–18). Likewise, there was no association in the control group (considering all eggs, Days −5 to 18) or in the two subpopulations of control hens (see *Materials and Methods*).

Relationship Between Egg Mass and Egg Sex

Statistical parameters for all tested effects and associations in this paragraph are shown in Tables 1 and 2. In the feed restriction group, the effect of egg mass on sex of eggs was not significant for eggs laid before treatment started (eggs laid on Days −5 to −1) ($P = 0.32$) or for eggs that had meiosis during the treatment period (eggs laid on Days 2–18) ($P = 0.16$), but was significant for eggs laid on Days 9–18 (i.e., eggs that had meiosis in the period of more severe feed restriction) ($P = 0.02$) (Table 1). Likewise, the association of mean egg mass per hen with sex ratio per hen was not significant when considering eggs laid before treatment started (eggs laid on Days −5 to −1)

TABLE 1. Statistical parameters of relations between egg mass and sex of egg or sex ratio.[a]

	Prediction of sex of eggs by egg mass[b]					Association between sex ratio per hen and mean egg mass per hen[b]			
Period	N	Var	Est	z	P	df	Est	z	P
Days 2–18	523	<0.0001	−0.03	−1.40	0.16	41	−0.02	−1.12	0.26
Days 9–18	**282**	**<0.008**	**−0.06**	**−2.28**	**0.02**	**41**	**−0.07**	**−2.58**	**0.009**
Days −5 to −1	157	<0.0001	−0.03	−0.97	0.32	41	−0.04	−1.07	0.28
Days −5 to 18[c]						16	−0.04	−1.82	0.06

[a] All data pertain to feed restriction group hens except where indicated otherwise.
[b] Number of observations (N), variance of the hen as random factor (Var), degrees of freedom (df), estimate (Est), and z, and P values of effect or association tested. Bold font indicates significance.
[c] Control group hens with decreasing egg mass.

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TABLE 2. Statistical parameters of relations between egg mass and sex ratio.[a]

	Sex ratio over period					
	Days −5 to −1	Days 2–18	Days 9–18[b]			
Mass	*P*	*P*	df	Est	*z*	*P*
Egg mass Days −5 to −1			41	−0.04	−1.45	0.14
Δ Egg mass[c]	0.50	0.10	**41**	**−0.14**	**−2.44**	**0.01**

[a] All the data pertain to feed restriction group hens.
[b] Degrees of freedom (df), estimate (Est), and *z*, and *P* values of effect or association tested. Bold font indicates significance.
[c] (mean egg mass per hen Days 9–8) – (mean egg mass per hen Days −5 to −1).

($P = 0.28$) or for all eggs that had meiosis during the treatment period (eggs laid on Days 2–18) ($P = 0.26$), but was significantly negative for eggs laid on Days 9–18 ($P = 0.009$, see Fig. 2 and Table 1). The mean egg mass per hen before start of treatment (eggs laid on Days −5 to −1) also tended toward a negative association with egg sex ratio per hen of eggs laid in the second phase of the treatment (eggs laid in Days 9–18), but this was not significant ($P = 0.14$) (Table 2). The change of egg mass per hen (i.e., the mean egg mass per hen of Days 9–18 minus the mean egg mass of Days −5 to −1) was significantly negatively associated with the sex ratio per hen (for eggs laid on Days 9–18) ($P = 0.01$) (Table 2). In other words, a larger decline (more negative change) of egg mass predicts a higher sex ratio. The association was not significant when relating the same measure of egg mass change per hen with the sex ratio per hen for eggs of Days 2–18 ($P = 0.10$), and there was not even a tendency when considering the sex ratio of eggs laid before treatment (Days −5 to −1, $P = 0.50$) (Table 2).

In the control group, in the subpopulation of hens that showed a decrease of egg mass, there was a tendency toward a negative association between mean egg mass per hen and sex ratio per hen when considering all eggs (Days −5 to18, $P = 0.06$) (see Table 1), whereas in the subpopulation of hens that showed no decrease of egg mass over time, there was no relation between egg mass and sex of egg or egg ratio.

Results from Microarray Studies

On the level of individual genes, there was no significant differential expression comparing the group of hens with high sex ratio/low mean egg mass with the group of hens with low sex ratio/ high mean egg mass. Functional annotation clustering (DAVID) analysis over all hens (male and female sex ratio bias groups together) showed that general cellular metabolic processes were enriched in highly expressed genes (gene expression >10 in each sample) (Table 3). The number of enriched processes (gene sets) found was much higher when the human database was used instead of the chicken database (Supplemental Table S1). Therefore the human database was used during the subsequent GSEA. Compared to analysis of differential expression in individual genes, GSEA is more powerful because it makes use of combined results of many genes belonging to the same pathway or process. GSEA showed that cellular processes related to cell cycle progression, mitotic/meiotic apparatus, and chromosomal movement had a significant (negative) normalized ES (false discovery rate < 0.25, the default setting of GSEA) for male sex ratio bias/low mean egg mass (Table 4). This means that these gene sets were enriched in genes that negatively correlated with sex ratio, that is, the genes had a higher expression in hens with a female sex ratio bias compared with hens with a male sex ratio bias. Variation of gene expression level in these gene sets in relation to sex ratio can also be seen in Supplemental Figure S1 that shows heat maps for four selected gene sets (cell cycle, motor activity, spindle, and chromosome segregation), indicating the gene expression levels of the core (or leading edge) genes per gene set for all 15 hens (hens ranked in order of sex ratio). Also, for almost all enriched processes, the means of the expression levels of the core genes per gene set per hen were significantly negatively correlated with sex ratio per hen (eggs of Days 9–19) and positively correlated with egg mass per hen before feed restriction treatment (Days −5 to −1) or in the second half of the treatment (Days 9–19). Gene expression of the enriched gene sets was not significantly correlated with the decrease of egg mass per hen during treatment (difference between mean mass of the eggs of Days 9–18 and eggs of Days −5 to −1). *P*-values for all correlations are given in Supplemental Table S2, while graphical representations are

TABLE 3. Gene sets (general metabolic processes) significantly enriched in highly expressed (expression level >10) genes in the germinal disks of follicles obtained on the last day of the feed restriction treatment (Day 16, 17, or 18).

No.[a]	Generalized term[b]	Enrichment score	*P* value[c]
1	Organelle lumen	10.43	0.47
2	Protein/ribosome	9.16	0.99
3	Membrane (inner/mitochondrial)	9.34	0.98
4	Envelope (organelle/mitochondrial)	9.62	0.06
5	Cellular respiration	6.78	0.17
6	Protein (ubiquitination, ligation, modification)	5.07	0.52
7	Ribosome	4.52	1.00
8	NADH dehydrogenase	4.10	0.83
9	Protein localization/transport	4.04	0.60
10	mRNA processing	3.95	0.61

[a] Numbered in order of enrichment score. These numbers are used in the legend of Figure 4.
[b] DAVID functional annotation analysis using (default) human database for data from all hens (male and female sex ratio bias groups together).
[c] *P* value for the comparison of mean gene expression per gene set of male-biased versus female-biased hens (no significant differences).

TABLE 4. Gene sets significantly enriched in genes that negatively correlated with sex ratio (significant negative normalized enrichment score [NES], false discovery rate [FDR] < 0.25), in the germinal disks of follicles obtained on the last day of the feed restriction treatment (Day 16, 17, or 18).

No.[a]	Cellular process[b]	Size[c]	NES	FDR
1	Cell cycle (KEGG)	95	−1.908	0.126
2	M phase	74	−1.710	0.163
3	Interphase	45	−1.669	0.166
4	Cell division	17	−1.673	0.169
5	Spindle	36	−1.681	0.170
6	Cell cycle	215	−1.717	0.173
7	Interphase of mitotic cell cycle	43	−1.685	0.177
8	M phase of mitotic cell cycle	55	−1.721	0.188
9	Motor activity	20	−1.686	0.192
10	Small GTPase mediated signal transduction	45	−1.641	0.198
11	Homologous recombination (KEGG)	22	−1.644	0.202
12	Regulation of mitosis	23	−1.741	0.209
13	Cell cycle phase	111	−1.724	0.211
14	Mitotic cell cycle	104	−1.753	0.227
15	Chromosome segregation	25	−1.611	0.249

[a] Numbered in order of FDR. These numbers are used in the legend of Supplemental Figure S2.
[b] Processes based on GO database unless KEGG database is indicated.
[c] Number of genes in the gene set.

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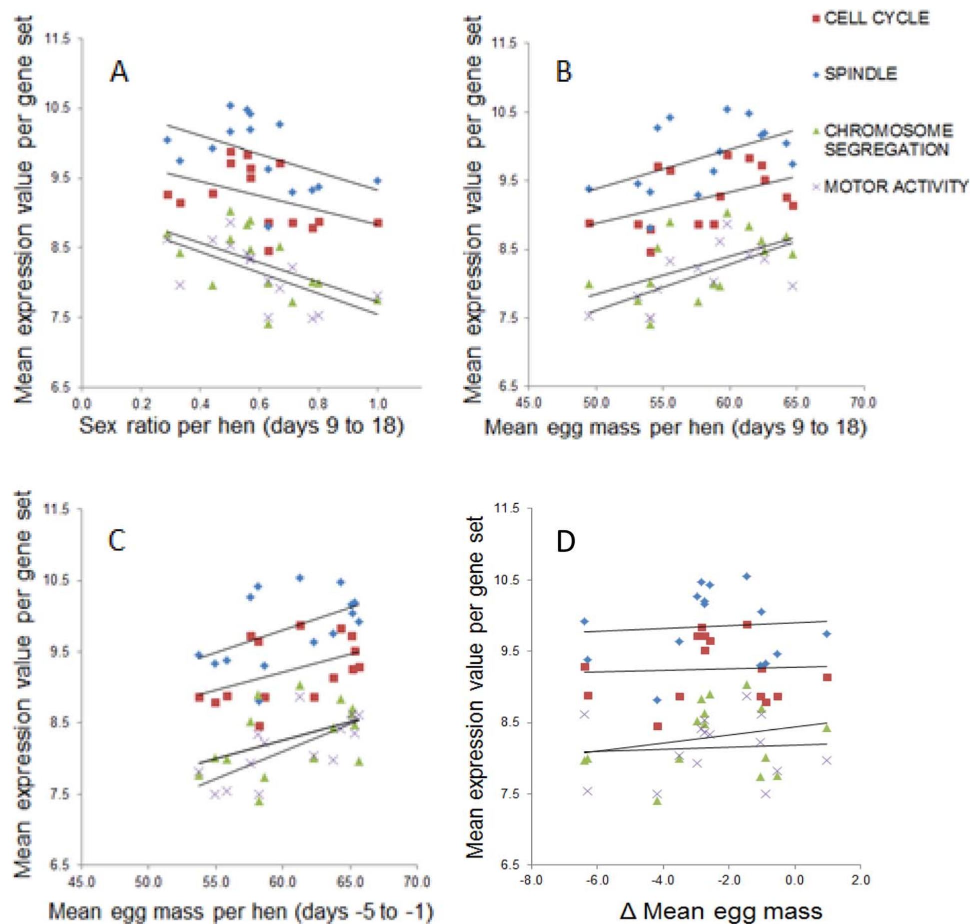

FIG. 3. Graphs showing mean expression levels per hen of selected gene sets shown in Table 4. Mean expression levels per hen of gene sets that were significantly enriched with genes that negatively correlated with sex ratio (see Table 4) are shown as a function of the sex ratio (proportion of male eggs) per hen (eggs of Days 9–18; **A**), the mean egg mass per hen of eggs of Days 9–18 (**B**) or of eggs of Days −5 to −1 (**C**), or the change of egg mass per hen during feed restriction, that is, Δ egg mass = (mean egg mass per hen Days 9–18) − (mean egg mass per hen Days −5 to −1) (**D**). All 15 significantly enriched gene sets show similar correlations, but for clarity, four selected gene sets are shown. All the gene sets are shown in Supplemental Figure S2.

shown in Figure 3 (selected enriched gene sets) and in Supplemental Figure S2 (all enriched gene sets). In contrast to the cell cycle-related processes, the mean expression level of general metabolic processes (processes/gene sets shown in Table 3) showed no correlation with sex ratio or mean egg mass per hen (Fig. 4).

DISCUSSION

Feed restriction led to an overall decline of egg mass (in almost all hens of the feed restriction group). In the second period of treatment (Days 9–18) with more severe feed restriction and a steeper decline of egg mass, the sex ratio per hen had a significant negative association with mean egg mass per hen. This association was largely due to differences between hens in the change of egg mass during treatment, which was significantly negatively associated with the sex ratio per hen (for eggs laid on Days 9–18). In other words, a larger decline (more negative change) of egg mass predicts a higher sex ratio. It may be that differences between hens in egg mass before the start of treatment has contributed somewhat to this association as there was a tendency toward a negative association of mean egg mass per hen before treatment with sex ratio of eggs of Days 9–18, but this was not significant. The analysis of sex ratio of eggs including eggs laid in the first phase of treatment, or only of eggs laid before the start of treatment, indicates that the sex ratio bias develops over time under conditions that lead to declining egg mass. The results in the control group corroborate these findings: In the subpopulation of hens that showed an egg mass decline during the experiment, a similar association appeared to exist as that found in the feed restriction group, albeit the association was just not significant ($P = 0.06$). This suggests that hens that for any reason showed a decline of egg mass develop a negative association of sex ratio per hen with mean egg mass per hen.

Sex allocation theory [2, 3] predicts that large mothers laying large eggs and producing large (costly) offspring in periods of reduced availability of resources would tend to produce less male offspring (more costly sex). Then, according to Fisher's principle [2], the resulting shortage of males would make it more advantageous for the more economic hens, that is, light mothers laying smaller eggs, producing lighter (less costly) offspring, to produce more males. This fits with our observation of an inverse relationship between egg mass and egg sex ratio in which hens with larger egg mass and a smaller decline of egg mass in response to feed restriction tend toward a female sex ratio bias, while hens with smaller egg mass and larger decline of egg mass in response to feed restriction tend to have a male sex ratio bias. The fact that the negative association between egg mass and egg sex was found only in the phase of more severe feed restriction, and not before starting feed restriction,

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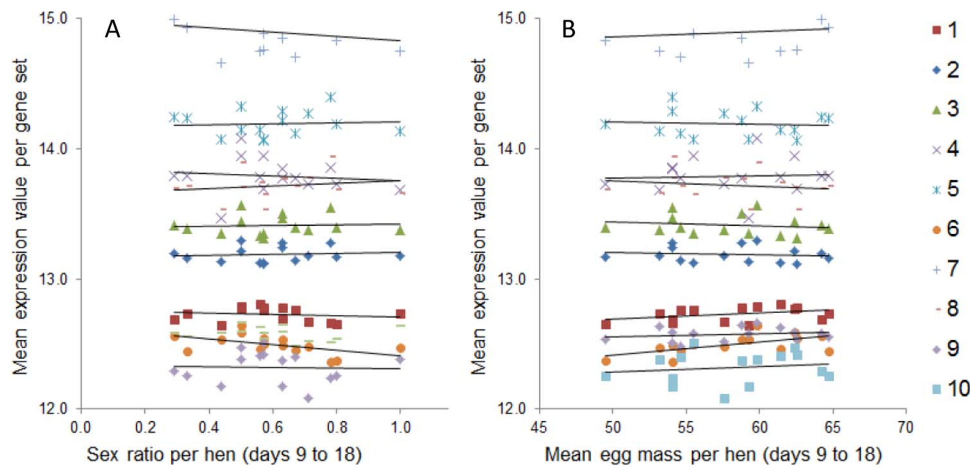

FIG. 4. Graphs showing mean expression levels per hen of gene sets shown in Table 3. Mean expression levels per hen of gene sets of general metabolic processes (gene sets given in Table 3) are shown as a function of the proportion of male eggs per hen (eggs of Days 9–18; **A**) or the mean egg mass per hen of eggs of Days 9–18 (**B**). Numbers 1–10 in the legend at the right side of the right panel identify the gene sets as listed in Table 3.

could be indicative of a strategic sex allocation response of the hens under the effect of feed restriction.

As explained in *Introduction*, many studies found an effect of food availability per se or resulting (change in) body condition on sex ratio. In the present study, we did not find an effect of feed restriction or decline of hen body mass on sex ratio, which could be due to the relatively minor decrease in hen body mass observed in the current study.

A number of mechanisms have been proposed for explaining sex ratio bias in birds, which include biased sex chromosome segregation during meiosis, selective follicular resorption, selective ovulation, differential survival or fertilization success of oocytes depending on their sex, and sex-dependent differential zygote or embryonic mortality until the sex ratio is determined (as reviewed by [4, 33, 48]). In the present study, we have used unincubated eggs. Therefore, the observed sex ratio bias cannot be a consequence of sex-specific embryonic death during incubation. Sex-specific infertility of eggs also appeared to play no role in the present study because fertility rate per hen was not associated with egg sex ratio per hen, and the fertility rate was very high (90.8%) anyway. Sex-specific follicle resorption, or selective ovulation, which would lead to lowered laying rate, is also not a likely mechanism in the present study. The laying rate in the feed restriction group was somewhat subdued but still quite high (mean 87%), and the laying rate per hen was not associated with sex ratio per hen. This would leave asymmetric sex chromosome segregation during meiosis in the preovulatory follicle (meiotic drive), proposed by other authors [4, 24, 29, 32–34, 37–39, 49], as a likely mechanism of sex ratio bias.

In this study, we found that a number of cellular processes (gene sets) related to cell cycle progression, mitotic/meiotic apparatus, and chromosomal movement were significantly enriched in genes that negatively correlated with sex ratio, and the mean expression levels of these gene sets negatively correlated with the sex ratio and positively correlated with the mean egg mass of eggs of Days 9–18. After isolation of the germinal disk region and removal of the theca interna, some granulosa cells may be included in the collected material. Therefore, we have to consider if it is possible that the found correlation of cell cycle-related gene expression could be due to contamination of granulosa cell mRNA in the germinal disk preparation. For instance, one could argue that the female-biased hens produce larger eggs with larger follicles that could have more, or more actively dividing, granulosa cells overlying the germinal disk region. However, it is not self-evident that larger follicles would have more granulosa cells per surface area over the germinal disk. Also, granulosa cells stop proliferating and dividing (mitosis) toward completion of follicle growth [50]. Furthermore, gene expression of the highly expressed general metabolic processes was not correlated at all with either female bias or egg mass per hen. Lastly, the germinal disk of chicken and other avian oocytes contain very high amounts of RNA, several orders of magnitude (microgram vs. picogram range, five or six orders of magnitude) more than somatic cells [31, 42]. Therefore the contribution of granulosa cell mRNA to the germinal disk mRNA preparation is likely to be small.

As mentioned above, meiotic drive has been proposed by many authors as mechanism for sex ratio bias in birds. Epigenetic factors, under the influence of different stimuli such as hormones, have been proposed to target meiosis and sex chromosome movement to cause sex ratio bias in a context-dependent manner under different environmental conditions (for more details, see [29, 34, 37]). As far as we are aware, our study is the first attempt to find cellular and molecular evidence supporting the concept of meiotic drive in birds. The observed differential expression of cell cycle-related gene sets was associated with both mean egg mass per hen and sex ratio per hen because these two variables were confounded. So the conclusion is that the hens that differed in sex ratio/egg mass differentially express cell cycle-related gene sets and differ in some mechanism of sex ratio adjustment. Thus, our results suggest that enrichment of cell cycle-related processes could be involved in meiotic drive, with a higher activity of these processes correlating with female sex ratio bias. The fact that differential expression was observed on the gene set level but not on the level of individual genes indicates that in these gene sets many genes show a small, but consistent, up- or down-regulation. Hence, these processes and the genes in these gene sets could be targets for further studies. Future experiments could first be aimed at reproducing our present findings in gene expression studies using perhaps different treatment methods to induce sex ratio bias than we have used. For instance, it would be interesting to see whether sex ratio adjustment induced by hormone treatment of hens (as has been reported previously [11, 32, 39, 40, 49, 51–58]) would show a similar association with differentially expressed gene sets as found in this study.

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Subsequently, these gene sets/cellular processes, or the genes in these processes, could be studied in more detail.

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